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EFECTO DE LA TEMPERATURA EN EL ESTADO REDOX Y LA CAPACIDAD FERMENTATIVA DE UNA LEVADURA TERMOTOLERANTE

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Resumen

Las levaduras tienen una amplia aplicación en la biotecnología, principalmente por su capacidad fermentativa; sin embargo, en el proceso de fermentación pueden generarse condiciones estresantes, como aumentos de temperatura. Lo anterior ha generado un interés por seleccionar levaduras termotolerantes que presenten viabilidad y crecimiento a temperaturas ≥40°C, así como capacidad para producir etanol. El estrés oxidativo es uno de los principales factores responsables del daño producido por la exposición a altas temperaturas. Este estrés es generado por un aumento de las especies reactivas de oxígeno (ERO), siendo la cadena transportadora de electrones mitocondrial (CTEm) la principal fuente productora en levaduras. Al igual que la concentración de ERO, la respuesta antioxidante influye en el estado redox celular, el cual es crítico para el apropiado desarrollo de los procesos celulares y por ende para la producción de etanol. Por lo anterior, el objetivo de este trabajo fue analizar la actividad de los sistemas antioxidantes y la CTEm, así como el estado redox y la capacidad fermentativa de una levadura termotolerante. Las levaduras *Saccharomyces cerevisiae* (MC4) y *Kluyveromyces marxianus* (OFF1 y SLP1), previamente aisladas de fermentaciones espontáneas, se crecieron a temperaturas de hasta 45°C. Con los resultados obtenidos se identificó a la levadura *K. marxianus* SLP1 como termotolerante, con una mayor actividad de sus sistemas antioxidantes y menor producción de ERO y lipoperoxidación. En las membranas de *K. marxianus* SLP1 se observó una mayor proporción de ácidos grasos saturados, por lo cual se estudió el efecto de la incorporación de ácidos grasos (oleico (C18:1), linoleico (C18:2), linolénico (C18:3) y araquídico (C20:0)), en la termotolerancia de *K. marxianus* SLP1, observando una relación inversa, entre su termotolerancia con el grado de insaturación de los ácidos grasos incorporados en su membrana citoplasmática. *K. marxianus* SLP1 presentó una disminución en la actividad de los complejos de su CTEm; sin embargo, conservó la función de su ATPasa, la concentración de NADH y glutatión total. La capacidad fermentativa de *K. marxianus* SLP1 se evaluó mediante los procesos de hidrólisis y fermentación separada (HFSe) e hidrólisis y fermentación simultánea (HFSi) a 40°C, utilizando bagazo de agave como fuente de carbono. *K. marxianus* SLP1 presentó un rendimiento máximo de etanol de 85%, correspondiente a 10.36 g/L de etanol, valores similares a los obtenidos con la levadura industrial *S. cerevisiae* Ethanol Red (83% y 10.23 g/L). En conclusión, *K. marxianus* SLP1 presenta termotolerancia y potencial para ser usada en procesos de fermentación a 40°C, ya que ante un incremento de temperatura aumenta la actividad de sus sistemas antioxidantes y disminuye la función de su CTEm, manteniendo su estado redox y capacidad fermentativa.

Abstract

Yeasts have a wide application in biotechnology, mainly by their fermentative capacity; however, in the fermentation process stressful conditions can be generated, such as temperature increases. This has generated an interest in selecting thermotolerant yeasts that present viability and growth at temperatures ≥40°C, with ethanol production. Oxidative stress is one of the main factors responsible for the damage produced by high temperature. This stress is generated by an increase in reactive oxygen species (ROS), being the mitochondrial electron transport chain (mETC) the main production source in yeast. Like the ROS production, the antioxidant response influences cellular redox status, which is critical for the proper development of cellular processes, and thus for the production of ethanol. Therefore, the objective of this work was to analyze the activity of antioxidant systems and mETC of a thermotolerant yeast, as well as its redox state and fermentative capacity. The yeasts *Saccharomyces cerevisiae* (MC4) and *Kluyveromyces marxianus* (OFF1 and SLP1), previously isolated from spontaneous fermentations, were grown at temperatures up to 45°C. *K. marxianus* SLP1 was identified as thermotolerant, wich showed a higher activity of its antioxidant systems, and lower production of ROS and lipoperoxidation. In *K. marxianus* SLP1 membranes, a higher proportion of saturated fatty acids was observed. For the last, the effect of the incorporation of fatty acids (oleic (C18:1), linoleic (C18:2), linolenic acid (C18:3) and arachidic (C20:0)), on the thermotolerance of *K. marxianus* SLP1 was studied. Results showed an inverse relationship between thermotolerance and unsutaration degree of the fatty acids incorporated in the cytoplasmic membrane. *K. marxianus* SLP1 showed a decrease in the activity of the complexes of its mETC, however, retained the ATPase function, NADH concentration and total glutathione. The fermentation capacity of *K. marxianus* SLP1 was evaluated by separate fermentation and hydrolysis (SFH) and simultaneous hydrolysis and fermentation (SHF) at 40°C, using agave bagasse as a carbon source. *K. marxianus* SLP1 presented a maximum yield of ethanol of 85%, corresponding to 10.36 g /L of ethanol, values similar to those obtained with the industrial yeast *S. cerevisiae* Ethanol Red (83% and 10.23 g/L). In conclusion, *K. marxianus* SLP1 showed thermotolerance and potential to be used in fermentation processes at 40°C, since in an increase of temperature it showed a higher activity of its antioxidant systems and a reduced in their mETC function, maintains without changes its redox state and fermentative capacity.

Palabras clave: Levaduras, termotolerancia, estado redox, bioetanol

1. Introducción

1.1. Levaduras

Las levaduras son hongos unicelulares con tamaños variados, dependiendo de la especie y las condiciones de crecimiento. La longitud puede ser de 2 a 50 μm, mientras que su diámetro de 1 a 10 μm. La mayoría de las levaduras pueden crecer en medios con pH de 4.5 a 6.5 y en temperaturas de 20 a 30°C. Las especies de levaduras son caracterizadas por criterios basados en su morfología, fisiología, inmunología y biología molecular. Algunas de las características fisiológicas utilizadas para diferenciar las especies, incluyen los diferentes carbohidratos que son capaces de asimilar, si presentan metabolismo en condiciones aerobias o semi-aerobias, su habilidad osmotolerante y/o capacidad de hidrólisis de lípidos. En 1996 se estimó que únicamente el 0.22% de las especies de levaduras (alrededor de 1500), de un total aproximado de 669 000, se encontraban aisladas y caracterizadas. Generalmente las levaduras se encuentran en plantas como comensales, y en animales pueden ser parásitos (Walker, 1998; Boekhout *et al*., 2002).

1.2. Metabolismo de las levaduras

En función de la concentración de oxígeno y el tipo de sustrato en el medio de crecimiento, las levaduras pueden llevar a cabo diferentes tipos de metabolismo (Tabla 1). La levadura *Saccharomyces cerevisiae* es fermentativa facultativa, por lo que puede realizar la fosforilación oxidativa o la fermentación para la síntesis de ATP (Figura 1) (van Dijken *et al*., 2002). En *S. cerevisiae,* la glucosa y la fructosa son adquiridas principalmente por difusión facilitada, más que por transporte activo. Los transportadores de hexosas consisten de más de 20 proteínas, las cuales funcionan como punto de control en el metabolismo de compuestos de carbono durante la fermentación. En cuanto a la selectividad por los azúcares, *S. cerevisiae* es principalmente glucofílica (Schü ltz y Gafner, 1995), capaz de cambiar su metabolismo de oxidativo a fermentativo, desde una concentración de glucosa en el medio de 0.14 g/L. Lo anterior, debido a la regulación de la expresión de genes para enzimas glucolíticas y transportadores de hexosas, e inhibición de genes involucrados en la respiración y funciones mitocondriales. Después de la entrada de glucosa a la célula, ésta es

fosforilada para comenzar la glucólisis. El producto final de la glucólisis es el piruvato, el cual puede ser incorporado en dos vías metabólicas; oxidativa o fermentativa (Figura 1). Ambas vías son reguladas por la concentración de sustrato y oxígeno presentes en el medio.

Tabla 1. Metabolismo de las levaduras

Metabolismo	Levadura	Características
Fermentación obligada	*Candida pintolopesii*	Únicamente fermentan, incluso en presencia de oxígeno
Crabtree-positiva	*Saccharomyces cerevisiae*	Principalmente fermentan en alta concentración de azúcares, aún en presencia de oxígeno
Crabtree-negativo	*Kluyveromyces marxianus*	No forman etanol en condiciones aerobias y no pueden crecer anaeróbicamente
No fermentativa	*Rhodotorula rubra*	No producen etanol en presencia o ausencia de oxígeno

Walker y White, 2005

Aún cuando en la fermentación alcohólica el rendimiento de ATP por mol de glucosa es menor comparada con la respiración, la fermentación es una vía metabólica que permite a las levaduras competir en condiciones anaerobias, además de que el etanol producido inhibe el crecimiento de otros microorganismos (D"Amore *et al*., 1990). Para levaduras no convencionales, el tipo de transporte de hexosas puede ser diferente, por ejemplo, a bajas concentraciones de azúcares, *Candida sutilis* transporta los azúcares a través de un mecanismo simporte de protones (van de Broek *et al*., 1997). Las levaduras Crabtree-negativo presentan bajos niveles de enzimas fermentativas y altos niveles de enzimas oxidativas, permitiendo la generación de altas concentraciones de biomasa y dióxido de carbono, pero bajas de etanol. No obstante ello, las levaduras del género *Kluyveromyces*

clasificadas como Crabtree-negativo, son consideradas buenas productoras de etanol, debido a su tolerancia a los condiciones de estres generadas en los procesos de fermentación (Postma *et al*., 1989). La vía de las pentosas o hexosas monofosfato, es considerada una vía metabólica alternativa a la degradación de glucosa. Esta vía permite la formación de NADPH y pentosa fosfato. El NADPH es usado en reacciones de biosíntesis de lípidos, así como para la biosíntesis de otros compuestos donde la ribosa 5-fosfato es un precursor de nucleótidos y ácidos nucleicos (Figura 1). Otras levaduras como *Candida tropicalis* y *Torulopsis* pueden utilizar alcanos como fuente de carbono, así como ácidos grasos (Fickers *et al*., 2005).

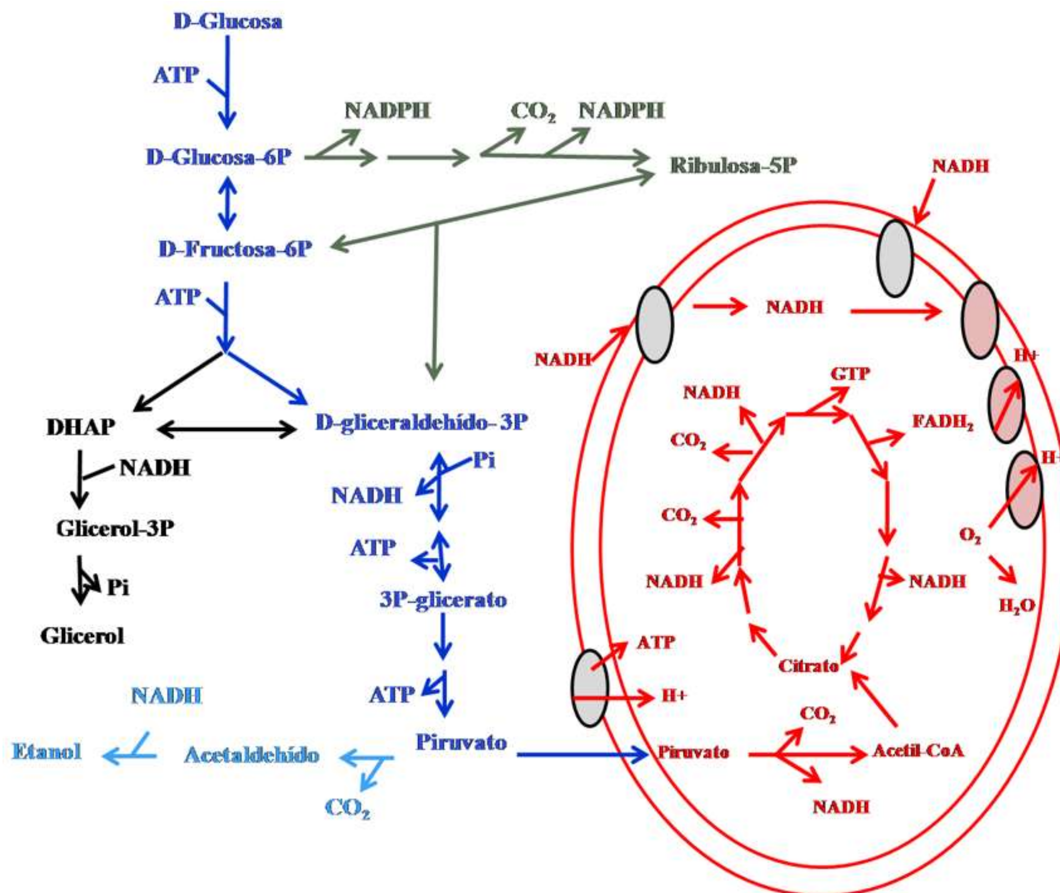

Figura 1. Vías metabólicas de levaduras. Azul oscuro: Glucólisis; Gris: Ciclo de las pentosas; Negro: Producción de glicerol; Azul claro: Fermentación; Rojo: Fosforilación oxidativa. Figura modificada de Walker, 1998.

1.3. Principales géneros de levaduras

Dos de las levaduras más estudiadas y utilizadas en la biotecnología son *Saccharomyces cerevisiae* y *Kluyveromyces marxianus*. A continuación se describen algunas de sus características.

1.3.1. *Saccharomyces cerevisiae*

La levadura *S. cerevisiae* (Figura 2) generalmente clasificada como segura, GRAS por sus siglas en inglés (generally regarded as safe), utilizando como fuente de carbono glucosa presenta un tiempo de duplicación de 90 min, mientras que con una fuente de carbono no fermentable este se amplía de 3.5 a 4 h. *S. cerevisiae* puede metabolizar azúcares como glucosa, fructosa, sacarosa, galactosa y manosa. Esta levadura es generalmente de forma elipsoide con un diámetro de 5 a 10 µm, y un genoma de 15 Mpb, el cual se considera altamente compacto. *S. cerevisiae* crece tanto en condiciones aerobias como anaerobias, con un óptimo pH ácido de 4 a 5, y temperatura de crecimiento de 30 a 35°C. Esta levadura puede producir hasta 18% (v/v) de etanol, por lo que es la levadura más utilizada para los procesos de fermentación (Walker y White, 2005; Walker, 2009; Zhao y Bai, 2009).

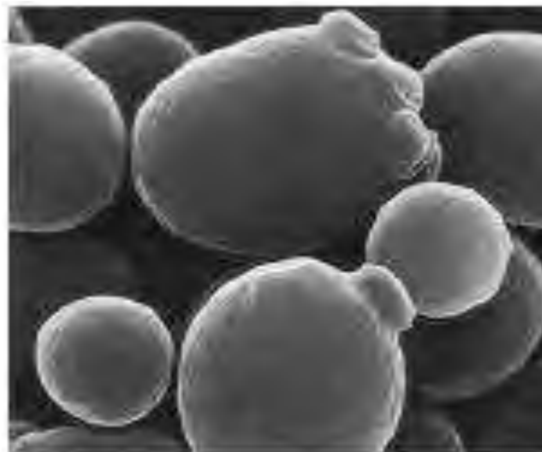

Figura 2. Morfología celular de *Saccharomyces cerevisiae* (1000X). Figura tomada de Kurtzman y Fell, 1997.

1.3.2. *Kluyveromyces marxianus*

La levadura *K. marxianus* también clasificada como GRAS, puede ser ovoide, elipsoide o cilíndrica (Figura 3). Esta levadura presenta un metabolismo Crabtree-negativo, respiro-fermentativo. *K. marxianus* es capaz de fermentar xilosa, xilitol, celobiosa, lactosa y arabinosa, tanto en medio líquido como sólido, lo cual contrasta con *S. cerevisiae* (Stambuk *et al.,* 2003; Nonklang *et al.,* 2008; Wilkins *et al.,* 2008; Signori *et al.,* 2014). *K. marxianus* es la célula eucariota con la mayor velocidad de crecimiento con un tiempo de duplicación de 52 min (Groeneveld *et al.,* 2009), produce etanol eficiente a 45°C, y sobrevivencia hasta 52°C. Además, presenta tolerancia osmótica (22% w/v) y a etanol (10% v/v), con una alta producción de compuestos volátiles (López-Álvarez *et al*., 2012). Recientemente se ha descrito la secuencia de genomas de algunas de sus cepas con un tamaño de 11 Mpb (De Souza *et al.,* 2012; Jeong *et al.,* 2012; Suzuki *et al.,* 2014; Inokuma *et al.,* 2015; Lertwattanasakul *et al.,* 2015).

Figura 3. Morfología celular de *Kluyveromyces marxianus* (1000X). Figura tomada de Kurtzman y Fell, 1997.

1.4. Aplicaciones de levaduras

Las levaduras son consideradas como los microorganismos con más usos dentro de la biotecnología (Johnson, 2013), por lo que a continuación se describen algunas de sus principales aplicaciones.

1.4.1. Levaduras como modelo biológico de células eucariotas

Las levaduras presentan características celulares similares a las células eucariotas de organismos superiores. Lo anterior, además de su rápido crecimiento y utilidad para análisis bioquímicos, genéticos y moleculares, permite que las levaduras sean un modelo de estudio de biología celular de células eucariotas. Las células de *S. cerevisiae* durante la fase estacionaria, son utilizadas para el estudio de los daños ocurridos durante periodos de estrés oxidativo y envejecimiento, ya que durante esta fase las células de *S. cerevisiae* se asemejan a las células de los organismos multicelulares, en aspectos como producción de energía, acumulación de daños celulares y mecanismos de defensa antioxidante (Smith y Snyder, 2006).

1.4.2. Levaduras para la obtención de metabolitos en procesos industriales

Las levaduras son usadas en los procesos industriales para la obtención de diversos productos como los que se enlistan en la tabla 2.

Tabla 2. Productos generados por las levaduras en los procesos industriales

Producto	Ejemplos
Bebidas alcohólicas	Tequila, mezcal, cerveza, vino, sidra, sake, whisky, vodka, coñac, entre otras
Alimentos	Pan, factores de crecimiento y pigmentos
Compuestos químicos	Alcoholes como metanol, etanol, butanol, así como ácidos orgánicos
Enzimas	Invertasa, inulasa, pectinasa, lactasa y lipasa
Proteínas recombinantes	Insulina, vacuna de hepatitis B, anticuerpos, y albúmina de suero humano

Eijk y Johannes, 1995; Wang *et al*., 2001; Gatto y Torriani, 2004

La producción de etanol mediante la fermentación alcohólica es el principal uso industrial de las levaduras. En este proceso los azúcares son convertidos principalmente a etanol y CO_2, con la formación de otros compuestos orgánicos.

1.4.3. Compuestos orgánicos producidos durante la fermentación alcohólica

La vía metabólica principal para la síntesis de compuestos orgánicos durante la fermentación alcohólica es la vía "Ehrlich" (Hazelwood *et al.*, 2008). En la figura 4 se observan los intermediarios en la biosíntesis de los principales compuestos orgánicos generados en la fermentación alcohólica.

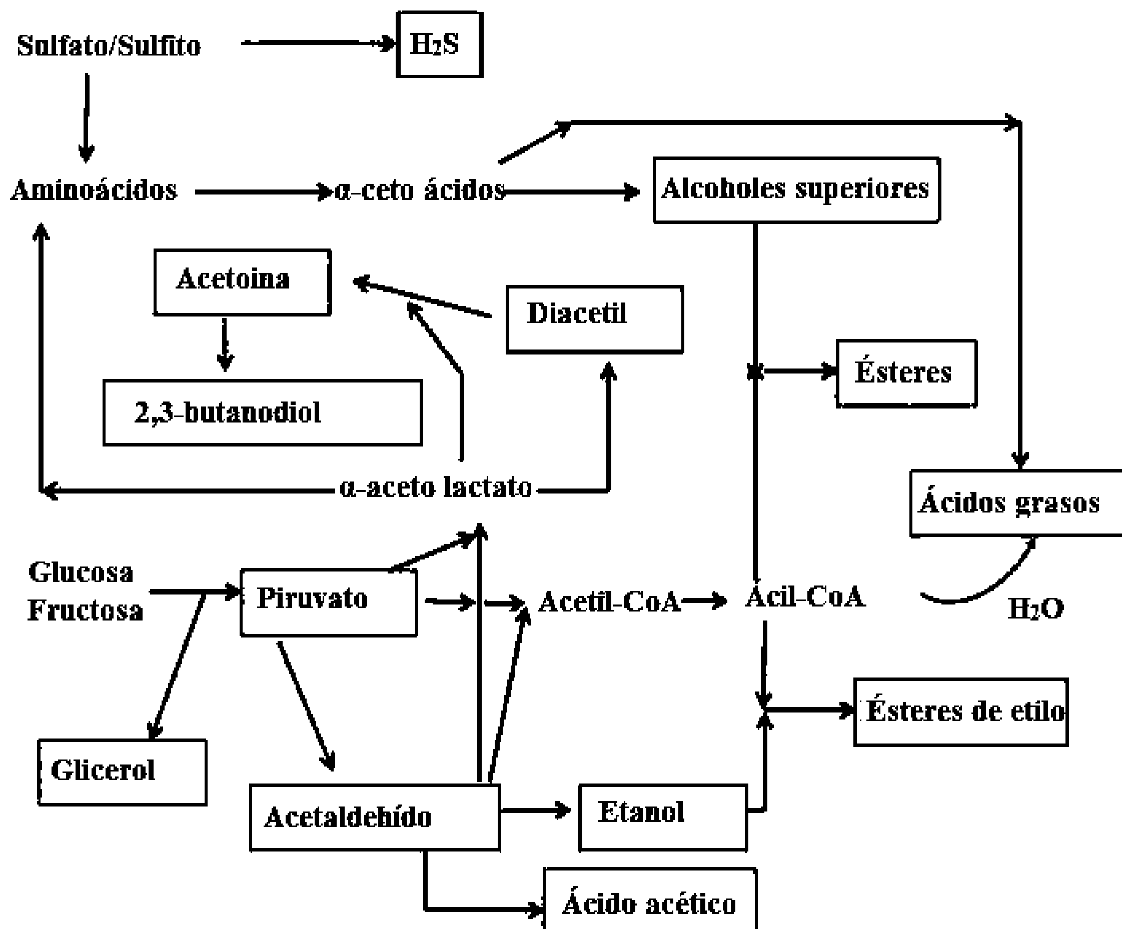

Figura 4. Compuestos orgánicos producidos por las levaduras. Figura modificada de Styger *et al.*, 2011.

Algunas de las características de estos compuestos es que son metabolitos secundarios, que pueden ser tóxicos o inhibidores del crecimiento de las levaduras, y que son generalmente obtenidos en concentraciones menores a 100 mg/L (Vandamme y Soetaert, 2002). Estos compuestos son importantes en la industria de las bebidas alcohólicas, ya que contribuyen en la percepción del sabor por los consumidores, además de ser intermediarios de reacciones químicas con potencial uso en la industria alimentaria, cosmética y farmacéutica (Carlquist *et al.*, 2014). En fermentaciones para la producción de bebidas alcohólicas estos compuestos son ampliamente evaluados; sin embargo, en fermentaciones de residuos lignocelulósicos han sido poco estudiados, aún cuando estos compuestos pueden ser tóxicos para las levaduras, ampliando el tiempo necesario para que se lleve a cabo la fermentación o deteniéndola completamente (Urit *et al.*, 2013; Morrissey *et al.*, 2015). Los principales compuestos orgánicos generados durante la fermentación alcohólica se describen a continuación.

1.4.3.1. Ácidos orgánicos

El principal ácido producido en la fermentación alcohólica es el acético. Este ácido es generado en las levaduras como intermediario en la conversión de piruvato a acetil-CoA, a través de una serie de reacciones catalizadas por la piruvato descarboxilasa, acetaldehído deshidrogenasa y acetil-CoA sintetasa. La producción de succinato y acetato restablece el NADH a partir del NAD^+. La concentración producida de este compuesto en la fermentación es influenciada principalmente por el género de la levadura y la temperatura de la fermentación (Ravasio *et al.*, 2014).

1.4.3.2. Ésteres

Los ésteres constituyen uno de los grupos más importantes de compuestos orgánicos volátiles producidos por las levaduras, ya que son responsables de la formación de los aromas deseados en las bebidas fermentadas. Estos compuestos se pueden dividir en dos grupos: 1) ésteres de acetato y 2) etil-ésteres. El acetato de etilo es el éster más abundante, el cual incrementa su concentración a bajas temperaturas. Los géneros *Kluyveromyces* y *Saccharomyces,* producen 2-feniletil-acetato (2-PE) a través de la vía del shikimato. Las

vías de síntesis de este compuesto también son usadas para el crecimiento celular, por lo cual la producción de 2-PE es muy baja (Saerens *et al*., 2010).

1.4.3.3. Etanol

El etanol es el compuesto orgánico producido en mayor concentración durante la fermentación alcohólica. Teóricamente 100 g de glucosa producen 51 g de etanol y 48 g de CO_2; sin embargo, en la práctica el rendimiento es menor al 100%, ya que la glucosa se utiliza también para el crecimiento de las células, así como para la producción de otros compuestos. En México, la producción de etanol proviene principalmente de la fermentación de los azúcares de la caña de azúcar, produciendo 15.3 millones de litros del año 2011 al 2012 (Comité Nacional para el Desarrollo sustentable de la Caña de Azúcar, 2015). Aproximadamente el 91% de la producción de etanol es mediante la fermentación, mientras que el 9% restante es obtenido de manera sintética (Wheeler *et al*., 1991). Aún cuando el 68% de la demanda de producción de etanol es para combustibles, el etanol que se produce se usa principalmente en las industrias de bebidas, farmacia, química y cosmética. En la industria de los combustibles el etanol puede mezclarse con gasolina, proporcionando ventajas como aumento del cambio de volumen de gases, mejora de la combustión y reducción de la emisión de CO_2 (Prasad *et al*., 2007; Talebnia *et al*., 2010). El etanol obtenido mediante la fermentación de la biomasa (bioetanol), se considera una alternativa con potencial para sustituir a los combustibles fósiles (Baeyens *et al*., 2015).

1.5. Bioetanol

La producción de bioetanol representa el proceso fermentativo industrial de mayor crecimiento mundialmente. El bioetanol es el etanol producido a partir de la fermentación de biomasa (Figura 5), el cual presenta características como baja temperatura de combustión, alto número de octano y menor pérdida por evaporación en comparación con la gasolina (Balat *et al.,* 2009; Ashfaq *et al.,* 2015; Awasthi *et al.,* 2015). Este tipo de etanol es uno de los biocombustibles más utilizados, con una producción mundial de alrededor de 25 billones de toneladas anuales (Dias *et al.,* 2013; Baeyens *et al.,* 2015). El bioetanol puede ser mezclado como oxigenante con gasolina (gasohol) en una proporción del 10%,

20% o 22%, sin necesidad de hacer modificaciones mecánicas en los vehículos de combustión (Bullen *et al.,* 2006; Balat *et al.,* 2011). Una limitante para el uso del bioetanol es su costo de producción, ya que como oxigenante de gasolina, Petróleos Mexicanos (PEMEX) produce metil ter-butil éter (MTBE), por lo que el precio del bioetanol debe ser comparable con el precio del MTBE (1.15 USD/ por galon) (Favela, 2006). Mientras que el bioetanol de primera generación se obtiene fermentando azúcares de insumos alimenticios, el bioetanol de segunda generación es obtenido utilizando biomasa lignocelulósica. Ya que la biomasa lignocelulósica se encuentra presente en residuos agroindustriales (Zabaniotou *et al*., 2008), su utilización puede reducir los costos de producción del bioetanol, por lo que la investigación en la producción de bioetanol se ha dirigido hacia la utilización de esta biomasa como fuente de carbono.

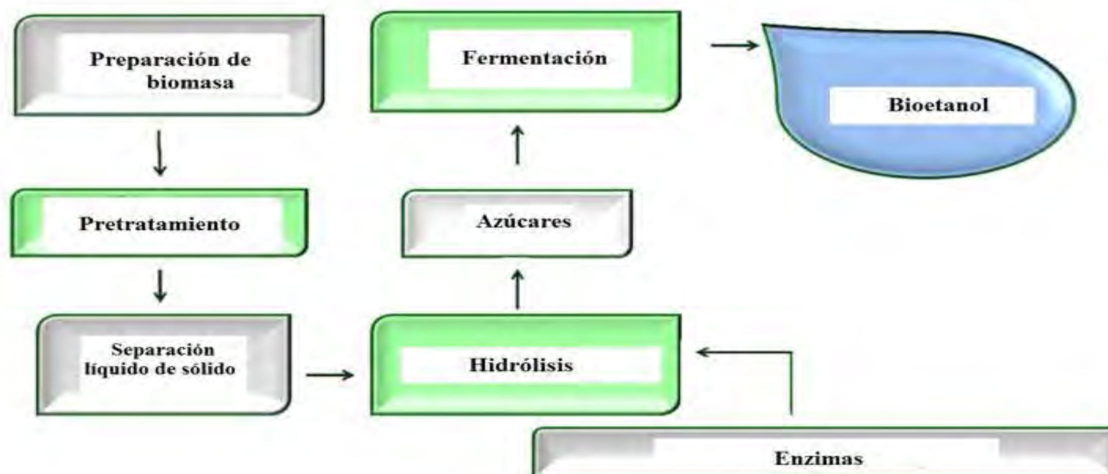

Figura 5. Proceso general para la producción del bioetanol. Figura modificada de Liguori *et al*., 2016.

1.5.1. Biomasa lignocelulósica para la producción de bioetanol de segunda generación

La biomasa lignocelulósica es renovable ya que continuamente es regenerada mediante la reducción del CO_2 en la fotosíntesis, por ello es considerada la fuente más prometedora para la producción de energía, alimentos y compuestos químicos (Sun y Cheng, 2002; Kim y Dale, 2004). Los materiales lignocelulósicos están formados principalmente por celulosa, hemicelulosa y lignina, los cuales en conjunto representan hasta un 75% en peso seco

(Lynd *et al.*, 2008). Las proporciones de los componentes de la biomasa lignocelulósica varían de acuerdo al material lignocelulósico (Mussatto *et al.*, 2010). El bagazo de agave es uno de los materiales lignocelulósicos que presenta una de las mayores proporciones de celulosa (Tabla 3), y uno de los más producidos en México.

Tabla 3. Composición de residuos agroindustriales lignocelulósicos

Fuente de biomasa lignocelulósica	Celulosa (%)	Hemicelulosa (%)	Lignina (%)
Bagazo de agave	42.0	20.0	15.0
Bagazo de caña	40.0	27.0	10.0
Tallo de maíz	35.0	14.4	21.5

Mussatto y Teixeira, 2010

1.5.1.1. Bagazo de agave

El bagazo de agave es obtenido principalmente del agave azul (*Agave tequilana Weber variedad azul*) el cual es utilizado para la producción de tequila. Las piñas del agave azul sin hojas son cocinadas en hornos, hidrolizando la inulina presente en las piñas a monosacáridos. Después del cocimiento las piñas son molidas y presionadas para extraer su jugo. El residuo de este proceso es el bagazo de agave (Figura 6). Durante el año 2015 el consejo regulador del tequila de México estimó que el consumo de agave para la producción de tequila fue de 859 000 toneladas, generando aproximadamente 343 600 toneladas de bagazo de agave (Consejo Regulador del Tequila, 2016).

Figura 6. Generación de bagazo de agave. Proceso explicado en el texto. Figuras modificadas de Iñiguez *et al*., 2001.

1.6. Procesos de fermentación para la producción de bioetanol

Los principales procesos de fermentación para la producción de bioetanol a partir de biomasa lignocelulósica, como la del bagazo de agave, son la hidrólisis y fermentación separada (HFSe), hidrólisis y fermentación simultánea (HFSi) y el bioproceso consolidado (BC) (Liguori *et al*., 2016). De estos procesos, el más utilizado es el de HFSe; sin embargo, el proceso de HFSi es considerado el método con mayor potencial para aumentar los rendimientos en la producción de bioetanol. Los procesos de HFSe y HFSi se describen a continuación.

1.6.1. Hidrólisis y fermentación separada

El proceso de HFSe consiste en el pretratamiento de la biomasa lignocelulósica, seguido de una hidrólisis enzimática y posteriormente la fermentación y destilación del etanol (Figura 7). En este proceso se puede generar una optimización de la función catalítica de las enzimas y la levadura utilizada, ya que las dos faces del proceso necesitan diferentes condiciones de temperatura y pH. Las desventajas de este proceso es que los azúcares producidos en la hidrólisis pueden ocasionar inhibición enzimática, que es necesario el empleo de dos biorreactores, y que los tiempos para concluir la fermentación son largos, comparados con otros procesos de fermentación (Choudhary *et al*., 2016).

1.6.2. Hidrólisis y fermentación simultánea

La HFSi (Figura 8) reduce la inversión de energía, así como los costos de operación e inhibición enzimática por los azúcares liberados de la biomasa lignocelulósica, ya que el proceso de fermentación se realiza en el mismo bioreactor de la hidrólisis enzimática (Olsson *et al*., 2006; Sánchez y Cardona, 2008); sin embargo, la temperatura óptima para las levaduras etanologénicas es de 30 a 37°C, mientras que para las enzimas hidrolíticas (celulasas) es de 45 a 50°C (Krishna *et al*., 2001; Abdel-Banat *et al*., 2010). Estas temperaturas pueden generar una disminución de la capacidad fermentativa de las levaduras, resultado del daño generado en su fisiología (Barak *et al*., 2014).

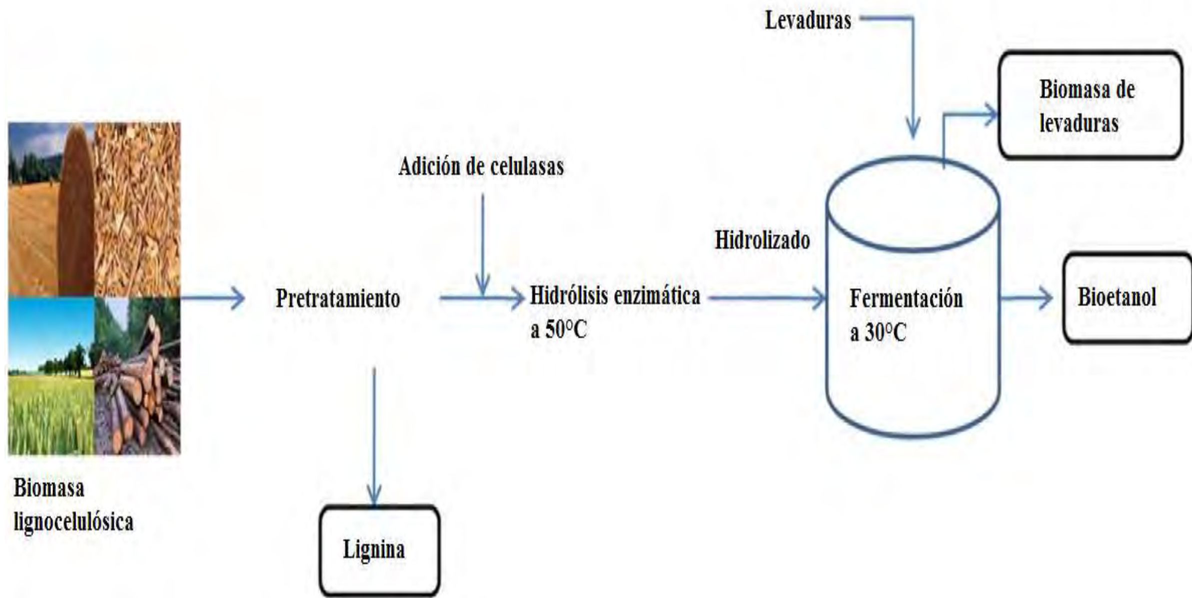

Figura 7. Proceso de hidrólisis y fermentación separada (HFSe) explicado anteriormente. Figura modificada de Choudhary *et al*., 2016.

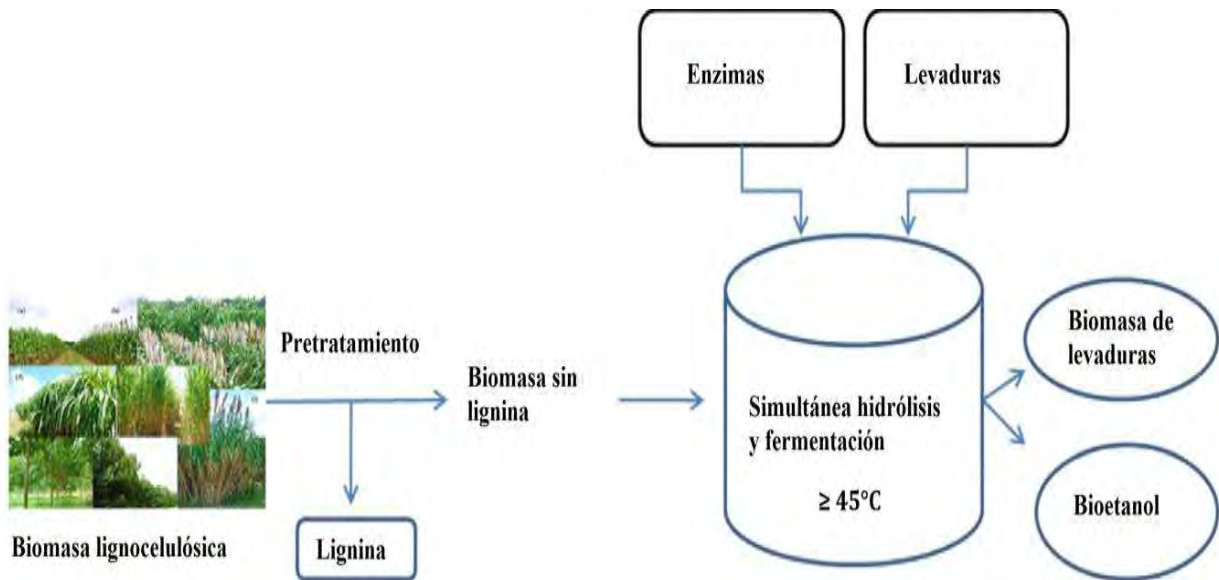

Figura 8. Proceso de hidrólisis y fermentación simultánea (HFSi) explicado anteriormete. Figura modificada de Choudhary *et al*., 2016.

1.7. Efecto de temperaturas >37°C en levaduras

Las levaduras mesófilas presentan una temperatura óptima de crecimiento de 25 a 35°C (Choudhary *et al.*, 2016). Estas levaduras reducen la eficiencia de la fermentación alcohólica a altas temperaturas (>37°C), debido a los cambios fisiológicos que experimentan (Tabla 4).

Tabla 4. Cambios fisiológicos en las levaduras mesófilas por la exposición a altas temperaturas

Incremento de la producción de trehalosa y de la actividad de la superóxido dismutasa
Incremento de la fluidez y reducción de la permeabilidad de las membranas
Reducción de la proporción de los ácidos grasos insaturados de la membrana plasmática
Inhibición de la síntesis de proteínas e inducción de la síntesis de proteínas de choque térmico
Incremento de la frecuencia de mutaciones e ineficiente reparación del ADN dañado
Reducción de la respiración mitocondrial y generación de levaduras "petite"
Estimulación de la actividad de la ATPasa y disminución del pH intracelular
Inducción de estrés oxidativo

Walker, 1998; Araque *et al.,* 2008; Choudhary *et al.,* 2016

Independientemente de un metabolismo oxidativo o fermentativo, en un aumento de temperatura como ocurre en el proceso de HFSi, las levaduras presentan un incremento de especies reactivas de oxígeno (ERO) e inducción de la expresión de genes de respuesta antioxidante. El aumento de las ERO puede generar estrés oxidativo, el cual es uno de los factores claves en la reducción de la viabilidad de las levaduras, y por ende en la disminución de la eficiencia de la fermentación alcohólica (Sugiyama *et al.*, 2000; Davidson y Schiestl, 2001).

1.7.1. Estrés oxidativo en las levaduras generado por altas temperaturas

El estrés oxidativo se produce cuando los sistemas antioxidantes son incapaces de reducir los niveles de las ERO o el daño generado por éstas. Las moléculas de oxígeno al sufrir una reducción parcial forman las ERO que pueden oxidar lípidos y proteínas, así como dañar el ADN. Existe una relación entre la concentración de ERO y la tolerancia a altas temperaturas por levaduras, ya que a concentraciones basales las ERO actúan como segundos mensajeros, modulando cascadas de señalización a través de la activación de factores de transcripción como Hsf1, Yap1, Msn2 y Msn4, que controlan la expresión de genes (Kim *et al.*, 2013). Un ejemplo es cuando el H_2O_2 generado por el incremento de la temperatura oxida tiorredoxinas citoplasmáticas induciendo la acumulación del factor de transcripción Msn2 en el núcleo celular, donde interactúa con el sitio de unión del elemento de respuesta a estrés (STRE) activando la expresión de genes (Figura 9).

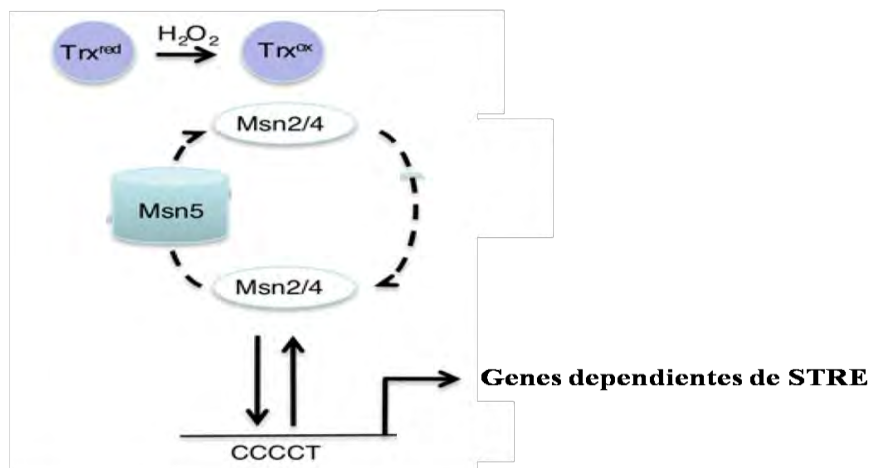

Figura 9. Regulación del factor Msn2/4 en respuesta al estrés oxidativo (Morano *et al.*, 2011). Tiorredoxina reducida (Trx^{red}) u oxidada (Trx^{ox}). Figura explicada en el texto.

1.7.1.1. Producción de especies reactivas de oxígeno en levaduras a altas temperaturas

En las levaduras la principal fuente de generación de ERO es la cadena transportadora de electrones mitocondrial (CTEm). El aumento de la temperatura induce la producción del anión superóxido ($O_2^{\bullet-}$), el cual es dismutado a H_2O_2 por la superóxido dismutasa. El H_2O_2

al reaccionar con fierro (Fe^{2+}) produce el radical hidroxilo ($OH^{\cdot}$), radical que es más reactivo que las anteriores ERO (Figura 10). Este aumento en la concentración de ERO puede dañar los complejos de la CTEm o ácidos grasos de las membranas, aumentando la concentración de biomoléculas oxidadas dentro de la célula (Cortés-Rojo *et al.*, 2009).

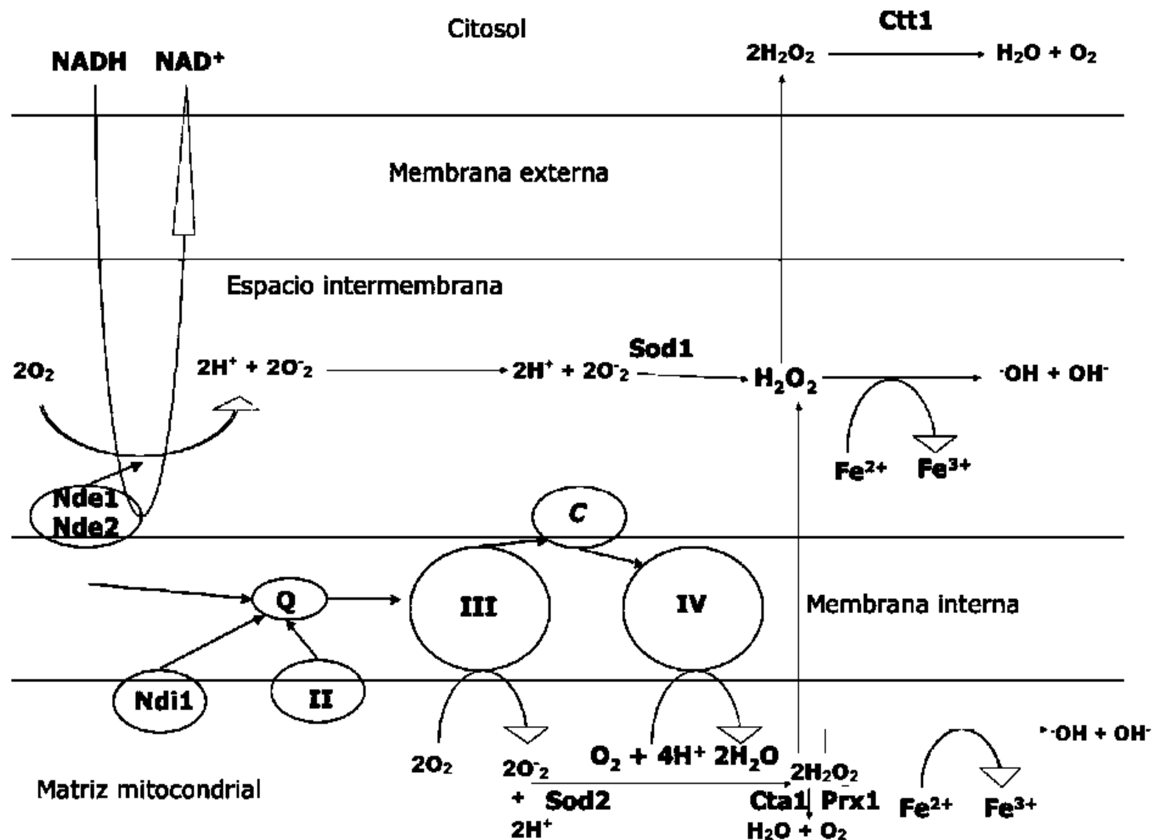

Figura 10. Producción de ERO en las levaduras por daño en la CTEm (Macedo-Márquez, 2012). Figura explicada en el texto.

El mantenimiento del estado redox celular por los sistemas antioxidantes es crucial para la viabilidad celular y la formación de productos de la fermentación con levaduras, ya que el estado redox afecta procesos biológicos como regulación del ciclo celular y transducción de señales, así como la estructura, función y activación de proteínas.

1.8. Sistemas antioxidantes en las levaduras

Los sistemas antioxidantes con los que cuentan las levaduras se presentan en la tabla 5.

Tabla 5. Sistemas antioxidantes en las levaduras

	Sistemas enzimáticos	Referencias
Catalasa	Descompone el H_2O_2 mediante una reacción de dismutación. Se conocen la catalasa A y T	Martínez-Pastor *et al.,* 1996
Superóxido dismutasa	Conversión del anión superóxido a H_2O_2. Existen dos tipos: Sod1p (Cu/Zn) en el citosol y Sod2p (Mn) en la matriz mitocondrial	Culotta *et al.,* 2006
	Sistemas no enzimáticos	
Ubiquinona	Actúa como radical lipídico con capacidad de unirse a radicales libres	Moradas-Ferreira *et al*., 1996
Glutatión reducido	Es el tripéptido γ-L-glutamil-L-cistinilglicina. El residuo de cisteína confiere las propiedades redox	Folch-Mallol *et al*., 2004
Metalotioneínas	Son proteínas ricas en cisteína	Morano *et al.,* 2012

Mientras que las levaduras con una sobreexpresión de los genes que codifican las enzimas antioxidantes catalasa y superóxido dismutasa, presentan un incremento en la tolerancia a la temperatura, la sobreexpresión del activador de respuesta antioxidante Msn2, redujo los niveles de ERO contribuyendo al mantenimiento del estado redox celular (Davidson *et al*., 1996; Davidson *et al*., 2001).

1.9. El estado redox en las levaduras

La celula cuenta con diferentes compuestos que funcionan como indicadores del estado redox, algunos de estos compuestos son GSSG/GSH, $NADP^+$/NADPH, y NAD^+/NADH. Los nucleótidos de piridina NAD^+/NADH y $NADP^+$/NADPH tienen una función central en el estado redox celular, puesto que el cofactor NADH es usado ampliamente en el catabolismo y el NADPH es requerido en las reacciones anabólicas. Mientras que en concentraciones elevadas de glucosa el balance redox es conservado principalmente por la formación de glicerol y etanol (Rigoulet *et al.*, 2004), en condiciones limitadas de oxígeno los equivalentes reducidos NADH productos de la glucólisis, deben transferir sus electrones al acetaldehído para regenerar el NAD^+ consumido por la glucólisis. El balance celular NADH/NAD^+ también se ha relacionado con las variaciones en la producción de ácidos y alcoholes, ya que el NADH generado durante la producción de ácido acético es oxidado durante la producción de los alcoholes (Figura 11).

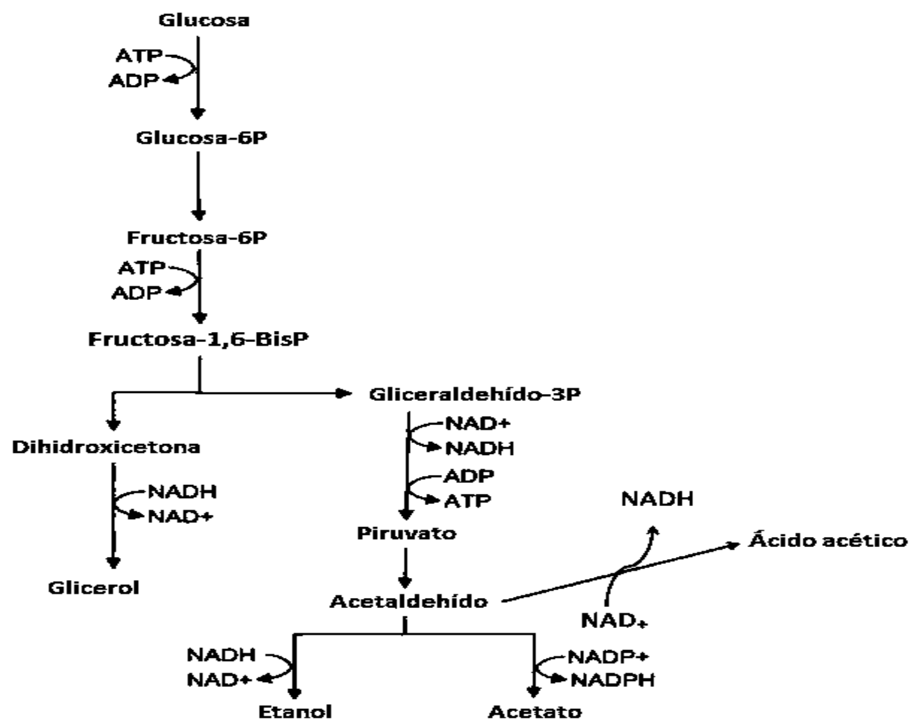

Figura 11. Estado redox celular en la producción de compuestos orgánicos por las levaduras (Almeida *et al.*, 2011). Figura explicada en el texto.

En presencia de oxígeno el NADH es oxidado en la CTEm, por lo que existe una relación entre el mantenimiento del estado redox y la función mitocondrial. Las células con una actividad mitocondrial deficiente incrementan sus niveles de glutatión oxidado (GSSG), disminuyendo el estado reducido de la célula, lo cual es importante ya que el incremento en las concentraciones de ésteres y ácidos grasos de cadena media, son producidos en un estado redox reducido (Kieronczyk *et al*., 2006; Martin *et al*., 2011).

La información antes descrita se ha obtenido a partir del estudio de levaduras mesófilas; sin embargo, existen levaduras termotolerantes de las cuales se conoce poco respecto a estos aspectos.

1.10. Levaduras termotolerantes

Las levaduras termotolerantes son aquellas que presentan un óptimo crecimiento a temperaturas ≥40°C (Koedrith *et al.,* 2008). Estas levaduras además de que pueden generar una mayor producción de bioetanol a temperaturas superiores que las levaduras mesófilas (Murata *et al*., 2015), presentan ventajas en el proceso de HFSi, tales como la reducción de contaminación del proceso y costos de enfriamiento, mayor viabilidad celular y actividad metabólica, así como mayor velocidad de fermentación. A mayores temperaturas también se reduce la viscosidad del medio de fermentación, por lo que la energía para mantener la agitación del proceso se reduce (Roehr, 2001; Choudhary *et al*., 2016). La termotolerancia en las levaduras puede ser generada mediante su exposición a altas temperaturas por tiempos prolongados. Shui *et al*. (2015) realizaron un análisis proteómico de una levadura con termotolerancia inducida, observando una disminución de las enzimas involucradas en la biosíntesis del glicerol, ciclo de los ácidos tricarboxilicos, vía de las pentosas y componentes de la CTEm, como citocromo b, c, y subunidades de la ATP sintasa. La termotolerancia inducida en las levaduras se atribuye a la activación de diferentes respuestas celulares, tales como la síntesis de proteínas de choque térmico y trehalosa, lo cual detiene el ciclo celular en la fase G1 y reduce la actividad de la enzima adenosina-3,5-monofosfato cinasa (cAMP-PK) asociada con el flujo glucolítico. Por lo anterior, se considera que la termotolerancia inducida no es una técnica viable en la selección de

levaduras termotolerantes para la producción de etanol (Choudhary *et al*., 2016). Por lo que una opción es la identificación de levaduras con termotolerancia intrínseca.

1.10.1. Levaduras termotolerantes con mayor potencial para la producción de bioetanol

La mayor proporción de levaduras termotolerantes aisladas ha sido a partir de cepas de *S. cerevisiae* y *K. marxianus* (Spindler *et al.,* 1989; Gough *et al.,* 1996; Barron *et al.,* 1997; Boyle *et al.,* 1997; Lark *et al.,* 1997; Bollók *et al.,* 2000). Se considera que *K. marxianus* es la especie más prometedora para la producción de bioetanol, así como de otros metabolitos como el 2-fenil etanol (Lyubomirov *et al.,* 2013) debido a su capacidad para metabolizar glucosa, xilosa, manosa y galactosa a temperaturas >40°C (Fonseca *et al*., 2008; Suryawati *et al*., 2008; West y Kennedy, 2014; Choudhary *et al.,* 2016).

2. Antecedentes

En nuestro grupo de trabajo, Arellano-Plaza *et al*. (2013) evaluaron la resistencia al estrés oxidativo de las levaduras *K. marxianus* SLP1 y OFF1 y *S. cerevisiae* MC4, previamente aisladas de fermentaciones espontáneas de zonas mezcaleras de los estados de San Luis Potosí, Guerrero y Oaxaca, respectivamente (Gschaedler *et al*., 2004). Estas levaduras presentaron resistencia al estrés oxidativo inducido por menadiona y H_2O_2. Esta resistencia fue atribuida a las condiciones cambiantes y estresantes del medio ambiente de donde se aislaron las levaduras. Por otro lado, Flores *et al*. (2013) utilizando las mismas levaduras *K. marxianus* SLP1 y OFF1, así como otras 13 cepas del mismo género, evaluaron la producción de bioetanol usando fructanos presentes en el jugo de agave como fuente de carbono. Las levaduras SLP1 y OFF1 presentaron una eficiencia en la producción de etanol del 96 y 97%, respectivamente.

3. Justificación

En la generación de bioetanol el proceso de HFSi es una de las mejores opciones; sin embargo, en este proceso se utilizan temperaturas ≥40°C que inducen la producción de ERO, modificando el estado redox celular y disminuyendo la viabilidad y productividad de las levaduras mesófilas. Debido a que la CTEm es la principal fuente generadora de ERO y que los sistemas antioxidantes pueden evitar la generación del estrés oxidativo, contribuyendo al mantenimiento del estado redox celular, es importante estudiar la actividad de estos parámetros en levaduras con potencial uso para la producción de etanol a temperaturas ≥40°C, como son las levaduras termotolerantes.

4. Hipótesis

Las levaduras termotolerantes ante un incremento de temperatura, aumentan la actividad de sus sistemas antioxidantes y disminuyen la función de su cadena transportadora de electrones mitocondrial, manteniendo su estado redox y capacidad fermentativa.

5. Objetivos

5.1. Objetivo general

Analizar el efecto de un incremento de temperatura en el estado redox y capacidad fermentativa de una levadura termotolerante.

5.2. Objetivos particulares

1.- Analizar la actividad de los sistemas antioxidantes de una levadura termotolerante.

2.- Estudiar el efecto de un incremento de temperatura en la cadena transportadora de electrones y estado redox en la levadura termotolerante.

3.- Conocer la capacidad fermentativa de la levadura termotolerante.

6. Estrategia experimental general

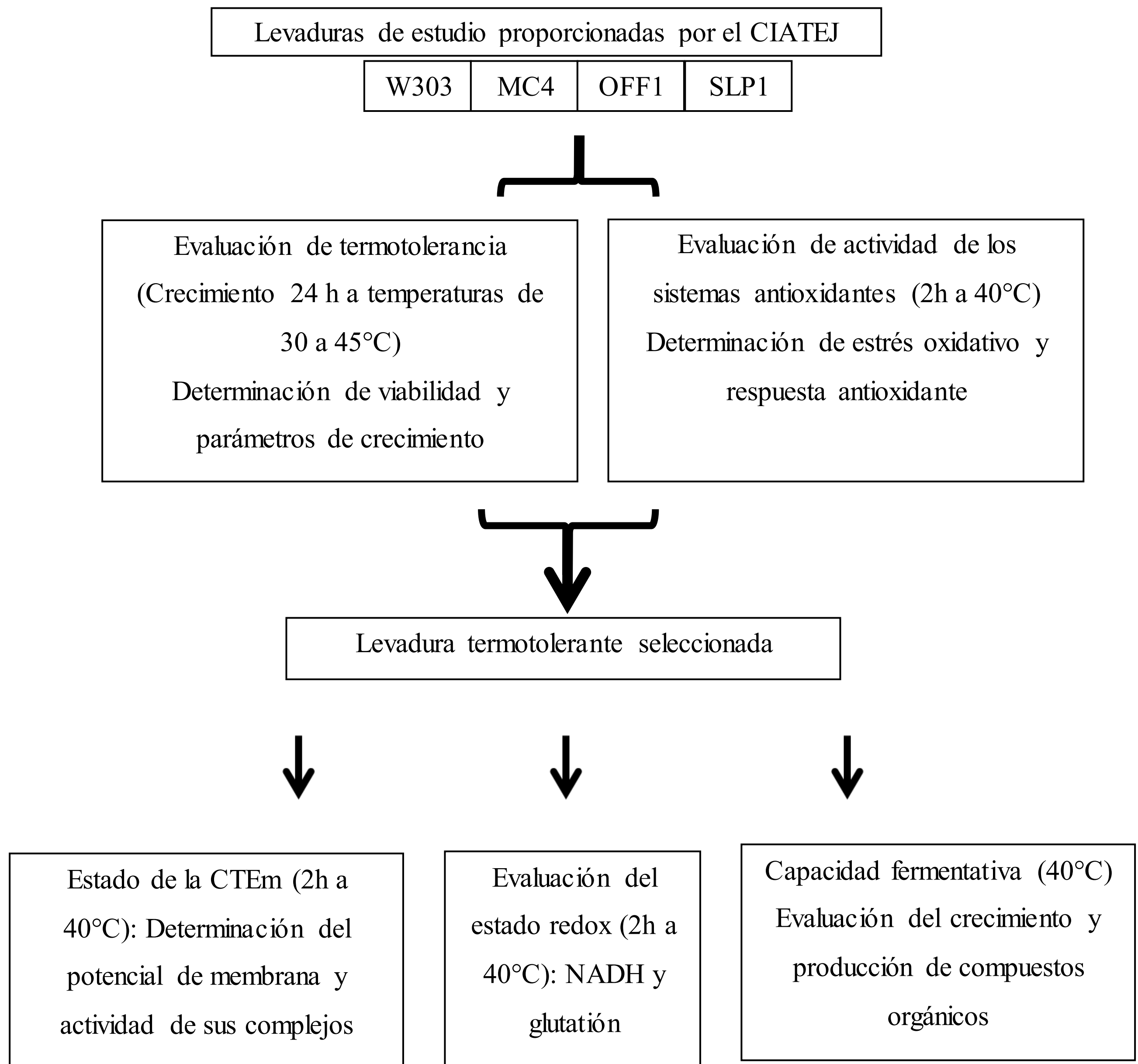

Figura 12. Estrategia experimental general

Levaduras: *S. cerevisiae*: W303 y MC4; *K. marxianus*: OFF1 y SLP1

CIATEJ: Centro de Investigación en Asistencia y Diseño del Estado de Jalisco

CTEm: Cadena transportadora de electrones mitocondrial

NADH: Nicotinamida adenina dinucleótido

7. Resultados

Los resultados generados se presentan en 6 capítulos.

Capítulo1:

Oxidative stress and antioxidant response in a thermotolerant yeast.

Este capítulo ha sido publicado como:

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Oxidative stress and antioxidant response in a thermotolerant yeast

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ABSTRACT

Stress tolerance is a key attribute that must be considered when using yeast cells for industrial applications. High temperature is one factor that can cause stress in yeast. High environmental temperature in particular may exert a natural selection pressure to evolve yeasts into thermotolerant strains. In the present study, three yeasts (*Saccharomyces cerevisiae*, MC4, and *Kluyveromyces marxianus*, OFF1 and SLP1) isolated from hot environments were exposed to increased temperatures and were then compared with a laboratory yeast strain. Their resistance to high temperature, oxidative stress, and antioxidant response were evaluated, along with the fatty acid composition of their cell membranes. The SLP1 strain showed a higher specific growth rate, biomass yield, and biomass volumetric productivity while also showing lower duplication time, reactive oxygen species (ROS) production, and lipid peroxidation. In addition, the SLP1 strain demonstrated more catalase activity after temperature was increased, and this strain also showed membranes enriched in saturated fatty acids. It is concluded that the SLP1 yeast strain is a thermotolerant yeast with less oxidative stress and a greater antioxidant response. Therefore, this strain could be used for fermentation at high temperatures.

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Introduction

Yeasts are eukaryotic unicellular fungi that are widely distributed in natural environments. They are used in many industrial processes, such as the production of alcoholic beverages, biomass, and metabolic products. Currently, the majority of yeast biotechnology applications are with the species *Saccharomyces cerevisiae*. However, the limited stress resistance of *S. cerevisiae* has led to an increased focus on the potential of the

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non-*Saccharomyces* spp. yeasts, such as the *Pichia* spp., *Debaryomyces* spp., and *Kluyveromyces* spp. The *Kluyveromyces* spp. is usually considered to be a thermotolerant yeast, with important commercial relevance to high temperature fermentation during ethanol production. High temperature is one of the most important factors affecting microbial activity, microbial growth rate and biomass yield.[1] The capacity to tolerate high temperatures is related to oxidative stress and the antioxidant response. High temperature increases oxidative stress and overexpression of antioxidant enzyme genes in *S. cerevisiae*.[2,3] However, this effect has not been studied in thermotolerant yeasts. Even less is known about yeasts isolated from damaged environments, where several types of stress affect communities, such as osmotic, temperature, pH, and oxidative stress. These conditions could apply a natural selection pressure on yeast to evolve into thermotolerant strains. Arellano-Plaza et al.[4] reported that the *Saccharomyces cerevisiae* (MC4) and *Kluyveromyces marxianus* (OFF1 and SLP1) yeast strains were able to resist oxidative stress for a long period of time compared with W303-1A (*S. cerevisiae* reference strain). The MC4, OFF1, and SLP1 yeast strains were isolated from spontaneous mezcal fermentation carried out at handcraft mezcal distilleries in Oaxaca, San Luis Potosí, and Guerrero (all Mexican states). Mezcal production occurs between October and May, when the environmental temperature decreases to −5 °C in winter, and can increase to 45 °C in spring. Little information is currently available regarding oxidative stress induction and the antioxidant response to increased temperatures in thermotolerant yeasts. The aim of this work was to select a thermotolerant yeast (yeasts that were isolated in regions of Mexico with high-temperature environments) and study its oxidative stress and antioxidant response.

Materials and methods

Yeast strains

Yeast strains were obtained from the culture collection of the CIATEJ (*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México*)[5] and from the ATCC (American Type Culture Collection, Rockville, MD, USA). Two *K. marxianus* yeast strains, SLP1 and OFF1, were isolated at handcraft mezcal distilleries in the Mexican State of San Luis Potosi and Guerrero, respectively, and one *S. cerevisiae*, MC4, was isolated at an Oaxaca state distillery. The ATCC yeast, W303-1A, was also used for comparison.

Growth conditions

Yeast growth was studied using YPD media containing 1% yeast extract, 2% peptone, and 2% glucose as the carbon source. Cells were grown at 30 °C and shaken at 180 rpm for 24 h.

Cell viability

Yeast strains were grown as mentioned above. The yeasts were then collected and inoculated 1×10^7 cell/mL in fresh YPD medium. The culture was incubated for 24 h under the same conditions. The cells were quantified, and yeast suspensions were cultured on YPD agar plates and incubated for 24 h at temperatures from 30 to 45 °C. After 24 h, the colony forming units (CFU) were determined.[6] The CFU at room temperature were taken as 100%.

Effect of temperature on specific growth rate, biomass yield, volumetric productivity, and duplication time.

The specific growth rate (μ) was calculated by cell growth, measured by optical density (OD) of the cell suspensions, and estimated using the Lineweaver–Burk equation. For biomass determination yeast cells in broth were harvested, washed with distilled water, and dried in an oven at 80 °C until reaching a constant weight. The biomass (Dw) was reported in dry cell mass (g/L). The volumetric productivity of biomass (Qp) was calculated by dividing the biomass yield by the corresponding culture time. The duplication time (Td) was calculated with the equation ln(2)/K, where K is the rate constant.

Temperature increase

An increase in temperature was applied as described by Kim et al.,[7] with some modifications. Yeast strains were grown in a 10 mL YPD (2%) medium for 24 h at 30 °C and 180 rpm. A concentration of pre-cultured cells of $A_{600} = 0.03$ was transferred to fresh YPD (2%) media, incubated at 30 °C and 180 rpm until the stationary phase. Then, the cultured cells were incubated for 2 h at 40 °C.

Intracellular reactive oxygen species

Intracellular reactive oxygen species in yeast cultures were determined using fluorescence assays with 2′,7′-dichlorodihydrofluorescein diacetate.[8] The cultured cells obtained from a pre-culture were incubated for 2 h at 40 °C, cells were counted and diluted in YPD medium to a final concentration of 0.5×10^7 cells. A 5-mM stock solution of dichlorofluorescein diacetate was added to each sample and incubated in the dark for 15 min at 30 °C. Afterwards, cells were harvested by centrifugation, washed, and re-suspended in 1930 μL 50 mM Tris/HCl buffer (pH 7.5). The cells were permeabilized by adding chloroform and SDS and vortexing at high speed for 20 s. The tubes were left to settle for 10 min. Cells were pelleted by centrifugation, and the supernatant fluorescence was measured using a Shimadzu RF-5301 fluorometer (excitation, 502; emission, 521 λ).

Lipid peroxidation

The extent of lipid peroxidation was determined through the thiobarbituric acid (TBA) assay.[9] Temperature increase was generated as previously described. Cells were re-suspended in Tris–HCl buffer, pH 7.4, containing 10% trichloroacetic acid, and glass beads were added. The samples were broken by agitation on a vortex. After centrifugation at 300 g, supernatants were mixed with EDTA 0.1 M and 1% (w/v) thiobarbituric acid in NaOH 0.05 M. The reaction mixture was heated for 15 min in a boiling water bath, and then centrifuged. The absorbance at 532 nm was measured in a Perkin–Elmer spectrophotometer.

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The results were calculated using the molar extinction coefficient for malondialdehyde (1.56×10^5 M^{-1} cm^{-1}).

Lipid extraction and fatty acid analysis

Lipids were extracted from yeast homogenates using the Bligh and Dyer method.[10] For fatty acid analysis, methyl esters were generated by the BF_3-methanol assay of Morrison and Smith.[11] After extraction with n-hexane, samples of methyl esters were separated by gas chromatography (Perkin Elmer Clarus 500) on a 30 m × 0.25 mm Omega wax capillary column using high-purity N_2 as the carrier gas. Fatty acids were identified and quantified by comparison of their retention time with those of authentic standards. The unsaturated/saturated index was determined. The values under 1 indicate that the percentage of saturated fatty acids is higher than the percentage of unsaturated fatty acids.

Determination of catalase activity

Catalase activity was quantified by the oxygen production rate using H_2O_2 (50 mM) as substrate; cells were placed in MES-TEA buffer (pH 6.0) in a sealed glass chamber with constant stirring, the oxygen generation rate was quantified with a Clark-type oxygen electrode coupled to a biological oxygen monitor.[12]

Statistical analysis

All values are means of three separate experiments. Differences in means were analyzed using Student's *t* test with independent measures. Differences were considered statistically significant with $p < 0.05$, $p < 0.01$, and $p < 0.001$ as ^{b}, ^{c}, and ^{d}, respectively.

Results

The *K. marxianus* SLP1 strain displayed a normal viability pattern at a high temperature (40 °C). Fig. 1 shows cell viability results at different temperatures (30, 35, 40, and 45 °C). Cell survival decreased in a temperature-dependent manner from 30 to 40 °C in the W303-1A, MC4, and OFF1 strains. The *K. marxianus* SLP1 strain showed viability at 40 °C, identical to that observed at 30 °C (Fig. 1). These findings indicate that the SLP1 strain was more tolerant than the other strains at 40 °C. At 45 °C the four yeast strains showed less than 50% viability.

The *K. marxianus* SLP1 strain showed the best kinetic pattern at temperatures from 35 to 45 °C. Temperature markedly affected the kinetic parameters. In the W303-1A and MC4 strains, specific growth rate (μ), biomass (Dw), volumetric productivity of biomass (Qp), and the duplication time (Td) evaluated were reduced when the temperature was raised, showing the best results at 30 °C. The SLP1 strain had the best yield in the kinetic parameters evaluated between 35 and 40 °C (Table 1).

Temperature increase elicits oxidative stress through ROS generation. There was direct molecular evidence of *in vivo* intracellular oxidation using the oxidant-sensitive 2′,7′-dichlorofluorescein diacetate probe. Measurements of oxidation development were carried out after increased

Table 1 – Effect of temperature on specific growth rate, biomass yield, volumetric productivity, and duplication time.

T (°C)	W303-1A				MC4				OFF1				SLP1			
	μ	DW	Qp	Td	μ	DW	Qp	Td	μ	DW	Qp	Td	μ	DW	Qp	Td
30	2.46 ± 0.1	1.54 ± 0.8	0.19	2.72	6.20 ± 0.2	1.75 ± 0.8	0.21	3.77	9.51 ± 0.1	2.16 ± 0.6	0.27	2.73	8.83 ± 0.1	5.47 ± 1.3	0.68	3.49
35	0.15 ± 0.1	2.34 ± 1.9	0.29	3.77	0.30 ± 0.2	1.52 ± 1.9	0.19	4.0	3.22 ± 0.1	4.06 ± 1.7	0.50	0.85	9.73 ± 0.1	4.48 ± 1.7	0.56	1.31
40	0.01 ± 0.1	0.21 ± 0.0	0.02	7.08	0.05 ± 0.0	0.37 ± 0.2	0.04	5.22	1.20 ± 0.3	0.95 ± 0.6	0.11	1.64	3.09 ± 0.1	3.87 ± 1.2	0.48	0.99
45	0.00 ± 0.1	0.21 ± 0.0	0.02	9.73	0.00 ± 0.0	0.36 ± 0.0	0.04	27.65	1.05 ± 0.1	0.40 ± 0.1	0.05	1.60	3.36 ± 0.1	0.93 ± 0.6	0.11	1.53

μ (h^{-1}); DW (gL^{-1}); Qx (gL^{-1} h^{-1}); (Td). Each value represents the mean ± SEM (Standard error of the mean) ($n = 3$).

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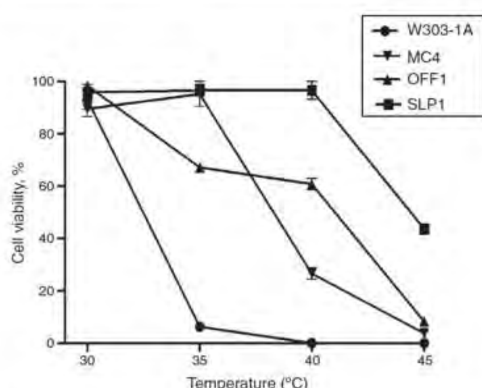

Fig. 1 – Cell viability. Dilutions of yeast suspensions were cultured on YPD agar plates and incubated 24 h at different temperatures. Other conditions are as described in '*Materials and methods*' section. Each value represents the mean ± SEM (Standard Error of the Mean) ($n = 3$).

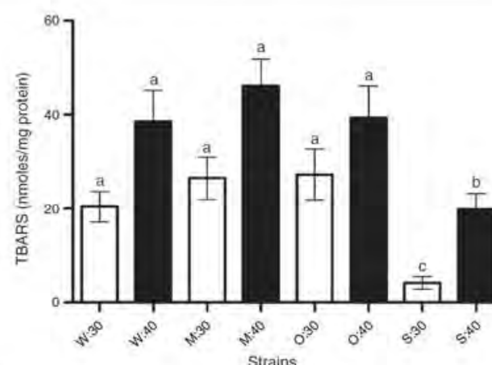

Fig. 3 – Lipid peroxidation in yeast strains (W: W303-1A, M: MC4, O: OFF1, and S: SLP1) under normal temperature condition 30 (30 °C) and increased temperature 40 (40 °C for 2 h). Other conditions are as described in '*Materials and methods*' section. Each value represents the mean ± SEM (Standard Error of the Mean) ($n = 3$). Significant differences ($^{b}p < 0.05$ and $^{c}p < 0.01$), with respect to W303-1A.

temperature from 30 to 40 °C for 2 h. At room temperature, only the SLP1 strain showed low ROS production compared with the reference strain W303-1A (Fig. 2). We could see a direct correlation between the increase in temperature and ROS generation. The increase in ROS by temperature was more significant in the reference strain, W303-1A, while the isolated strains showed less ROS generated by the temperature increase. The SLP1 strain had the lowest ROS production under 40 °C. To examine whether the increased ROS in the strains was enough to damage cellular components, such as lipids, lipoperoxidation was determined.

There was a lower degree of lipid peroxidation in the SLP1 strain following increase in temperature. The method based on the reaction of thiobarbituric acid with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA), was used to determine lipid peroxidation generated by the exposure to increased temperature. No differences in the degree of lipid peroxidation were observed in the MC4 and OFF1 strains at temperatures of 30 or 40 °C, with respect to the reference yeast strain (W303-1A). On the other hand, the SLP1 strain showed less lipid peroxidation at normal temperatures and increased temperature (Fig. 3). The degree of lipid peroxidation was directly correlated with the lower ROS production observed (Figs. 3 and 2, respectively). The lower degree of lipid peroxidation in the SLP1 strain could be due to the types of lipids present in their cell membrane or could be a result of more antioxidant system activity; both possibilities were evaluated.

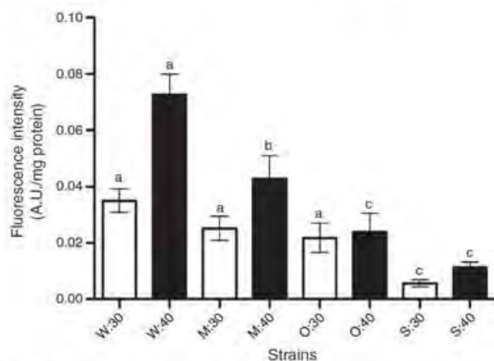

Fig. 2 – Reactive oxygen species (ROS) in yeast strains (W: W303-1A, M: MC4, O: OFF1, and S: SLP1) under normal temperature condition 30 (30 °C) and increased temperature 40 (40 °C for 2 h). Each value represents the mean and SEM (Standard Error of the Mean) values and is indicated as bars ($n = 3$). Other conditions are as described in '*Materials and methods*' section. Significant differences ($^{b}p < 0.05$, $^{c}p < 0.01$), with respect to W303-1A are indicated.

The most temperature-tolerant yeast strain (SLP1) showed the highest concentration of saturated fatty acids under increased temperature. The fatty acid membranes were identified by comparison with authentic standards. The fatty acid composition percentage of phospholipids is presented in Table 2. The yeast strains showed a saturated fatty acid composition of less than 50%. The percentage of the sum of saturated fatty acids was 45, 48, and 47% for the W303-1A, MC4, and OFF1 strains, respectively (Table 2). The saturated fatty acids found in the yeast strains were myristic, palmitic, stearic, and arachidic. The strain with more viability at higher temperature (SLP1) showed the highest concentration of arachidic fatty acid (Table 2). When the temperature was increased, all of the unsaturated fatty acids increased their concentration. Palmitoleic acid was the unsaturated fatty acid with the highest concentration at 40 °C in the W303-1A and OFF1 strains, whereas in the MC4 and SLP1 yeast, the oleic acid had the highest concentration.

When cells were exposed to 40 °C, the unsaturated/saturated index changed for the W303-1A and MC4

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Table 2 – Fatty acid composition of lipid membranes of the yeast strains, under normal temperature condition, and increased temperature.

	Myristic (C14:0)		Palmitic (C16:0)		Palmitoleic (C16:1)		Stearic (C18:0)		Oleic (C18:1)		Linoleic (C18:2)		Linolenic (C18:3)		Arachidic (C20:0)	
	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
W	2 ± 0.3^a	2 ± 0.4^a	39 ± 0.4^a	31 ± 2.0^c	18 ± 1.3^a	29 ± 0.2^d	15 ± 0.4^a	10 ± 0.5^a	20 ± 0.5^a	23 ± 1.8^a	2 ± 0.4^a	2 ± 0.0^a	1 ± 1.2^a	N.D.	N.D.	N.D.
M	7 ± 4.0^a	1 ± 0.7^a	35 ± 15.0^a	26 ± 5.0^a	3 ± 6.0^a	1 ± 1.0^a	19 ± 12.0^a	20 ± 2.0^a	11 ± 10.0^a	37 ± 2.0^b	7 ± 6.0^a	13 ± 2.0^a	6 ± 4.0^a	2 ± 2.0^a	13 ± 5.0^a	1 ± 0.8^b
O	4 ± 0.7^a	4 ± 0.6^a	32 ± 2.0^a	33 ± 4.0^a	23 ± 0.7^a	24 ± 1.0^a	9 ± 0.5^a	9 ± 0.5^a	20 ± 1.0^a	20 ± 0.9^a	10 ± 0.7^a	8 ± 6.0^a	2 ± 0.2^a	2 ± 0.4^a	N.D.	N.D.
S	5 ± 0.5^a	4 ± 0.2^a	32 ± 9.0^a	28 ± 1.0^a	13 ± 3.0^a	14 ± 0.3^a	14 ± 0.9^a	14 ± 0.6^a	17 ± 12.0^a	24 ± 0.2^a	3 ± 0.7^a	3 ± 0.0^a	N.D.	0 ± 0.2^a	16 ± 2.0^a	13 ± 0.5^b

W303-1A (W), MC4 (M), OFF1 (O), and SLP1 (S); (30 °C (3); (40 °C for 2 h (4). No determinate (N.D.). Other conditions are as described in '*Materials and Methods*' section. Each value represents the mean ± SEM (Standard error of the mean) ($n = 3$). Significant differences ([a]not significant, $^b p < 0.05$, $^c p < 0.01$ and $^d p < 0.001$), with respect to every strain without increased temperature are indicated.

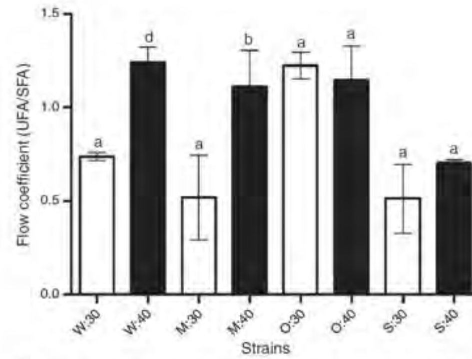

Fig. 4 – Flow coefficient from the lipid membranes of the yeast strains (W: W303-1A, M: MC4, O: OFF1, and S: SLP1) under normal temperature condition 30 (30 °C) and increased temperature 40 (40 °C for 2 h). UFA, unsaturated fatty acids; SFA, saturated fatty acids. Other conditions are as described in '*Materials and methods*' section. Each value represents the mean ± SEM (Standard Error of the Mean) ($n = 3$). Significant differences ($^b p < 0.05$ and $^d p < 0.001$), with respect to every strain without increased temperature, are indicated.

strains that showed less resistance to heat. The coefficient of the OFF1 and SLP1 yeast strains remained unaffected. The most heat-tolerant yeast (SLP1) showed a fluidity coefficient without changes under 1, meaning that its saturated fatty acid concentration was higher than the unsaturated fatty acid concentration under both conditions (30 and 40 °C) (Fig. 4).

Catalase activity had a direct correlation with lower ROS production and lipid peroxidation produced in the yeast SLP1 by increased temperature. The pattern of catalase activity was directly correlated with the lower level of damage caused in the yeasts by increased temperature. Under normal conditions (30 °C) the OFF1 and SLP1 yeasts showed more catalase activity than the reference yeast, W303-1A ($p < 0.01$). Table 3 shows a significant ($p < 0.001$) difference in catalase activity between

Table 3 – Catalase activity in the yeast strains under normal temperature condition and the increased temperature condition.

	Catalase activity (natm O_2/min mg protein)	
	30	40
W	1511 ± 208^a	1778 ± 347^a
M	1778 ± 178^a	3200 ± 422^b
O	2933 ± 400^c	4533 ± 1108^b
S	4444 ± 627^c	$12{,}622 \pm 1808^d$

(W, W303-1A; M, MC4; O, OFF1; S, SLP1); 30 (30 °C); 40 (40 °C for 2 h). Other conditions are as described in '*Materials and Methods*' section. Each value represents the mean ± SEM (Standard error of the mean) ($n = 3$). Significant differences ([a]not significant, $^b p < 0.05$, $^c p < 0.01$ and $^d p < 0.001$), with respect to W303-1A are indicated.

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strains exposed and not exposed to increased temperature, particularly in the SLP1 strain.

Discussion

Thermotolerance is a desirable characteristic for yeast in industrial applications,[13] and high-temperature environments could exert a natural selection pressure, evolving yeasts into thermotolerant strains. The effect of high temperature on the viability and kinetic growth parameters of wild yeast isolated from mezcal processes was evaluated and compared with a lab yeast strain (W303-1A). This yeast was used as a control because the stress resistance of this strain is well characterized. Nevertheless, yeast strains with a higher stress resistance, such as the *K. marxianus* species, may be more useful in terms of their industrial applications. The two *K. marxianus* strains (OFF1 and SLP1) were able to grow at 40 °C, whereas the *S. cerevisiae* MC4 and W303-1A strains showed low viability (<40%). Specific growth rate (μ) and biomass yield (DW) are physiological features of major importance for a "cell factory" organism to reach high volumetric productivity. The highest viability and DW at 40 °C by the SLP1 strain showed the resistance and ability of this yeast to convert substrate into biomass at a high temperature (40 °C) for mesophilic yeast. The SLP1 yeast showed a lower duplication time (Table 1), indicating it has a better physiological state. The μ and DW decreased in all of the strains when the temperature was raised (45 °C), suggesting that cell reproduction at this temperature generates metabolic cost, affecting the biomass yield. This study showed that the *K. marxianus* SLP1 strain has 100% viability and the best performance in terms of growth rate, biomass yield, volumetric productivity, and duplication time at 40 °C. These results indicate that the *K. marxianus* SLP1 is a thermotolerant yeast. Arellano-Plaza et al.[4] reported that MC4 showed the higher viability when the same yeast strains were exposed to oxidative stress. These results suggest that stress resistance differs in the same yeast strain depending on the stressor. The SLP1 yeast strain has shown that it has the ability to carry out simultaneous saccharification and fermentation of *Agave tequilana* fructans[14] at 30 °C. This quality, along with its thermotolerance, could be used as a principal advantage in simultaneous saccharification-fermentation at a high temperature, improving the process.

There is increasing evidence pointing toward oxygen-derived free radicals as one of the causes for thermal stress-associated damage to microorganisms. High temperature produces ROS,[2] and this causes damage to proteins, lipids, and nucleic acids and thereby compromises cell viability. In contrast, overexpression of antioxidant enzyme genes increases thermotolerance in *S. cerevisiae*. The intracellular oxidant level measured by the oxidation of 2′,7′-dichlorofluorescein was found to be increased following an increase in temperature, a result that correlates with findings reported by Davidson and Schiestl.[2] Fig. 2 shows the correlation between lower ROS production and resistance to increased temperature. Our results support the possibility that oxidative stress plays a major role in the lethal effect of heat on yeast strains. An electron leakage from the respiratory chain in the mitochondria, the major production site for superoxide, could initiate the cascade of ROS production.[15] It has also been reported that reduction in respiratory activity produced by the deletion of one of the respiratory enzymes strongly increases the thermotolerance of *S. cerevisiae*.[3] Thus, further studies analyzing the state of SLP1 yeast mitochondria and the electron transport chain are important. One of the best-described effects of oxidative stress-generated ROS on cells is the oxidation of membrane lipids. Membrane lipids participate in the interaction of proteins with the cell barrier.[16] Lipid peroxidation is a free-radical-mediated chain of reactions that, once initiated, results in an oxidative deterioration of polyunsaturated lipids. In general, cells subjected to increased temperature (40 °C for 2 h) showed an increase in the level of thiobarbituric acid reactive substances (TBARS) (Fig. 3). Cell survival is inversely correlated with TBARS levels. Thus, the yeast with less viability (Fig. 1) showed a higher TBARS level (Fig. 3), whereas the SLP1 yeast strain that had higher viability showed less lipid peroxidation at 40 °C. The susceptibility of lipids to oxidation is determined by the lipid class composition and degree of unsaturation.[17] Under heat shock, the extent of cellular damage has been correlated positively with increasing unsaturation of the phospholipid fatty acyl component.[18] Sudden changes in environmental conditions cause alterations in the organization and dynamic structure of membrane lipids[16] and alter the function of many cellular activities. To assess whether the differences observed in yeast lipid peroxidation could be related to the quantitative and qualitative lipid composition of the cell, these were analyzed. The extent of cellular damage correlated positively with the increased polyunsaturation of the phospholipid fatty acyl component (Table 2). In the SLP1 strain, a higher concentration of arachidic (C20:0) fatty acid was observed. Arthur and Watson[19] found that the thermophilic strains had an unusual phospholipid composition, such as that of the SLP1 strain that presented 20% arachidic acid. Our result concurred with the report of Steels et al.[20] They reported that the most stress-resistant yeast had membranes enriched in saturated fatty acids. Membrane fluidity is determined by the ratio of saturated *versus* unsaturated fatty acids,[21] and maintenance of membrane fluidity is essential for optimal functioning of membrane proteins.[22] Changes in the composition of the cell lipid fraction can influence the activity of many membrane-associated proteins and transporters, thus potentially leading to growth arrest and cell death.[15] Fig. 4 show that W303-1A and MC4 increased their fluidity coefficient by the gain of unsaturated fatty acids. The fluidity coefficient of the OFF1 and SLP1 strains remained unchanged, being lower in the SLP1 strain, owing to its higher saturated fatty acid percentage. A significant alteration of the membrane lipids such as in the W303-1A and MC4 yeasts strains, could affect the function of proteins, preventing their adaptation and reducing their viability. Kim et al.,[7] reported oxidative stress as a result of heat-shock in the *Saccharomyces* spp. KNU5377 strain, and this stress induced antioxidant enzyme stimulation. Catalase is one of the central components of the detoxification pathways that prevent the formation of the highly reactive hydroxyl radical by catalyzing the decomposition of H_2O_2 into water and oxygen through two electron transfers.[23] Our results support those of Davidson et al.[2] who reported that yeast mutants deficient in the antioxidant enzyme, catalase, are sensitive to heat exposure at a

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temperature of 50 °C, whereas overexpression of catalase provides protection from lethal heat-shock. The strains evaluated in this work showed higher catalase activity with respect to the reference strain, with and without the increased temperature (Table 3). The significant increase in the activity of this enzyme in the SLP1 yeast could reduce H_2O_2, preventing the production of another more toxic ROS, such as OH·, and therefore also preventing, together with the higher index of saturated fatty acids, lipid peroxidation in this strain. This effect could explain why this strain has more viability under increased temperature. This work is the first to compare the physiological state of wild yeast isolated from mezcal processes when exposed to increased temperature. The *K. marxianus* SLP1 is a thermotolerant yeast strain, with less oxidative stress, higher antioxidant response, and a higher percentage of saturated fatty acids in its cell membranes. This thermotolerant SLP1 yeast could be used for fermentation at high temperatures in ethanol production.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Capítulo 2:

Fatty acid addition improves the thermotolerance of a *Kluyveromyces marxianus* strain

Este capítulo ha sido sometido para su revisión como:

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Fatty acid addition improves the thermotolerance of a Kluyveromyces marxianus strain

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Abstract:	Membrane fatty acid composition has an important role in yeast stress resistance, particularly in temperature tolerance. The majority of the studies investigating temperature and membrane fatty acids use Saccharomyces cerevisiae without considering other yeasts, such as Kluyveromyces marxianus, which has physiological differences and industrial advantages with respect to S. cerevisiae. One of the primary traits of K. marxianus is its thermotolerance. The effect of fatty acid (FA) addition (oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and araquidic acid (C20:0)) on the thermotolerance of the K. marxianus SLP1 strain was evaluated. The SLP1 yeast exhibited temperature tolerance of up to 50°C, while at 55°C, its viability was reduced significantly, probably due to an increase in the generation of reactive oxygen species (ROS). Externally added FAs were incorporated in the yeast membrane, increasing their proportion to approximately 70%, thereby changing the membrane fluid coefficient. SLP1 cells supplemented with polyunsaturated FAs decreased their thermotolerance and increased the degree of lipoperoxidation, while arachidic acid addition exhibited a tendency to increase SLP1 thermotolerance. Based on these results, we suggest that membrane lipid engineering has the potential to increase temperature tolerance of thermotolerant K. marxianus yeasts.	
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Title: Fatty acid addition improves the thermotolerance of a *Kluyveromyces marxianus* strain

Running title: Fatty acids in yeast thermotolerance

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Abstract

Membrane fatty acid composition has an important role in yeast stress resistance, particularly in temperature tolerance. The majority of the studies investigating temperature and membrane fatty acids use *Saccharomyces cerevisiae* without considering other yeasts, such as *Kluyveromyces marxianus*, which has physiological differences and industrial advantages with respect to *S. cerevisiae*. One of the primary traits of *K. marxianus* is its thermotolerance. The effect of fatty acid (FA) addition (oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and araquidic acid (C20:0)) on the thermotolerance of the *K. marxianus* SLP1 strain was evaluated. The SLP1 yeast exhibited temperature tolerance of up to 50°C, while at 55°C, its viability was reduced significantly, probably due to an increase in the generation of reactive oxygen species (ROS). Externally added FAs were incorporated in the yeast membrane. increasing their proportion to approximately 70%, thereby changing the membrane fluid coefficient. SLP1 cells supplemented with polyunsaturated FAs decreased their thermotolerance and increased the degree of lipoperoxidation, while arachidic acid addition exhibited a tendency to increase SLP1 thermotolerance. Based on these results, we suggest that membrane lipid engineering has the potential to increase temperature tolerance of thermotolerant *K. marxianus* yeasts.

Keywords: Membranes; Fatty acids; Thermotolerance; *Kluyveromyces marxianus*

Introduction

Exposure to high temperatures can cause cell damage in yeasts in different ways and are among the most serious causes of membrane disruption (Singer and Linquist 1998). The cytoplasmic yeast membrane is composed of glycol sphingolipids, ergosterol, proteins, and phospholipids (Daum et al. 1998). The phospholipids are based on a glycerol-3-phosphate backbone with two esterified fatty acid (FA) chains. The predominant FAs in *Saccharomyces cerevisiae* yeasts are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1); however, their proportions can vary according to the yeast and environmental conditions (Papanikolaou and Aggelis 2003; Pedroso et al. 2009). *S. cerevisiae* are capable of incorporating FAs from their environment (Córtes-Rojo et al. 2009; Landolfo et al. 2010; Duan et al. 2015);

this incorporation is affected by the synthesis, metabolism, and degradation of the FAs (McDonough and Roth 2004). Because *S. cerevisiae* is an experimental model organism and a major manufacturer of biotechnological products (Pichler et al. 2001; Sorger and Daum 2003; Johnson, 2013), most studies about yeast membrane lipids have used *S. cerevisiae,* without considering other yeasts such as *Kluyveromyces marxianus. K. marxianus* has physiological differences with respect to *S. cerevisiae* (Lane et al. 2011), and are emerging as yeasts with industrial potential for diverse applications including broad-spectrum sugar utilization, rapid growth rate, and ethanol production at high temperatures (Lane and Morrisey, 2010; Choudhary et al. 2016). A major impediment to using *K. marxianus* as a cell factory has been the limited fundamental knowledge of its physiology (Morrisey et al. 2015). Altering the composition of the cytoplasmic membrane yeasts has recently been proposed to increase yeast thermotolerance (Choudhary et al. 2016). In this work we studied the effect of adding the FAs oleic, linoleic, linolenic and araquidic, on the thermotolerance of the *K. marxianus* SLP1 strain. The viability and reactive oxygen species (ROS) production was evaluated by exposing the yeast cells to a 55°C treatment. After growing the SLP1 strain with external FAs, its thermotolerance was reevaluated, and we observed a direct relation between unsaturated FAs proportions and lipoperoxidation. The SLP1 yeast cells with the higher saturated fatty acids proportions in their membranes showed a tendency to reduce their temperature damage and to increase their thermotolerance.

Materials and Methods

Yeast strain

The thermotolerant *K. marxianus* SLP1 yeast strain (Mejía-Barajas et al. 2016b) was isolated from a mezcal distillery in the Mexican State of San Luis Potosi and it was obtained from the culture collection of the CIATEJ (*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México*) (Gschaedler et al. 2001).

Growth conditions and fatty acid addition

Yeasts were grown using YPD media (1% yeast extract, 2% peptone, and 2% glucose). Pre-inoculated cells were grown at 30°C and shaken at 180 rpm for 24 h. The cells were then collected and inoculated at 1 x 10^7 cell/mL in fresh YPD medium with 1 mM of fatty acids (FAs) solubilized with 5% (v/v) Igepal CA-630 (Cortés-Rojo et al. 2009). After FA addition the cells were incubated at rest for 24 h at 30°C, with oxygen limitation according to Steels et al. (1994). The external FAs added were oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) and araquidic acid (C20:0).

Cell viability after temperature exposure

Temperature exposure was applied as described in Kim et al. (2006) with several modifications. Yeast cells were grown at 30°C as mentioned before, with or without external FA addition. The cells were collected by centrifugation and diluted at 1 g/mL, then incubated for 2 h from 40 to 55°C. The viability of the cells was evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method according to Hodgson et al. (1994). 50 µl of MTT (5 mg/mL) were added to the cells that were temperature exposed, and the mix was incubated at 30°C for 2 h. Then 500 µl of 2-propanol in 0.04 M HCl were added and the mix was vortexed and centrifuged at 11 600 x g for 2 min. The absorbance of the supernatants was measured at 570 nm against a sample control.

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species in yeast cells were evaluated after temperature exposure using the fluorescence assays with 2′, 7′-dichlorodihydrofluorescein diacetate (Wang and Joseph 1999). After cells were exposed to the temperature treatment, 5-mM stock solution of dichlorofluorescein diacetate was added and the mix was incubated in the dark for 15 min at 30°C. Afterwards, cells were harvested by centrifugation, washed, and re-suspended in 1,930 µL of 50 mM Tris/HCl buffer (pH 7.5). The cells were permeabilized with chloroform and SDS, pelleted by centrifugation, and the supernatant fluorescence was measured using a Shimadzu RF-5301 fluorometer (excitation, 502 λ; emission, 521 λ).

Lipid extraction and fatty acid analysis

Lipids were extracted from yeast homogenates according to Bligh and Dyer (1959). For fatty acid analysis, methyl esters were generated by the BF_3-methanol assay of Morrison and Smith (1964). The extraction was done with n-hexane and the samples of methyl esters were separated by gas chromatography (Perkin Elmer Clarus 500) on a 30 m × 0.25 mm Omega wax capillary column using high-purity N_2 as the carrier gas. Fatty acids were identified and quantified by comparison of their retention time with those of authentic standards. The flow coefficient was determined by dividing the unsaturated/saturated fatty acid proportions.

Lipid peroxidation

The extent of lipid peroxidation was determined through the thiobarbituric acid (TBA) assay (Buege and Aust 1978). Temperature exposure was done as described above. Yeasts cells were suspended in Tris-HCl buffer, pH 7.4, containing 10% trichloroacetic acid, then glass beads were added. The samples were broken by agitation using vortexing. After centrifugation at 300 x g, supernatants were mixed with EDTA 0.1 M and 1% (w/v) thiobarbituric acid in NaOH 0.05 M. The reaction mixture was heated for 15 min in a boiling water bath and then centrifuged. Supernatant absorbance was measured at 532 nm in a Perkin-Elmer spectrophotometer. The molar extinction coefficient for malondialdehyde ($1.56 \times 10^5 M^{-1} cm^{-1}$) was used.

Statistical analysis

All values are means of three separate experiments. Differences in means were analyzed using ANOVAs.

Results

Temperature yeast survival

After temperature exposure the *K. marxianus* SLP1 cells without added fatty acids (FAs) showed 100% survival at 50°C with respect to survival at 40°C; at 55°C comparative cell survival was less than 20% (figure 1).

Reactive oxygen species production

Increased temperature can generate overproduction of reactive oxygen species (ROS); therefore, we also evaluated ROS production in the *K. marxianus* SLP1 strain after temperature exposure. When the temperature was increased, a higher ROS generation was observed as compared to the 40°C exposure (figure 1). The highest ROS production was at 55°C (figure 2).

Membrane fatty acids

Unsupplemented FAs cultures of the *K. marxianus* SLP1 strain (control cells) revealed the presence of the FAs myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidic. As the dominant FAs, C16:0 and C16:1 accounted for 27 and 29% of the total FAs, respectively (Table 1). The flow coefficient of the unsupplemented FA yeast was 1.23, indicating a ratio of 55% of unsaturated fatty acids (UFAs) to 45% of saturated fatty acids (SFAs).

Fatty acid supplementation

A change in the FAs profile of the SLP1 cells was generated when the medium was supplemented with external FAs. When the monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) were added, palmitic acid was kept as the second in proportion; but when arachidic saturated fatty acid (SFA) was added, the palmitoleic acid was kept as the second FA in proportion. When the FAs were supplemented the membrane yeast flow coefficient changed, primarily in yeast supplemented with oleic acid, where the flow coefficient was 5.11. In contrast, after addition of the arachidic acid, the flow coefficient was as low as 0.32 (Table 1).

Temperature yeast survival after fatty acid supplementation

Yeasts cells grown in the presence of external FAs were evaluated for survival at temperatures from 40 to 55°C. When the yeast cells grown with external PUFAs were exposed to high temperature, the viability was less than 20% with respect to control cells (cells without FAs addition) at 40°C; viability decreased further when the temperature increased, decreasing to less than 5% at 55°C. Cells grown with MUFAs had decreased survival with respect to control cells but only at 55°C. In contrast, cells with a higher proportion of SFAs

(mainly with arachidic acid added) tended to resist the temperature increase more than unsupplemented control cells, mainly at 40°C (figure 3).

Lipoperoxidation

The increase in the proportion of PUFAs in membrane yeast cells was accompanied by an increase in their lipoperoxidation. Cells supplemented with PUFAs (C18:2 and C18:3) at 40°C had two times more lipoperoxidation compared with control yeast cells (figure 4). Cells grown in the presence of MUFAs or SFAs (C18:1 and C20:0, respectively) showed the same degree of lipoperoxidation with respect to control cells. In the control cells supplemented with MUFA or SFA, the lipoperoxidation degree was conserved even when temperature increased (figure 4).

Discussion

High temperatures affect yeast viability and limit yeast performance in terms of industrial applications (Mejía-Barajas et al. 2016a). While *Saccharomyces cerevisiae* heat sensitivity is related to membrane lipid composition (Steels et al. 1994), thermotolerant *S. cerevisiae* cells can alter their membrane composition to maintain their fluidity at high temperatures (Caspeta et al. 2014). Most studies on the effect of high temperatures on yeast membrane fatty acids (FAs) have been carried out with cells of *S. cerevisiae*; however, recent analysis of membrane fatty acid composition showed that total lipid content varies widely among yeasts, even in the same yeast genus (Arous et al. 2016). Due the possible differences in the type and concentration of FAs among yeasts, and because *K. marxianus* is emerging as competitors to *S. cerevisiae* (Morrissey et al. 2015) due to their biotechnological applications (Fonseca et al. 2008; Lane and Morrissey 2010; Lachance, 2011), safe use in food industry, and knowledge of their genome sequence (Lertwattanasakul et al. 2015, we studied the relation of membrane FAs and yeast thermotolerance using the native thermotolerant *K. marxianus* yeast SLP1 strain (Mejía-Barajas et al. 2016b).

Our results show that the SLP1 strain could maintain viability up to 50°C, while at 55°C viability was reduced significantly (figure 1). One of the main traits of *K. marxianus* strains is their thermotolerance (Lane and

Morrissey 2010); as the SLP1 strain showed the same viability at 50°C as well as at 40°C. With this fact has been identified as thermotolerant previously (Koedrit et al. 2008; Mejía-Barajas et al. 2016b).

Arous et al. (2016) reported that the more common and abundant yeast FAs are palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid, with less proportions than other FAs (C12:0, C14:0, C20:0 and C22:0). In the SLP1 strain the main FAs were C16:1, C16:0 and C18:1, while the lowest proportion was C18:3. When the membrane FAs of the *K. marxianus* yeast CC1 strain were analyzed, linoleic acid was observed as the main FA, while the oleic acid was detected in a lower proportion (Arous et al. 2016). These results show that FA proportions can vary within every yeast strain, including yeasts of the same genus.

When the incorporation of exogenous FAs by a *Schizosaccharomyces pombe* yeast was evaluated, the FAs supplied in the growth medium were preferentially incorporated into the cells (McDonough and Roth 2004). Selective incorporation of exogenous FAs has been proposed as an energy-saving strategy during FAs production and membrane function in stressful environmental conditions (McDonough and Roth 2004). The external FAs incorporated by the *K. marxianus* yeast SLP1 strain were fewer than those reported for *S. cerevisiae* strains. Córtes-Rojo et al. (2009) reported an increase in the proportion of linolenic acid to 90% when the *S. cerevisiae* yeast Foam strain was grown in 1 mM of this FA. In the same conditions, the *K. marxianus* yeast SLP1 strain incorporated linolenic acid at a level of approximately 70%, confirming that external FA incorporation could vary in every yeast strain. Temperature during growth influences external FA incorporation; cells grown at 20°C decreased levels of FA incorporation compared to cells grown at 30°C (McDonough and Roth 2004). In this work the temperature used to grow yeast was 30°C; because SLP1 strain is a thermotolerant yeast, higher temperatures during growth could promote higher FA incorporation.

The UFAs are necessary for yeast adaptation during alcoholic fermentation (You et al. 2003; Rupčić and Jurešić 2010). During alcoholic fermentation, *K. marxianus* cells responded to temperature rise by increasing the levels of SFAs and by decreasing the levels of UFAs (Banat et al. 1998; Le et al. 2013). Steels et al. (1994) reported that *S. cerevisiae* cells with membranes enriched in PUFAs had higher lipoperoxidation than cells enriched with SFAs, and Cortés-Rojo et al. (2009) found that PUFAs are susceptible to ROS oxidation. Considering our results, we suggest that high temperatures induce ROS overproduction (figure 2), generating lipoperoxidation (figure 4) in the yeast cells with enriched PUFAs (Table 1), a fact that decreased cell survival (figure 3).

The arachidic acid previously reported in yeast membranes (Pedroso et al. 2009; Patel et al. 2014) was decreased in *S. cerevisiae* yeasts adapted to H_2O_2 (Pedroso et al. 2009); however, in the current study, this FA promoted a higher survival at 40°C with respect to the SLP1 yeast cells without FA supplementation (figure 3). When the C20:0 FA was incorporated in the SLP1 yeast membrane, the proportions of other UFAs increased, probably to keep their membrane fluidity. A higher degree of unsaturation in cell membranes maintains fluidity and guarantees the activity of membrane-associated enzymes and transporters (Rosa and SaCorreia 1992).

Duan et al. (2015) evaluated the effects of adding UFAs in *S. cerevisiae*. The UFAs improved yeast growth, fermentation activity, and concentration of volatile compounds. These results could be relevant to the study of the fermentative capacity and aroma compounds produced by the *K. marxianus* yeast SLP1 strain with external FAs addition; the yeasts strains from *K. marxianus* are some of the better yeast volatile compound producers (Morrissey et al. 2015).

Conclusions

Our results indicate that the addition of a mixture of SFAs and UFAs improve *K. marxianus* yeast thermotolerance.

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Figures legends

Fig. 1 *K. marxianus* SLP1 strain survival after 2 h of temperature exposure. Survival was determined in comparison to 100% survival in cells at 40°C. Survival was determined as described in material and methods. Each value represents the mean ± SD

Fig. 2 Reactive oxygen species (ROS) production in the *K. marxianus* SLP1 strain after 2 h of temperature exposure. The ROS production was determined as described in material and methods. Each value represents the mean ± SD

Fig. 3 *K. marxianus* SLP1 strain survival after growth in media supplemented with fatty acids (oleic, linoleic, linolenic or araquidic) and after 2 h of temperature exposure. Cells without fatty acid additions were considered to have 100% survival. Each value represents the mean ± SD

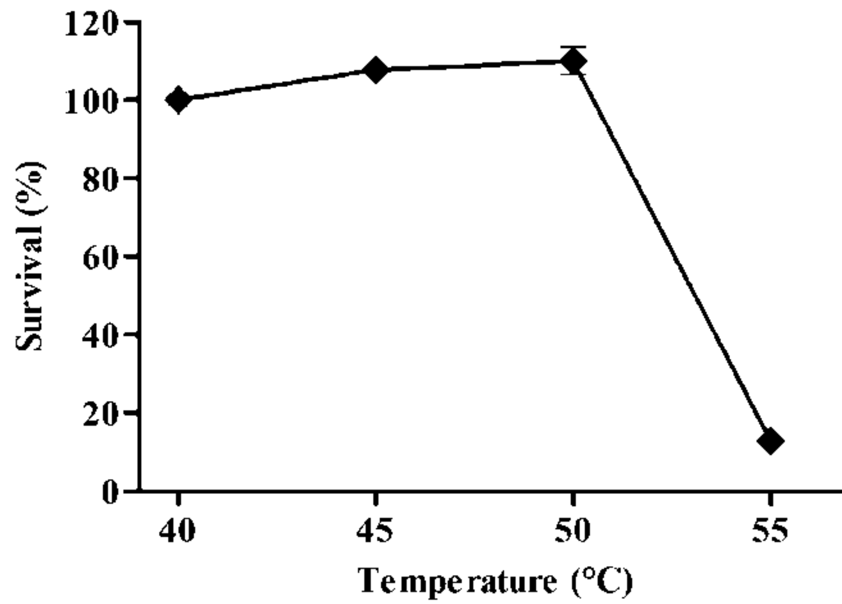
Fig. 4 *K. marxianus* SLP1 strain lipoperoxidation after growth in media supplemented with fatty acids (oleic, linoleic, linolenic or araquidic) and after 2 h of temperature exposure. Each value represents the mean ± SD

Table 1. Fatty acid composition and flow coefficient of the *K. marxianus* SLP1 strain supplemented with fatty acids.

	Proportions (%) of membrane fatty acids (FAs) in control yeast								Flow coefficient
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	UFAs/SFAs
Control yeast	8.86	27.56	29.10	3.91	13.71	11.91	0.49	4.47	1.23
Fatty acids added	Proportions (%) of membrane FAs in yeast with external FAs added								UFAs/SFAs
C18:1	4.16	11.01	4.80	0.83	74.07	4.72	0	0.37	5.11
C18:2	4.82	14.91	2.93	1.82	1.47	72.76	1.31	0.59	3.54
C18:3	4.23	16.17	0.75	4.25	2.20	1.53	70.25	0.59	2.96
C20:0	3.66	10.80	12.90	1.40	5.00	5.86	0.27	60.07	0.32

1 mM of the oleic acid, linoleic acid, linolenic acid and araquidic acid. Other fatty acids detected were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), and stearic (C18:0). Cells were cultured as mentioned in materials and methods. Ratios of unsaturated fatty acids (UFAs) to saturated fatty acids (SFAs) are also reported.

Figure 1:



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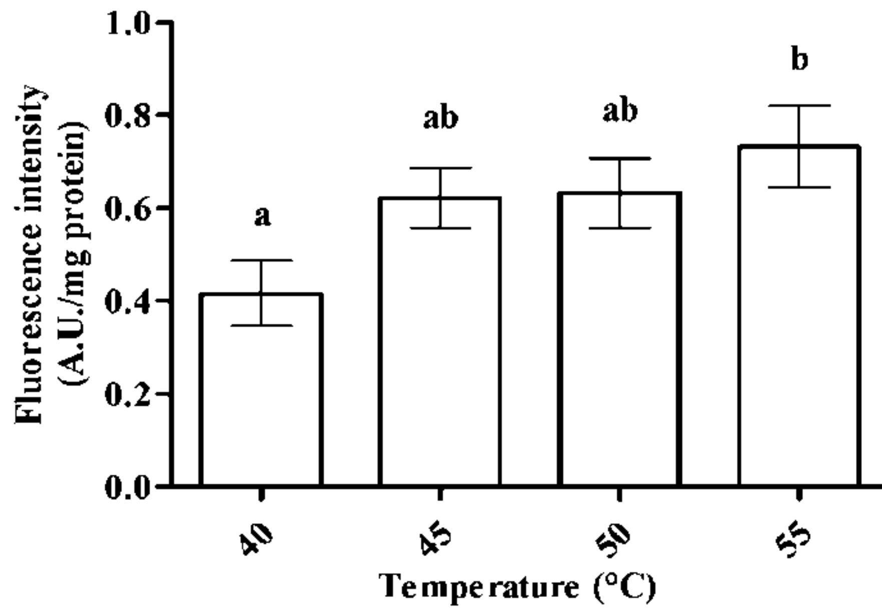

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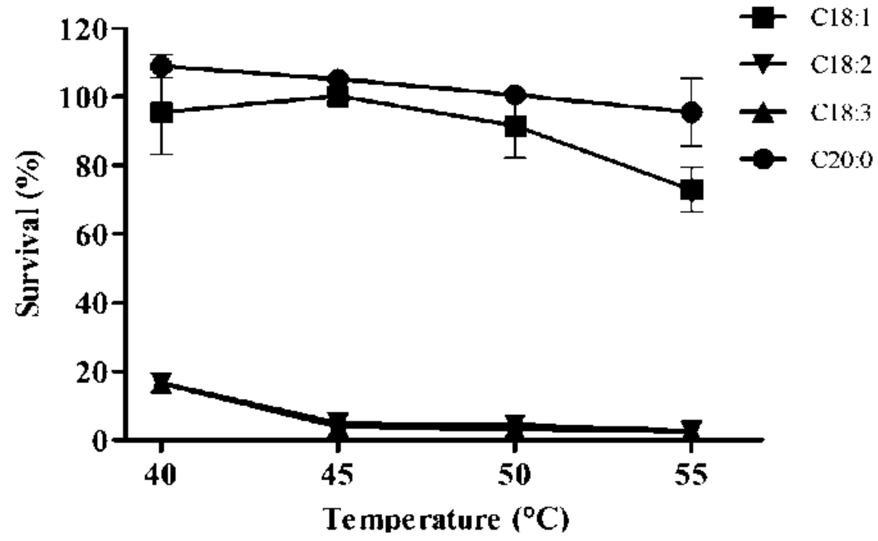
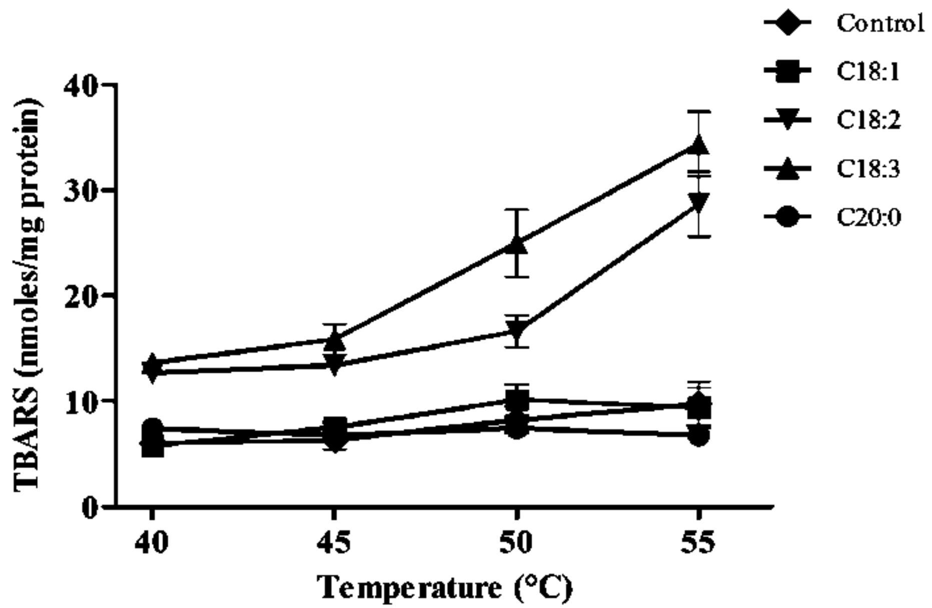


Figure 4:



Capítulo 3:

Electron transport chain in a thermotolerant yeast

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Electron transport chain in a thermotolerant yeast

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Abstract Yeasts capable of growing and surviving at high temperatures are regarded as thermotolerant. For appropriate functioning of cellular processes and cell survival, the maintenance of an optimal redox state is critical of reducing and oxidizing species. We studied mitochondrial functions of the thermotolerant *Kluyveromyces marxianus* SLP1 and the mesophilic OFF1 yeasts, through the evaluation of its mitochondrial membrane potential ($\Delta\Psi_m$), ATPase activity, electron transport chain (ETC) activities, alternative oxidase activity, lipid peroxidation. Mitochondrial membrane potential and the cytoplasmic free Ca^{2+} ions (Ca^{2+} cyt) increased in the SLP1 yeast when exposed to high temperature, compared with the mesophilic yeast OFF1. ATPase activity in the mesophilic yeast diminished 80% when exposed to 40° while the thermotolerant SLP1 showed no change, despite an increase in the mitochondrial lipid peroxidation. The SLP1 thermotolerant yeast exposed to high temperature showed a diminution of 33% of the oxygen consumption in state 4. The uncoupled state 3 of oxygen consumption did not change in the mesophilic yeast when it had an increase of temperature, whereas in the thermotolerant SLP1 yeast resulted in an increase of 2.5 times when yeast were grown at 30°, while a decrease of 51% was observed when it was exposed to high temperature. The activities of the ETC complexes were diminished in the SLP1 when exposed to high temperature, but also it was distinguished an alternative oxidase activity. Our results suggest that the mitochondria state, particularly ETC state, is an important characteristic of the thermotolerance of the SLP1 yeast strain.

Keywords Yeast mitochondria · Thermotolerant yeast · Redox state · Electron transport chain · Alternative oxidase

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Introduction

In yeasts, there is a direct correlation between fermentative behavior and stress resistance (Zuzuarregui and del Olmo 2004). In the prototypical yeast *Saccharomyces cerevisiae*, its survival during stressing conditions like thermal stress is related to the maintenance of a high mitochondrial membrane potential $\Delta\psi$ and a decreased rate of respiration as the dissipation of $\Delta\psi$ is a key event leading to apoptosis (Bonnet et al. 2007; Davidson and Schiestl 2001).

In stress resistance the maintenance of appropriate cellular redox environments is crucial, since alterations in redox can adversely affect signal transduction (Janssen-Heininger et al. 2008), protein structure and function, DNA and RNA synthesis (Muller 1995), enzyme activation and regulation of the cell cycle (Burhans and Heintz 2009). The term *redox state* is used to describe the ratio of the interconvertible oxidized and reduced form of a specific redox couple. Sir Hans Krebs focused on the NAD^+/NADH couple and defined the redox state of this couple in a cell (Schafer and Buettner 2000). The NADH produced in the metabolism is oxidized to NAD^+ by the electron transport chain (ETC) in the mitochondria, generating a connection between mitochondrial function and redox homeostasis. Nevertheless, a decreased respiratory rate and an enhanced $\Delta\psi$ might be deleterious to yeast as this can stimulate electron leak and ROS production. Thus, the levels of ROS must be tightly regulated as temperature increase can affect a variety of cellular processes and impact cell growth

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(Lee et al. 2012), fermentation yield and product quality via increased ROS production (Salvado et al. 2011).

During fermentative processes utilizing yeast, the increase in bioreactor temperature is a challenging issue to address because this impose the need to expend an important amount of energy in order to control fermentation temperature to avoid an impairment on yeast fermentative performance. Thus, it would be desirable to utilize thermotolerant yeast aimed to decrease the cost and the energy utilized in fermentative processes. Among the thermotolerant yeasts, the Kluyveromyces marxianus SLP1 yeast has been reported to possess high temperature resistance (Mejia-Barajas et al. 2016) and is suitable for bioethanol production (Flores et al. 2013). Whether the thermotolerance of *Kluyveromyces marxianus* SLP1 is related to an enhanced ability of mitochondria to maintain a high $\Delta\psi$ and a decreased respiratory rate while controlling oxidative stress is an unknown issue. Therefore, the aim of this study was to compare mitochondrial function, oxidative damage and calcium transients in the thermotolerant yeast *Kluyveromyces marxianus* SLP1 and the mesophilic strain OFF1 after being submitted to a heat shock by inducing a temperature shift in the culture. To the best of our knowledge, this is the first report evaluating the activity of the individual ETC complexes in a thermotolerant yeast. Our results show that the thermotolerance of the SLP1 yeast correlated with their mitochondrial ETC state.

Materials and methods

All the chemicals were of reactive grade, acquired either from Sigma-Aldrich (St. Louis, MO USA) or JT Baker (Center Valley, PA, USA).

Yeast strains

The *K. marxianus* SLP1 yeast was used as the thermotolerant yeast (Mejia-Barajas et al. 2016) and the OFF-1 yeast was used as the mesophilic comparative yeast. Both yeast strains were of the same species and were obtained from the culture collection of the CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, Mexico) (Gschaedler et al. 2004).

Temperature increase

Temperature shift was applied as described by Kim et al. (2006), with some modifications. Yeast strains were grown until the late-log phase was achieved, as reported by Mejía-Barajas et al. (2016). In order to elicit the temperature increase, the late-log cultured cells previously grown at 30 °C were exposed to a temperature of 40 °C for 2 h.

In situ mitochondrial membrane potential ($\Delta\Psi_m$) determination

This parameter was assayed using the methodology described by Peña et al. (2010) with some modifications. Briefly, 25 mg of cells (wet weight) were placed in a quartz cuvette containing MES-TEA buffer, 20 mM glucose, 15 μM $BaCl_2$ and 1 μM of the dye $DiSC_3(3)$ in a final volume of 2 mL. Measuring basal fluorescence for 180 s started membrane potential monitoring. Then, 15 μM CCCP (carbonyl cyanide m-chlorophenyl hydrazone) was added and the changes in the fluorescence signal were followed for 90s. Following, 25 mM KCl was added and the fluorescence was monitored for an additional 60s. The changes in $DiSC_3$ (3) fluorescence (λex = 540 nm; λem = 590 nm) were evaluated in a Shimadzu RF5301 fluorometer.

Determination of in situ mitochondrial respiration

Mitochondrial respiration was analyzed polarographically with a Clark-type oxygen electrode connected to a YSI 5300 biological oxygen monitor and a computer for data acquisition. 125 mg of cells (wet weight) were placed in 2.5 mL MES-TEA buffer (10 mM 2-(N morpholino)ethanesulfonic acid, pH 6.0 with triethanolamine) in a closed chamber with constant stirring. Oxygen consumption measurement was started in state 4 by adding 10 mM glucose, and uncoupled respiration (U state) was stimulated by adding 15 μM of the uncoupler CCCP, as reported (Aguilar-Toral et al. 2014).

Mitochondria isolation

For mitochondria isolation the thermotolerant *K. marxianus* SLP1 yeast and mesophilic OFF-1 yeast strains were grown and exposed to the above mentioned temperature shift. After the temperature increase was generated, the yeasts were harvested and the mitochondria were isolated from the spheroplasts, as previously described (Guérin et al. 1979; Avéret et al. 1998). Zymolyase 20 T was used instead of cytohelicase. For optimal determination of ETC reactions, the mitochondria that were previously isolated from the yeast exposed to the temperature shift were permeabilized with Triton X-100 (Hallberg et al. 1993). Mitochondrial protein concentration was measured by the Biuret assay.

Succinate-DCIP oxidoreductase activity

This enzymatic activity was measured at room temperature following the succinate-stimulated secondary reduction of DCIP (Uribe et al. 1985). The reaction mixture contained 50 mM KH_2PO_4 buffer (pH 7.6), 0.3 mg/ml permeabilized mitochondria, 80 μM DCIP, 1 mg antimycin A and 0.75 mM KCN in 1 ml as the final volume. After a 5-min

incubation with inhibitors, the reaction was initiated with 10 mM sodium succinate (pH 7.6). Absorbance changes were recorded in a Perkin Elmer Lambda 18 UV/vis spectrophotometer at 600 nm. The rate of DCIP reduction was calculated from the slope of the absorbance plot using the molar extinction coefficient of 21 mM^{-1} cm^{-1} for DCIP. The activity was calculated by knocking off the background reduction rate of DCIP in the absence of succinate from the reduction rate of DCIP stimulated with succinate.

Succinate cytochrome *c* oxidoreductase activity

The antimycin A-sensitive succinate-mediated reduction of cytochrome *c* was followed by the room temperature measuring the reduction of cytochrome *c* in the permeabilized mitochondria of the yeast exposed to the above mentioned temperature shift, as reported (Cortés-Rojo et al. 2009).

Cytochrome *c* oxidase activity

This activity was evaluated by measuring the cyanide-sensitive oxidation of reduced cytochrome *c* in the permeabilized mitochondria, as reported (Cortés-Rojo et al. 2009).

Alternative oxidase

The alternative oxidase (AOX) activity was evaluated according to Veiga et al. (2000). As mentioned before oxygen consumption was measured with Clark-type oxygen electrode connected to an YSI 5300 biological oxygen monitor.

Assessment of F1Fo-ATPase activity

The activity was measured by quantifying the oligomycin-sensitive release of phosphorus from ATP hydrolysis using the methodology described by Fiske and Subbarow (1925).

Mitochondrial lipid peroxidation measurement

Changes in the lipid peroxide levels were determined with the thiobarbituric acid (TBA) assay (Buege and Aust 1978).

Cytoplasmic calcium measurement

The yeasts were grown and exposed to a modified temperature, as mentioned above. They were then harvested and 100 mg were suspended in 1 ml of PBS, pH 7.4, after which 5 μL of Fluo-3-acetoxymethyl ester (Fluo-3/AM; Biotium, Hayward, CA, USA) was added. Fluo-3 AM was dissolved in 20% Pluronic F-127 DMSO solvent (Sigma) at a concentration of 1 mg/ml (Wang et al. 2013). The suspension was incubated for 50 min at 37 °C. The yeasts were harvested and washed twice with PBS, pH 7.2. The excitation wavelengths were 506 nm and fluorescence was recorded at 526 nm, using a Shimadzu RF5301 fluorometer with constant stirring.

Statistical analysis

All experiments were independently performed at least 3 times and results were expressed as the mean ± standard error (SE).

Results

The effect of temperature on the in situ mitochondrial potential ($\Delta\Psi_m$) and the cytoplasmic membrane potential ($\Delta\Psi_c$).

A rise in $DiSC_3$ (3) fluorescence after the addition of CCCP was observed only in the thermotolerant SLP1 yeast grown at 30 °C (Fig. 1a, b, black lines). This effect indicates that only the SLP1 yeast has a functional $\Delta\Psi_m$. When the cells were exposed to 40 °C for 2 h, the OFF1 mesophilic yeast remained without changes in its fluorescence (Fig. 1a, red line), whereas the SLP1 thermotolerant yeast exhibited an increase in the fluorescence corresponding to the $\Delta\Psi_m$ (Fig. 1b, red line). The percentage of $\Delta\Psi_m$ was estimated by taking the potential displayed by the yeast strains grown at 30 °C (Fig. 2a, b, respectively) as 100%.

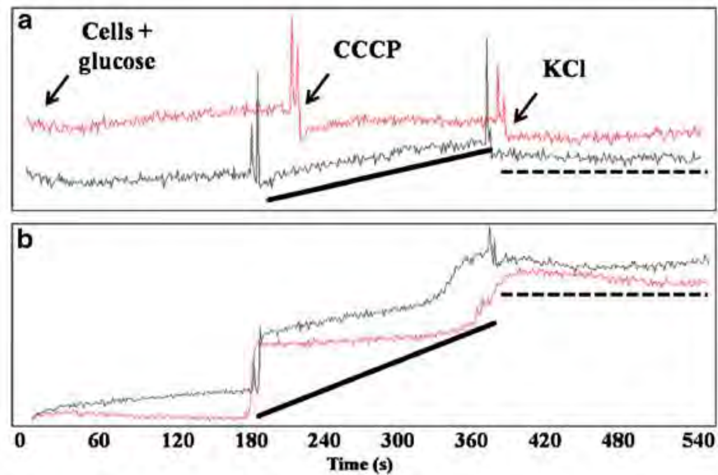
Effect of temperature shift on yeast respiration

No changes were observed in state 4 respiration during the temperature shift in the OFF1 yeast (Fig. 3a). The same was observed when uncoupled respiration (State U) was stimulated by adding CCCP (Fig. 3b). Indeed, the respiration of this yeast was almost fully insensitive to CCCP addition as a negligible increase in oxygen consumption was detected, indicating that mitochondria from OFF1 were uncoupled before CCCP addition. In contrast, beside state 4 respiration was ostensibly higher in the SLP1 yeast than in the OFF1, respiration in the SLP1 yeast was stimulated ~2-fold by CCCP at 30 °C, indicating a higher coupling in mitochondria from this yeast (Fig. 3b). Unexpectedly, respiration in both states 4 and U was notably inhibited by the shift to 40 °C in the SLP1 cells, decreasing in this way the apparent coupling observed at 30 °C.

Effect of temperature increase on electron transport chain (ETC)

In yeasts, the ETC is composed of the II, III and IV complexes, without the complex I, with some exceptions like *Yarrowia lypolitica*, *Pichia membranaefaciens*, *Saccharomyces kluyveri*, *Saccharomyces exiguus* and *Candida maltosa* (Kitano et al. 1995). The complex II or succinate-DCIP oxidoreductase was measured following the

Fig. 1 Effect of temperature on the in situ mitochondrial membrane potential ($\Delta\Psi_m$). Mesophilic OFF1(**a**) and thermotolerant SLP1(**b**) yeasts. Yeasts were grown at 30 °C (black line) and exposed at 40 °C for 2 h (red line). Representative traces of $n \geq 3$ are shown. The uncoupler CCCP was added in respiration traces to stimulate the maximum respiration rate (uncoupled state). The changes in $DiSC_3$ (3) fluorescence by CCCP and KCl estimated the mitochondrial potential ($\Delta\Psi_m$; solid line) and the plasma membrane potential ($\Delta\Psi_c$; slash line), respectively



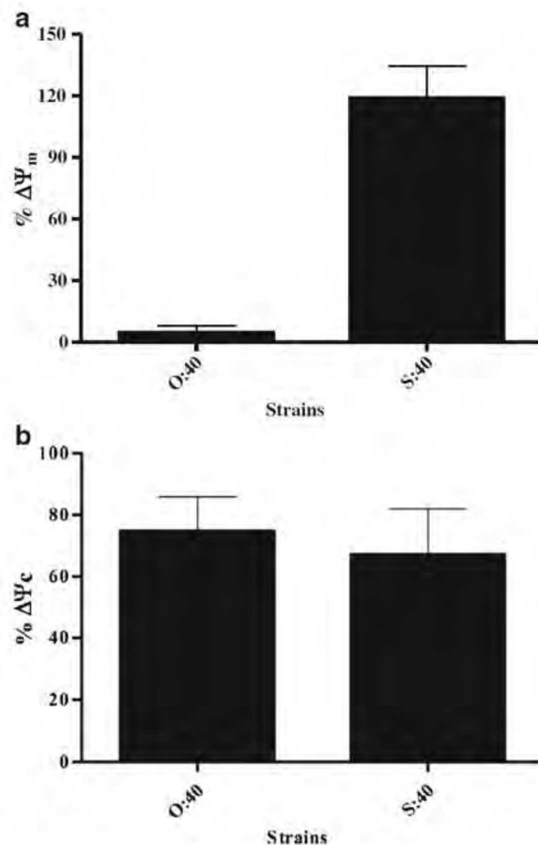


Fig. 2 Effect of temperature on the in situ mitochondrial (**a** [$\Delta\Psi_m$]) and cytoplasmic (**b** [$\Delta\Psi_c$]) membrane potentials of the mesophilic (OFF1 [O]) and thermotolerant (SLP1 [S]) yeasts. Cells grown at 30 °C were taken as 100 % to compare with cells exposed to 40 °C for 2 h

succinate-stimulated secondary reduction of DCIP (Uribe et al. 1985). The activities of the reactions comprising the electron transport chain are depicted in the Fig. 4. When the OFF1 and SLP1 yeasts were grown at 30 °C, the complex II activity of the latter was three times higher. In contrast, when exposed to the temperature shift, the activity of both yeasts was completely abolished, with OFF1 displaying a negligible activity (Fig. 4a). The activity of the complex II-complex III segment of the ETC is shown in the Fig. 4b. No differences were observed in this activity between both yeasts at 30 °C. Temperature shift decreased severely this activity at a similar degree in both yeasts, in such way that no differences were detected between them. Regarding the activity of the complex IV, a similar trend was observed with respect to complex II-complex III, with no differences between both types of yeasts at 30 °C and both experiencing a similar degree of inhibition after the temperature shift (Fig. 4c).

Effect of temperature on cytosolic Ca^{2+} levels

Table 1 show that the SLP1 yeast had a significant increase in its cytosolic Ca^{2+}, whereas the OFF1 yeast had a tendency to reduce its concentration, with no significant changes.

Alternative oxidase

Experiments showed that 1 mM cyanide decreased the oxygen rate consumption of the OFF1 yeast at about hundred times (Fig. 3b compared to Fig. 5). The effect of cyanide in the oxygen consumption of SLP1 yeast exposed to temperature increase, was half of the observed with the OFF1 and SLP1 grown at 30 °C, and a decrease in the oxygen rate consumption around fifty times (Fig. 5).

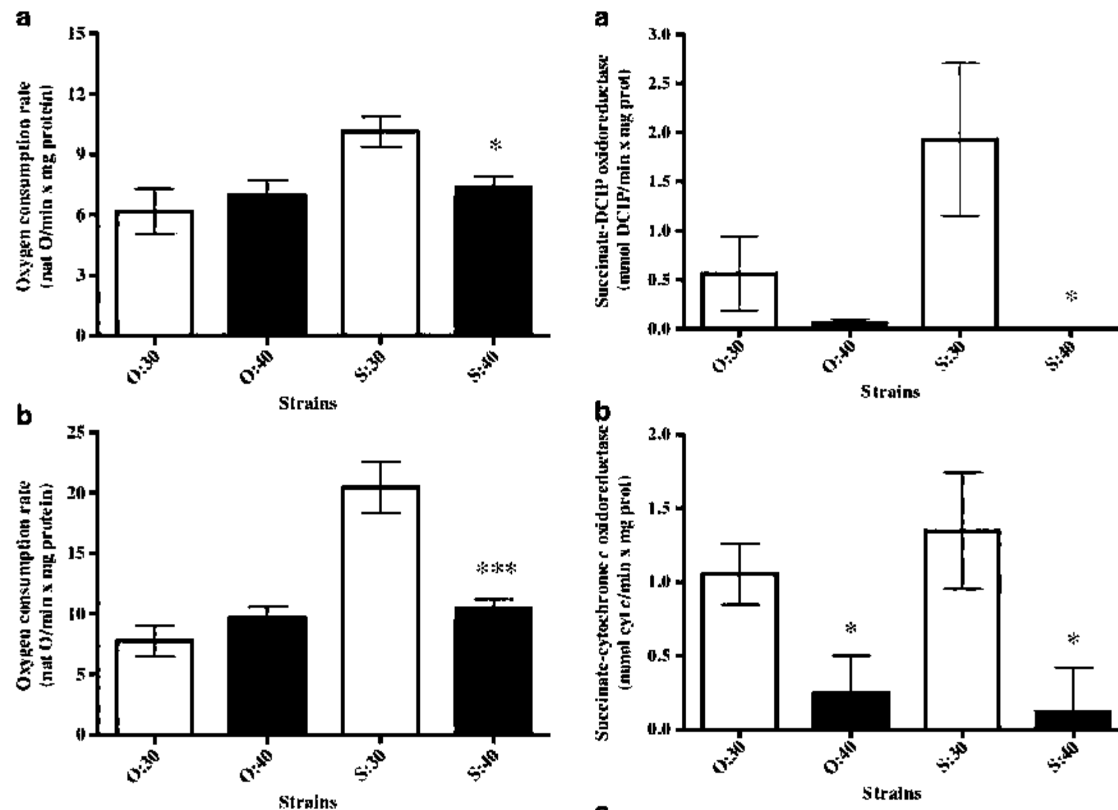


Fig. 3 Effect of temperature increase on the rate of O_2 uptake in state 4 (**a**) and the uncoupled state (**b**) in mesophilic OFF1 (O) and thermotolerant SLP1 (S) yeasts grown at 30 °C (30: white bars) or exposed to 40 °C for 2 h (40: black bars). The cells were incubated for 30 min at 4 °C in a respiration medium. After pre-incubation, oxygen uptake was measured, as described in Materials and Methods. Data are presented as mean ± SE from >3 independent experiments. There was a statistically significant difference when compared with the control (*$P < 0.05$; ***$P < 0.001$)

Effect of the temperature shift on F_1F_0-ATPase activity

The OFF1 yeast had higher F_1F_0-ATPase activity than the SLP1 yeast when grown at 30 °C (Fig. 6). The temperature shift did not have any effect on F_1F_0-ATPase activity in the SLP1 yeast, whereas it was inhibited in the OFF1 yeast.

Effect of temperature on mitochondrial lipid peroxidation

Lipid peroxidation occurs in the polyunsaturated hydrocarbon chain of the lipids of the membranes, in this case, the mitochondrial membranes. Both yeasts showed a significant increase in the lipid peroxidation of their mitochondrial membranes when they were exposed to temperature (Table 2). However, no differences in this parameter were observed

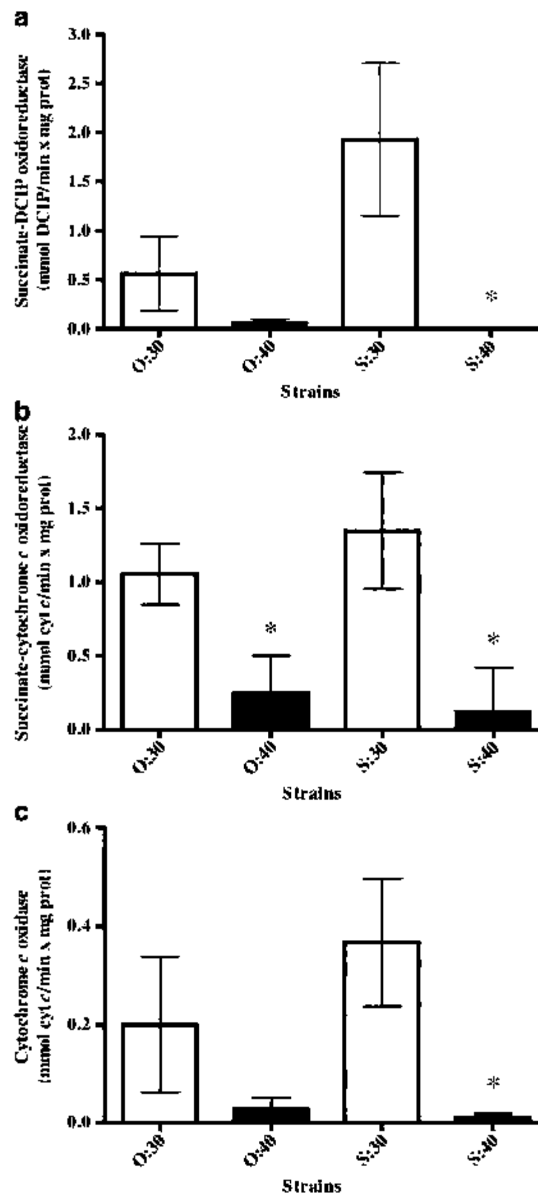


Fig. 4 Influence of temperature on the activities of the ETC complexes from the mitochondria isolated from the OFF1 (O) and SLP1 (S) yeasts grown at 30 °C (30: white bars) or exposed to 40 °C for 2 h (40: blacks bars). A) Succinate-DCIP oxidoreductase activity (complex II). B) Antimycin A-sensitive succinate-cytochrome *c* oxidoreductase activity (complex III (Matsuno-Yagi and Hatefi 1996)). C) Cytochrome *c* oxidase activity (complex IV). Data are presented as mean ± SE from >3 independent experiments. The Student's *t* test was used to compare the activities of the mitochondria isolated from cells grown at 30 °C (white bars) versus mitochondria isolated from cells exposed to 40 °C for 2 h (black bars). *$P < 0.05$

Table 1 Change at the cytosolic Ca^{2+} level induced by temperature in OFF1 (O) and SLP1 (S) yeasts grown at 30 °C (30) or exposed to 40 °C for 2 h (40)

Fluorescence intensity (A.U./mg protein)		
	30	40
O	5 ± 0.7	3 ± 0.4
S	5 ± 0.4	7 ± 0.5**

Data are presented as mean ± SE from >3 independent experiments (Student's *t* test: **$P < 0.01$)

between the two types of yeast when compared at the same temperature.

Discussion

The adaptations that thermotolerant yeast undergo in the function of the electron transport chain when exposed to increased temperature may have an impact on the yeast performance during fermentation high temperature. In first place, the increment in $\Delta\Psi_m$ observed in the SLP1 cells but not in the mesophilic OFF1 yeast in response to the temperature shift may have important implications for the bigger survival to high temperature of SLP1 (Figs. 1 and 2a), as it has been demonstrated that hyperpolarization of the inner mitochondrial membrane influences temperature tolerance in yeast (Rikhvanov et al. 2006). Moreover, the hyperpolarization of the mitochondria stall respiration and oxidative phosphorylation, essentially "turning off" the mitochondria and shifting energy production to the cytoplasm, with glycolysis as the primary source of ATP. A glycolytic phenotype is related to resistance to apoptosis in part because the "inactive" hyperpolarized mitochondria cannot induce apoptosis (Bonnet et al. 2007). The idea that SLP1 thermotolerance is related to hyperpolarization of the inner mitochondrial membrane and stalled respiration agrees well also with the notable decrement of respiration observed in this yeast after the temperature shift.

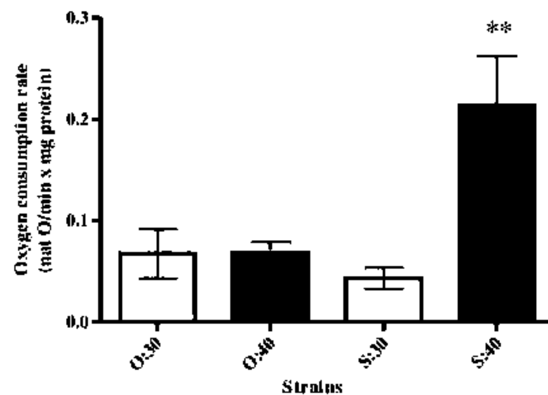


Fig. 5 Effect of cyanide on oxygen rate consumption. White bars: yeast grown at 30 °C; black bars: yeast exposed to 40 °C for 2 h. The results are expressed as the mean ± SE from >3 independent experiments. **$P < 0.01$ (Student's *t* test) compared the results of the yeasts with and without the temperature shift

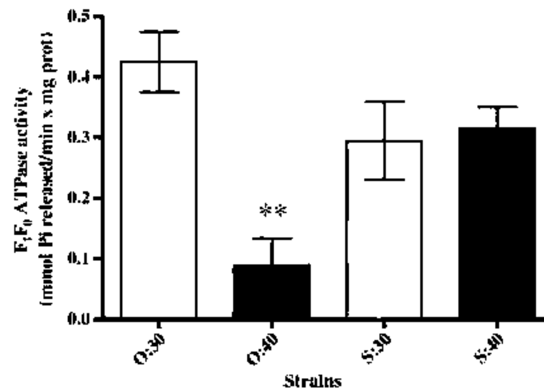


Fig. 6 Effect of the temperature shift on F_1F_0-ATPase activity. White bars: mitochondria isolated from yeast grown at 30 °C; black bars: mitochondria isolated from yeast exposed to 40 °C for 2 h. The results are expressed as the mean ± SE from >3 independent experiments. **$P < 0.01$ (Student's *t* test) compared the activities of the mitochondria from the yeasts with and without the temperature shift

The dissipation of the $\Delta\Psi_m$ is associated with ROS induction (Suski et al. 2012) and translocation of the apoptosis-inducing factor (AIF) to the cytosol, which, in turn, may lead to organelle swelling, ATP depletion and cell death (Kwong and Molkentin 2015). On the other hand, the incubation of *S. cerevisiae* at an elevated temperature (45 °C) stimulated its respiration and decreased their survival rate (Rikhvanov et al. 2001). In agreement with this, the mesophilic OFF1 yeast exhibited a dissipated membrane potential even before the temperature shift (Fig. 1a), which is in concordance with the negligible effect of CCCP the respiration observed in this strain. This indicates that respiration in this yeast was fully uncoupled. Therefore, based on the above antecedents, it is feasible to hypothesize that intolerance of OFF1 to higher temperatures might be related, in part, to a lack of plasticity of mitochondrial metabolism to avoid cellular death via the maintenance of a high mitochondrial membrane potential. A

Table 2 Lipid peroxidative damage induced by temperature in the mitochondria isolated from the OFF1 (O) and SLP1 (S) yeasts grown at 30 °C (30) or exposed to 40 °C at 2 h (40)

TBARS (nmol/mg protein)		
	30	40
O	8 ± 1.0	13 ± 1.0*
S	10 ± 0.7	14 ± 2.0*

Data are presented as mean ± SE from >3 independent experiments. There was a significant difference when the lipid peroxidation in the yeasts with (40) and without (30) the temperature change was compared (Student's *t* test: *$P < 0.05$)

higher stress response has been reported for this thermotolerant yeast with respect to the mesophilic yeast strains (Flores et al. 2013; Mejia-Barajas et al. 2016). The reduction in respiratory activity increases the thermotolerance of *S. cerevisiae* (Davidson and Schiestl 2001).

The activities of the ETC complexes were evaluated one-by-one in the isolated mitochondria. The effect of the ETC inhibitors on yeast thermotolerance has been explained by the different types of glucose utilization (Rikhvanov et al. 2002). To prevent this, the effect of the temperature on the ETC of the SLP1 and OFF1strains was evaluated in the stationary phase, after the diauxic shift. The OFF-1 yeast showed a reduced activity in complex III, whereas the thermotolerant SLP1 yeast reduced its complex activity in the three ETC complexes (Figs. 4a, b and c). Cells adapt to heat stress by the up-regulation of the genes for activating the metabolic pathways and by the down-regulation of the mitochondrial genes to avoid heat-induced ROS (Cao et al. 2013). It has even been suggested that the optimal thermotolerant yeast phenotype could not be generated through evolution as long as oxidative respiration is wholly functional, because it would possibly generate more ROS-inducing oxidative stress (Caspeta et al. 2014). Caspeta et al. (2015) observed that functions involved in controlling the growth rate decreased in the thermotolerant yeasts, which correlates with the loss of respiration capacity. Voinikov et al. (1989) and Chou et al. (1989) suggested that the energetic activity of the mitochondria changes under heat shock due to the thermo-sensitivity of their ETC.

The treatment of *S. cerevisiae* cells with amiodarone elevated the cytosolic calcium (Cyt Ca^{2+}) level in parallel with the $\Delta\Psi_m$, which led to the induction of Hsp104p synthesis, helping the mitochondria keep their integrity and metabolic homeostasis within the cells (Ueom et al. 2003). Heat shock causes an increase in the cytosolic Ca^{2+} level in plant (Saidi et al. 2011) and mammalian cells (Balogh et al. 2005) and a similar effect has been suggested in *S. cerevisiae* (Fedoseeva et al. 2012). In our results, we observed a Cyt Ca^{2+} rise when the cells were exposed to the temperature increase (Table 1). The elevation of the Cyt Ca^{2+} level might stimulate activity of yeast mitochondria and elevate the $\Delta\Psi_m$, regardless of the slow ETC complex activity (Lehninger et al. 1967). Petite yeasts, despite the lack of functionally active ETC, contain mitochondria, and the inner membrane of these mitochondria is capable of maintaining the electrochemical potential generated by F1ATPase, catalyzing hydrolysis of ATP and the electrogenic ADP/ATP exchange via the ADP/ATP translocator (Lefebvre-Legendre et al. 2003). Upon ETC dysfunction, F1F0 ATP synthase reverses its action and starts to hydrolyze ATP. It has been proposed that ATP hydrolysis in the F1 domain releases electric energy and the energy produced is transferred (through ATP synthase) from F1 to F0, where it is used to charge the $\Delta\Psi_m$ (−140 mV). Because the mutations that affect the ADP/ATP translocator and F1ATPase have been observed to be lethal in the petite strains (Kominsky et al. 2002), it was suggested that the maintenance of the $\Delta\Psi_m$ on the inner mitochondrial membrane is vital for cell survival. This occurred in the thermotolerant SLP1 yeast strain, but not in the mesophilic OFF1 yeast strain. The oxygen uptake observed with the reduced ETC complex activity in the SLP1 yeast, could be exerted by the residual tricarboxylic acid cycle activity and by oxidation of the cytosolic NADH (Caspeta et al. 2014). It could be argued that $\Delta\Psi_m$ is sustained by ATP hydrolysis by the reverse action of F_1F_0 ATPase, whose activity remained unaltered despite the temperature shift; however, this possibility can be discarded it because the high $\Delta\Psi_m$ may be having the opposite effect on F_1F_0 ATPase (i.e. the synthesis of ATP). In contrast, the thermal shift decreased severely the F_1F_0 ATPase activity of the OFF1 yeast, which suggests that neither the synthesis of ATP nor the establishment of $\Delta\Psi_m$ by ATP hydrolysis would not be sustained during the temperature increase, accounting this for events leading to a higher sensitivity of OFF1 to cell death during the temperature shift via a decreased availability of ATP and a decreased $\Delta\Psi_m$. Quantifying the release of phosphorus during ATP hydrolysis assessed the activity of the F1F0-ATPase. It is important to note that this technique lets us know if the F_1F_0-ATPase is active, but not if it is coupled with the ETC.

During heat shock, the cell loses intracellular ATP and tries to compensate for the loss by enhancing its respiration (Rikhvanov et al. 2001). This effect was observed in the OFF1 yeast strain (Fig. 3). The decreased ATP synthesis in the OFF1 yeast could be due to the reduction of its ATPase activity (Fig. 6), causing an alteration in cellular ion homeostasis because of the reduced activity of the ATP-dependent ion pumps (Cross et al. 2010). Moreover, the maintenance of normal ATP levels has to be an important physiologic fact, given that many heat-shock proteins involved in the reactivation of heat-denatured protein, working as molecular chaperones, require ATP for their release from the complex (Parsell and Lindquist 1993). The SLP1 yeast strain had no changes in its ATPase activity (Fig. 6), a fact that has been reported in temperature-tolerant yeasts (Piper 1993). The mitochondrial cell membrane plays a critical role in the activity of mitochondrial ATPase because this enzyme is embedded in it. Lipid peroxidation is a deleterious phenomenon for mitochondrial ATPase function and ETC (Cortés-Rojo et al. 2009). A high level of lipid peroxidation has been described as a common consequence of ROS accumulation (Dani et al. 2008). An increase in lipid peroxidation was observed in the two yeasts evaluated (Table 2). However, no reduction in ATPase function in the thermotolerant SLP1 yeast was observed, in contrast to the mesophilic OFF-1 yeast (Fig. 6). The reduction in F_1F_0-ATPase activity observed in the OFF1 yeast or the lower ETC complex activity in the SLP1 yeast could be explained as the result of the damage produced in the mitochondrial membrane by the ROS generated during the exposure to the

increased heat. Lipid peroxidation of the mitochondrial membranes was evaluated in relation to this.

The antioxidant response observed in the thermotolerant SLP1 yeast concurs with the reports of Kim et al. (2013). In their conclusion they mentioned that the thermotolerant *S. cerevisiae* KNU5377 activated antioxidant enzymes, minimizing oxidative damage from ROS, and improving redox homeostasis. However, they did not mention anything about the ETC state. Our results suggest that it is possible that the increase in the $\Delta\Psi_m$, due to a rise in Cyt Ca^{2+} and inverse ATPase function, along with reduced ETC complex activity, generated ROS production at a concentration that induced Hsp104p synthesis. The activity of this protein, together with the maintenance of the ROS level due to a higher catalase activity (Mejía-Barajas et al. 2016), could have contributed to the maintenance of the NADH level (data not shown) in the SLP1 yeast strain and its higher temperature tolerance, in contrast to the OFF1 yeast. At any rate, it is important to study the relation between the redox state, stress response factors (Hsf1, Yap1, and Msn2/4) and ETC state.

Conclusion

With these results we suggest that thermotolerance in SLP1 yeast is attributed to its reduced ETC activity due to sustaining its $\Delta\Psi_m$, together with the ATPase insensitibity compared with the mesophilic OFF1 yeast, important factors that could cause apoptosis in yeast.

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Capítulo 4:

Bioethanol production by simultaneous saccharification and fermentation of agave bagasse using the native thermotolerant *Kluyveromyces marxianus* SLP1 yeast

Este capítulo ha sido sometido para su revisión como:

Manuscript number	BJM_2017_253
Title	Bioethanol production by simultaneous saccharification and fermentation of agave bagasse using the native thermotolerant Kluyveromyces marxianus SLP1 yeast
Short title	Bioethanol production by thermotolerant yeast
Article type	Research paper

Abstract

Lignocellulosic residues and thermotolerant yeasts have been exploited for a wide range of applications, including bioethanol production. In bioethanol production, simultaneous saccharification and fermentation (SSF) appear as promising processes. In this study, agave bagasse from Agave tequilana Weber was obtained from tequila industry wastes. The agave bagasse residue (ABR) was exposed to thermo-acid treatment and subsequent hydrolysis using a mix of commercial cellulases (CTec2, HTec2 and Rapidase), allowing recovery of fermentable sugars at approximately 69%. After this treatment, the solid and soluble fractions were used for subsequent SSF at 40°C, using the thermotolerant Kluyveromyces marxianus SLP1 yeast, and compared with the K. marxianus OFF1 and the industrial Saccharomyces cerevisiae Ethanol Red. The maximum ethanol yield obtained with the SLP1 yeast by SSF was 85%, corresponding to 10.36 g/L of ethanol from 10% (w/v) of ABR. In comparison, the industrial Ethanol Red and the K. marxianus OFF1 yeasts were 83% and 80%, respectively, corresponding to 10.23 g/L, and 10.12 g/L of ethanol by 10% (w/v) of ABR dry weight. The ethanol yields obtained through SSF were higher than by separate hydrolysis and fermentation (SHF). Thus, bioethanol production using ABR from the thermotolerant K. marxianus SLP1 yeast using SSF at 40°C could be considered a promising process.

Keywords	Agave bagasse; bioethanol; Kluyveromyces marxianus; simultaneous saccharification and fermentation; thermotolerant yeast.
Manuscript category	Biotechnology and Industrial Microbiology

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Title: Bioethanol production by simultaneous saccharification and fermentation of agave bagasse using the native thermotolerant *Kluyveromyces marxianus* SLP1 yeast

Running title: Bioethanol production by thermotolerant yeast

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Abstract

Lignocellulosic residues and thermotolerant yeasts have been exploited for a wide range of applications, including bioethanol production. In bioethanol production, simultaneous saccharification and fermentation (SSF) appear as promising processes. In this study, agave bagasse from *Agave tequilana* Weber was obtained from tequila industry wastes. The agave bagasse residue (ABR) was exposed to thermo-acid treatment and subsequent hydrolysis using a mix of commercial cellulases (CTec2, HTec2 and Rapidase), allowing recovery of fermentable sugars at approximately 69%. After this treatment, the solid and soluble fractions were used for subsequent SSF at 40°C, using the thermotolerant *Kluyveromyces marxianus* SLP1 yeast, and compared with the *K. marxianus* OFF1 and the industrial *Saccharomyces cerevisiae* Ethanol Red. The maximum ethanol yield obtained with the SLP1 yeast by SSF was 85%, corresponding to 10.36 g/L of ethanol from 10% (w/v) of ABR. In comparison, the industrial Ethanol Red and the *K. marxianus* OFF1 yeasts were 83% and 80%, respectively, corresponding to 10.23 g/L, and 10.12 g/L of ethanol by 10% (w/v) of ABR dry weight. The ethanol yields obtained through SSF were higher than by separate hydrolysis and fermentation (SHF). Thus, bioethanol production using ABR from the thermotolerant *K. marxianus* SLP1 yeast using SSF at 40°C could be considered a promising process.

Key words: Agave bagasse; bioethanol; *Kluyveromyces marxianus;* simultaneous saccharification and fermentation; thermotolerant yeast.

Introduction

Ethanol production using lignocellulosic biomass has received increasing attention due to the low cost and abundance of the lignocellulosic biomass. The agave bagasse of *Agave tequilana* Weber is a lignocellulosic residue that results from the production of tequila. In Mexico, the tequila industry generates agave bagasse residues at the rate of approximately 360 thousand of dry tons per year.[1,2] The production of ethanol from agave bagasse residue (ABR) through saccharification and subsequent fermentation has been previously studied.[3,4,5,6] The process of producing ethanol from a lignocellulosic biomass, such as ABR, has four main steps: pretreatment, saccharification, fermentation and distillation.[7] One option is the simultaneous saccharification and fermentation (SSF) process,[8] in which the substrate, enzyme, and yeast are present in the same reactor, reducing investment costs and end product inhibition of the enzymatic hydrolysis; however, this process requires the utilization of microorganisms capable of working at high temperatures, due to different optimum temperatures for saccharification (45-50°C) and fermentation (25-35°C).[9] Therefore, thermotolerant yeast strains represent biocatalytic tools for carrying out fermentations at these process conditions. The potential applications of thermotolerant yeasts[10,11,12] and their ethanol production[13,14,15] have been studied previously. Fermentation with thermotolerant yeasts in SSF using lignocellulosic biomass has been described by Choudhary et al.[11], proposing that thermotolerant yeasts strains are suitable alternatives to overcome the limitations of separate hydrolysis and fermentation (SHF). *Kluyveromyces marxianus* yeast species grow at 45 52°C and can efficiently produce ethanol at temperatures from 38 to 45°C.[16,17], and have additional advantages, such as their broad spectrum of sugar utilization and rapid growth rates.[18] In this way, the use of the thermotolerant yeast *K. marxianus* SLP1, which can grow at 40°C[19] and produce ethanol with high efficiency at 30°C,[20] could generate higher ethanol yield through SSF than

production by the SHF process. The aim of this work was to evaluate the ethanol production using the thermotolerant *K. marxianus* SLP1 yeast through SSF and SHF processes at 40°C, using ABR collected from tequila elaboration industries, which use different juice extraction processes. This will be compared with the mesophilic yeast *K. marxianus* OFF1 and the commercial yeast *S. cerevisiae* Ethanol Red.

Materials and Methods

Yeast strains

Yeast strains were obtained from the culture collection of the CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México)[21] and from the ATCC (American Type Culture Collection, Rockville, MD, USA). Two *K. marxianus* yeast strains, SLP1 and OFF1, were isolated at handcrafted mezcal distilleries in the Mexican State of San Luis Potosi and Guerrero, respectively. The Ethanol Red yeast was acquired from the ATCC.

Agave bagasse residue source and preparation

Agave bagasse residue (ABR) from *A. tequilana* Weber was collected from tequila distilleries with different elaboration processes, where the ABR was obtained mainly from agave plants of 5-8 years of age. The first ABR was collected from the distillery "Casa de Piedra", which is in Camino Arenal, Jalisco, Mexico. This distillery obtains the agave juice by extraction using masonry ovens. The agave pines were cooked at a temperature approximately 100°C for 36 to 48 h. The cooked pines were milled and compressed using a roller grinder with blades to separate the juice from the wet bagasse, which is accumulated

in piles near to the factory. The second ABR was collected from the distillery "Gonzalez-Gonzalez", which is in Guadalajara, Jalisco, Mexico. The milling and juice extraction were the same as mentioned above, but the agave cooking was done in an industrial autoclave with a constant temperature of 121°C at 1.2 kg/cm² for 12 h. The last ABR was collected from the distillery "La Madrileña" in Tototlan, Jalisco, Mexico. In this process, the agave pines are not cooked; instead, the juice was processed and extracted using a diffusion process, as is mentioned by Casas.[22] In all three cases, after the ABR samples were collected, they were washed with distilled water and dried at 70°C in a convection oven. In this work, the ABR samples were named according to their cooking procedures, such as masonry oven, autoclave, and diffuser.

Characterization of the agave bagasse residue

The characterization of the initial agave bagasse composition was carried out by quantitative inorganic acid hydrolysis, as described Foster et al.[23]

Thermo-acid hydrolysis of the agave bagasse residue

This process was carried out using a 1:10 w/v proportion. The flashes were loaded with 3 g of ABR and 30 ml of 1 to 3% sulfuric acid, and then incubated at temperatures of 110°C, 130°C and 140°C for 30 min. Samples were taken every ten min and filtered using a 0.45 mm membrane (Millipore). The reduced sugar concentration was determined by the 3,5-dinitro salicylic acid (DNS) method.[24] The absorbance was measured with a microplate reader at 540 nm. Yields of sugars release were calculated using the formula:

$Y = (C*V/W)*100$

Where Y is the yield of sugar released expressed as a percent of the dry weight of the

reduced sugars in. C is the concentration of reducing sugars (g/L), V is the total volume of the liquid phase (L) and W is the dry weight of the corresponding lignocellulosic material (g).

Enzymatic hydrolysis of thermo-acid pretreated agave bagasse residue

Agave bagasse product (solid and liquid fraction) from thermo-acid hydrolysis was adjusted with NaOH (2 M) at a pH of 5. The enzymatic hydrolysis was done with three commercial cellulase complexes (CTec2, HTec2 and Rapidase (Novozymes, Sigma)). Enzyme mixes were prepared at 0.15%, 1.5% and 15% of dry ABR, incubating at 40°C with shaking at 100 rpm for 72 h. Each sample was centrifuged and the supernatant was collected, filtered through a 0.45 mm membrane (Millipore). The concentration and kind of sugars in the liquid fraction of the enzymatic hydrolysis were determined by the DNS method and high-performance liquid chromatography (HPLC) as mentioned below.

Compound analysis of samples by HPLC

The concentration and kind of sugars, organic acids, glycerol, ethanol and furfural in the liquid fraction of the thermo-acid, enzymatic hydrolysis, and fermentations processes were determined by HPLC using an Aminex HPX-87C column 300 mm x 7.8 mm (BioRad). The supernatants were first filtered using filters of 0.45 mm. A mobile phase of 5 mM H_2SO_4 was used at 0.5 mL/min and the column was used at 60°C. Calibration plots were obtained with standards compounds.

Separated hydrolysis and fermentation (SHF) of agave bagasse residue

The hydrolysates (solid and liquid fractions) obtained by thermo-acid and enzymatic

treatments were fermented with the thermotolerant *K. marxianus* yeast SLP1, *K. marxianus* OFF1, and *S. cerevisiae* Ethanol Red yeasts. After thermo-acid treatment and a subsequent 48 h of ABR enzymatic hydrolysis, the yeast was inoculated at 1 X 10^7cells/ml and incubated at 40°C with shaking at 100 rpm.

Simultaneous saccharification and fermentation (SSF) of agave bagasse residue

In this process, the yeast cells were inoculated while the enzyme mix was added. The fermentations conditions were the same as the SHF process. The SHF and SSF were carried out without the addition of nutrients or the adjustment of pH. Ethanol, glycerol, residual sugars and furfural determination were carried out by HPLC as mentioned above. The ethanol yield was calculated considering ethanol produced (g) by ABR (g) and by the sugars (g) released by the thermo-acid and enzymatic treatments.

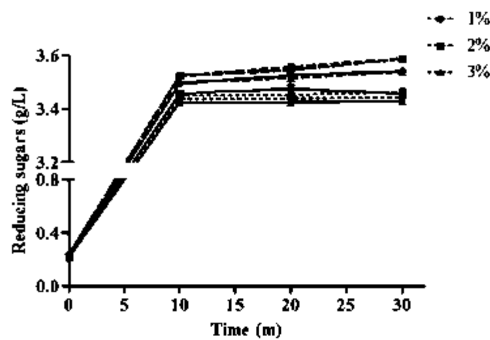
Results

Agave bagasse residue composition and thermo-acid hydrolysis

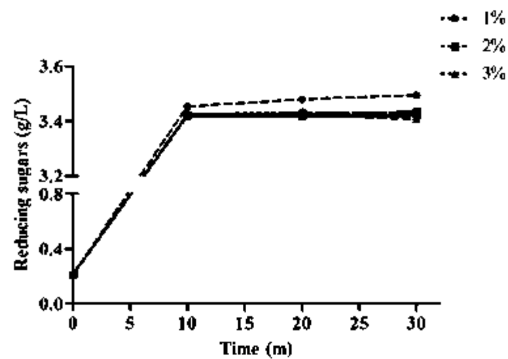
The ABR composition of the samples obtained from different tequila industries showed similar carbohydrate profiles, containing approximately 31 to 36% cellulose, and 23 to 27% hemicellulose (Table 1a). After thermo-acid hydrolysis, we determined the percentage composition of the solid ABR. The percentage of cellulose increased approximately 5% in the three ABR samples because the hemicellulose percentage decreased (between 4 and 14%) with respect to their original composition (Table 1b). The higher cellulose and hemicellulose proportion was found in the bagasse produced from tequila industry using the diffuser process. Thermo-acid hydrolysis of the ABR shows that release of reduced sugars was dependent on the exposition time, acid concentration, temperature, and bagasse

kind (Figure 1a-c). The thermo-acid conditions that generated the higher sugar release for each kind of ABR residue are shown in Table 1c. To address if a longer time generated a higher sugar release, the conditions that generated the high sugar release were prolonged to 60 min (Figure 1d). The amount of reducing sugars released by the thermo-acid pretreatment was different for each ABR (Table 1d). The total amount of reduced sugars detected by the DNS method was from 0.66 to 3.6 g/L (Table 1d). The ABR that showed less sugar release was from the distillery that used the diffuser process (3.5% yield). Its sugar release was a concentration of 0.66 g/L that was kept even at 60 min. The ABR samples from the autoclaving process showed the best sugar release with the thermo-acid pretreatment (22% yield).

a)



b)



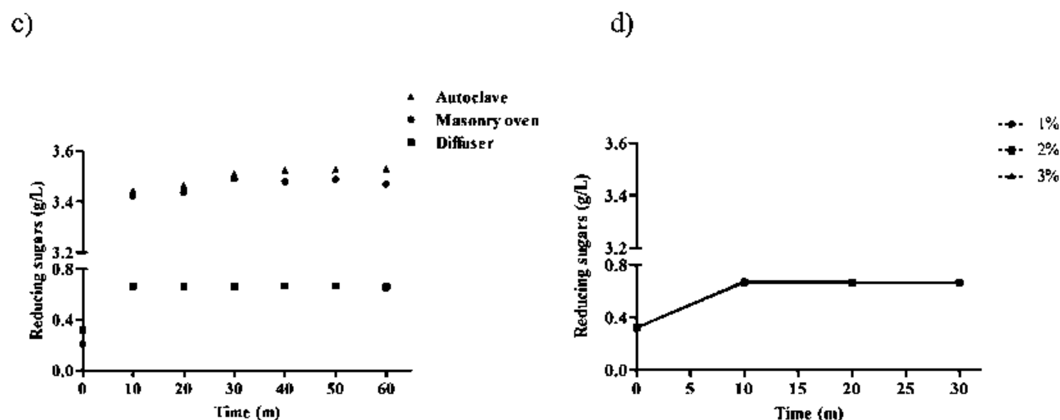


Figure 1. Effect of thermo-acid pretreatment on the release of reducing sugars from 1:10 w/v of ABR in H_2SO_4 (1, 2 and 3%, as mentioned in the figure) and temperature (°C): 110 (points lines); 130 (discontinuous lines); 140 (continuous lines). Exposure of 30 min to the autoclave (a), masonry oven (b), diffuser (c)) or 60 min with the best condition (d). The values are the means of three replicates + SD.

Table 1. Composition of dry ABR before (a) and after (b) thermo-acid hydrolysis. The conditions and concentration of higher sugar release (c and d, respectively). Percent of reducing sugars released (e).

a)	Dry matter before thermo-acid treatment (% w/w)		
	Autoclave	Masonry oven	Diffuser
Cellulose	31.43±0.93	33.33±0.54	36.03±1.4
Hemicellulose	26.16±2.2	23.88±2.6	27.12±1.5
b)	Dry matter after thermo-acid treatment (% w/w)		
Cellulose	36.19±1.3	37.62±1.6	40.06±1.2
Hemicellulose	12.83±0.59	15.55±0.84	18.71±0.76
c)	Conditions		
H_2SO_4 (%)	3	1	1
Temperature (°C)	110	130	110
Time (min)	40	30	10

d)	Higher sugars released (g/L) from 1:10 w/v of ABR in H_2SO_4		
	3.52±0.09	3.48±0.06	0.66±0.07
e)	Percent (% w/w)		
	22±0.38	16±0.53	3,5±0.22

The values are the means of three replicates ±SD.

Analysis of the liquid fraction after thermo-acid hydrolysis of the ABR by HPLC shows that the kind of sugars and organic acids produced was dependent on the ABR source (Figure 2a-b). Glucose was the main sugar released from ABR processing in the masonry oven and diffuser, while the main sugar was xylose for the autoclaved ABR. In all the samples the sugar concentration in the liquid fraction was higher after thermo-acid treatment (Figure 2a). After the thermo-acid treatment, the organic acids detected were succinic and acetic acids with concentrations of 2 to 4 g/L and 5 to 6 g/L, respectively. Citric, lactic, and malic acids were not detected. The masonry oven-processed ABR was the only sample with fumaric acid content (concentration <1 g/L). The ABR samples from the diffuser showed organic acid concentrations less than 1 g/L (Figure 2b).

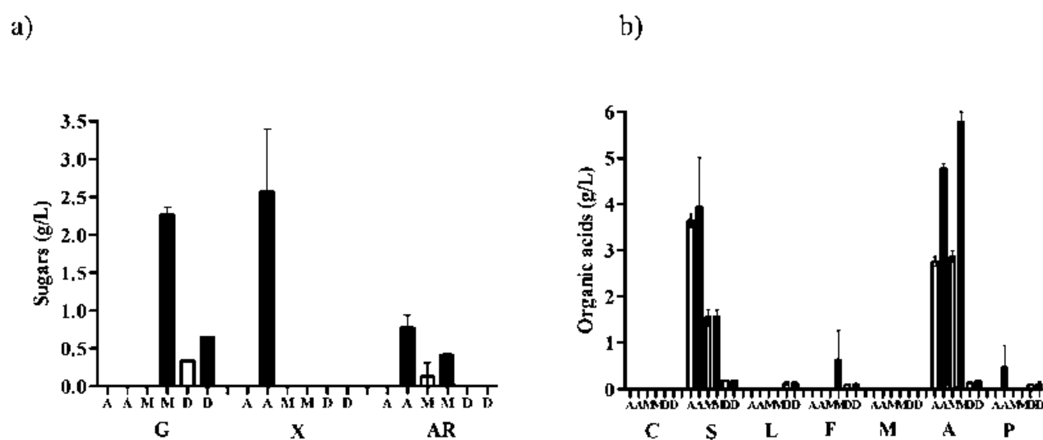


Figure 2. Kinds of sugars (a) and organic acids (b) released in the soluble fraction before

(white) and after (black) thermo-acid pretreatment of the ABR. ABR from the autoclave (A), masonry oven (M), and diffuser (D). Glucose (G); Xylose (X); Arabinose (AR). Citric (C); Succinic (S); Lactic (L); Fumaric (F); Malic (M); Acetic (A); Propionic (P). The values are the means of three replicates ±SD.

Enzymatic hydrolysis of agave bagasse residue after thermo-acid treatment

The solid and soluble fractions obtained from the thermo-acid treatment were used to carry-out enzymatic hydrolysis using commercial cellulases as described above. The combination of thermo-acid and enzymatic treatment resulted in the release of 10 to 15 g/L of reduced sugars detected by the DNS method. The higher yields were observed with the Rapidase enzyme (Table 2). The higher sugar release was at 48 h. In general, we did not observe a significant difference between the released sugar concentration in enzyme mix proportions of 1.5% and 15% at 48 h of treatment. Therefore, the enzymatic hydrolysis of the fermentation processes were run at 1.5% of the cellulase mix.

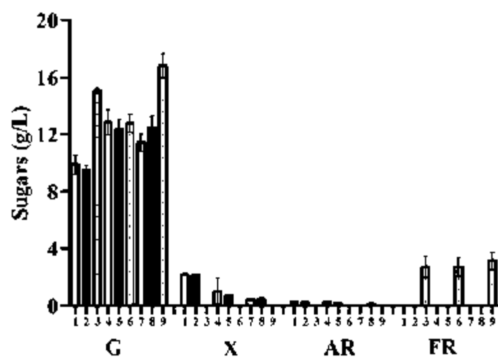
Table 2. Reduced sugars released after enzymatic hydrolysis of ABR pretreated with the thermo-acid method.

Reducing sugars (g/L) released at 48 h of enzymatic hydrolysis									
Enzyme	C			H			RAP		
ABR	A	M	D	A	M	D	A	M	D
0.15%	10±0.01	10±0.04	13±0.2	10±0.05	10±0.67	13±0.03	13±0.25	14±0.38	13±0.13
1.5%	12±0.32	12±0.25	13±0.03	12±0.08	11±0.46	13±0.01	14±0.69	15±0.11	13±0.05
15%	11±0.14	11±0.32	13±0.03	11±0.18	11±0.07	13±0.06	13±0.19	14±0.40	13±0.05

ABR from the autoclave (A), masonry oven (M), and diffuser (D). Enzymatic complex: CTec2 (C), HTec2 (H) and Rapidase (RAP) at a concentration of 0.15, 1.5 and 15 g of enzyme/g of ABR (dry weight). The values are the means of three replicates ±SD.

The kinds of sugars released at 48 h of the enzymatic hydrolysis are a function of the ABR source (Figure 3a). In the three kinds of ABR sources, the higher sugar proportions corresponded to glucose. In the samples obtained from tequila industries that use pine cooking treatment (in autoclave and masonry ovens), the second most abundant sugar was xylose, while in the samples treated industrially using the diffuser process, it was fructose. The arabinose sugar (less than 1 g/L) was detected in the autoclave and masonry samples, while no sugar was previously detected after the thermo-acid treatment (Figure 2a). The higher released glucose content was observed from the ABR of tequila industries using the diffuser process. The kinds of sugars and organic acids were determined in the soluble fractions rendered with thermo-acid and enzymatic hydrolysis. The organic acid accumulations at 48 h were citric, succinic, lactic, fumaric, acetic and propionic acids. These organic acids were found in the samples of the three ABR sources tested. The higher organic acid concentrations were observed in samples from ABR produced using the diffuser process, with acetic acid as the main organic acid with a concentration of 7 g/L, followed by succinic acid with 4 g/L (Figure 3b).

a)



b)

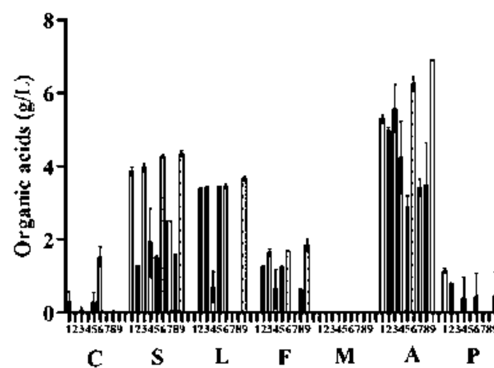


Figure 3. Sugars (a) and organic acids (b) present in the soluble fraction after thermo-acid and enzymatic hydrolysis of ABR at 48 h. Autoclave (white); Masonry oven (blank); Diffuser (with points). Enzymes: CTec2 (1,2,3); HTec2 (4,5,6); Rapidase (7,8,9). Sugars: Glucose (G); Xylose (X); Arabinose (AR); Fructose (FR). Organic acids: Citric (C); Succinic (S); Lactic (L); Fumaric (F); Malic (M); Acetic (A); Propionic (P). The values are the means of three replicates ± SD.

The percentages of cellulose and hemicellulose conversion to sugar released at 48 h of treatment using a cellulase mix at 1.5%, 40°C, and 100 rpm are shown in Table 3. In all the ABR samples, we observed an increase in the time dependent sugar release, whose content increased from 2 to 21% over 24 to 48 h of saccharification. With two of three enzymes used, the autoclave ABR showed that less theoretical sugars were released, at approximately 48%. The ABR from the masonry oven with the enzyme CTec2 showed higher theoretical sugar release of 69%. However, the other cellulase complexes were 51%, with an average of 57%. Independent of the cellulase complex used, the higher percent of theoretical reducing sugar release was with the ABR from the tequila industry where the diffuser process was used. This ABR showed a sugar release approximately 63% with respect to theoretical content (Table 3).

Table 3. Percent of reducing sugars released after thermo-acid and enzymatic hydrolysis of ABR. ABR from autoclave (A), masonry oven (M), and diffuser (D).

(%)									
Enzyme	C			H			RAP		
ABR	A	M	D	A	M	D	A	M	D
48 h	47±2.3	69±0.55	64±0.7	54±7.4	51±3.2	60±2.1	45±2.1	51±4.4	65±1.7

Enzymatic complex used: CTec2 (C), HTec2 (H) and Rapidase (RAP) at a concentration of 1.5 g of enzyme/g dry bagasse. The values are the means of three replicates ± SD.

Fermentation by SHF and SSF processes

After determination of the conditions for sugar saccharification with thermo-acid and enzymatic hydrolysis, the fermentations of hydrolysates were done. Since the OFF1 and Ethanol Red yeasts are not a thermotolerant as the SLP1 yeast, the non-thermotolerant yeasts were used in the comparative analysis. The fermentation processes were carried out at 40°C as mentioned in the materials and methods, and samples were taken at 48 h after yeast inoculation. The glycerol is a by-product of yeast fermentation. In our results, we observed variable glycerol production from 0 to 10 g/L. In the SHF and SSF processes where the CTec2 and HTec2 enzymes were used for the saccharification, the higher glycerol production was 2.6 g/L by the SLP1 yeast. In contrast, in the fermentations done with the product of the enzymatic hydrolysis by Rapidase, the glycerol production was from 3 to 10 g/L by the SLP1 yeast. The SLP1 yeast generated higher glycerol production, while that less was produced for the Ethanol Red yeast. Comparing between the SHF and SSF processes, a higher glycerol concentration was observed in samples taken from the SHF process (Table 4).

Table 4. Glycerol production (g/L) in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) after 48 h of yeast inoculation.

Yeast	SLP1								
Enzyme	C			H			RAP		
ABR	A	M	D	A	M	D	A	M	D

SHF	2.0±0.11	1.9±0.00	2.5±0.07	0.0±0.0	1.9±0.01	2.6±0.59	9.3±0.20	10±0.31	4.8±0.51
SSF	2.1±0.05	2.3±0.29	2.2±0.02	2.0±0.04	1.0±0.98	2±0.02	6.1±0.19	6.4±0.03	3±0.05
Yeast	OFF1								
SHF	0.97±0.97	0.97±0.97	2.60±0.2	2.1±0.20	0.97±0.97	2.4±0.23	9.1±0.24	9.9±0.87	4.2±0.02
SSF	2.0±0.02	2.2±0.05	2.4±0.01	2.0±0.01	2.2±0.19	2.5±0.04	6.9±0.17	6.2±0.18	3±0.08
Yeast	Ethanol Red								
SHF	1.9±0.0	0	0	2.0±0.05	1.0±1.0	2.4±0.18	8.5±0.27	6.9±2.0	3.3±0.41
SSF	2.3±0.05	2.2±0.22	2.4±0.02	2.2±0.04	2.2±0.15	2.2±0.09	8.4±0.33	7.9±0.80	3.2±0.14

ABR from the autoclave (A), masonry oven (M), and diffuser (D). Enzymatic complex: CTec2 (C), HTec2 (H) and Rapidase (RAP). The values are the means of two replicates ± SD.

The high ethanol concentration (10.36 g/L) generated at 48 h, whose ethanol yield was 85% with respect to the theoretical yeast, was obtained for the *K. marxianus* SLP1 yeast through SSF process, using autoclave ABR processing of the raw material and the CTec2 enzyme. Similar ethanol production was observed with the commercial *S. cerevisiae* yeast Ethanol Red (10.23 g/L), corresponding to 83% yield. The OFF1 yeast produced 10.12 g/L of ethanol with 80% yield. In most conditions, the *K. marxianus* yeast SLP1 through the SSF process showed higher ethanol yields with respect to that generated by the SHF process, which was approximately 80% (Table 5).

Table 5. Ethanol production (g/L), yield (%) and maximum theoretical sugars by g of ABR (g/g), in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) after 48 h of yeast inoculation.

Yeast	SLP1									
Enzyme		C			H			RAP		
ABR		A	M	D	A	M	D	A	M	D
SHF	Ethanol (g/L)	8.33±0.01	7.72±2.1	10±1.2	7.59±0.52	6.18±3.23	7.2±1.2	10.02±0.01	10.01±0.17	7.5±0.55
	Yield (%)	67±0.08	65±1.8	56±6.5	53±3.7	46±2.4	42±7	85±0.11	78±1.3	41±3

	Yield (g/g)	0.09	0.08	0.11	0.08	0.07	0.08	0.11	0.11	0.08
SSF	Ethanol (g/L)	10.36±0.77	9.63±2.03	7.5±0.2	8.74±1.22	7.12±1.18	8.3±0.47	6.47±1.65	9±0.49	10±0.88
	Yield (%)	85±6.6	84±1.8	40±0.1	80±1.1	71±1.0	52±2.9	74±1.3	80±4.4	59±4.9
	Yield (g/g)	0.11	0.11	0.08	0.1	0.08	0.09	0.07	0.1	0.12
Yeast	OFF1									
SHF	Ethanol (g/L)	8.06±0.41	7.32±1.62	9.8±3	8.75±1.14	7.67±0.32	8.6±4.9	9.76±1.84	10.12±1.53	6.4±0.24
	Yield (%)	65±3.4	62±1.4	54±1.6	61±8	58±2.4	51±1.9	83±1.6	80±1.2	35±1.3
	Yield (g/g)	0.09	0.08	0.11	0.1	0.08	0.09	0.11	0.11	0.07
SSF	Ethanol (g/L)	8.54±0.43	7.76±1.83	10±0.61	8.14±1.53	7±1.6	9.6±0.18	7.77±0.23	7.61±0.01	9.6±2.4
	Yield (%)	72±3.7	67±12	55±3.2	75±12	70±14	59±1.1	85±2.7	68±0.08	54±11
	Yield (g/g)	0.09	0.09	0.11	0.09	0.08	0.11	0.09	0.08	0.11
Yeast	Ethanol Red									
SHF	Ethanol (g/L)	8.25±0.03	8.49±0.18	4.8±0.04	8.12±1.06	6.47±0.57	8.7±2.3	8.66±0.82	6.74±1.15	6.9±1.7
	Yield (%)	66±0.26	72±1.6	26±0.2	57±7.4	49±4.3	51±12	73±7	52±8.8	37±7.2
	Yield (g/g)	0.09	0.09	0.05	0.09	0.07	0.1	0.1	0.07	0.08
SSF	Ethanol (g/L)	8.22±1.27	10.23±2.03	9±1.3	7.84±0.54	9.27±1.72	4.5±0.2	7.34±0.55	8.97±0.34	5.6±0.52
	Yield (%)	70±11	83±1.4	40±0.1	72±5	84±1.5	52±2.9	84±6.3	80±3.1	59±4.9
	Yield (g/g)	0.09	0.11	0.08	0.09	0.1	0.09	0.08	0.1	0.12

ABR from the autoclave (A), masonry oven (M), and diffuser (D). Enzymatic complex: CTec2 (C), HTec2 (H) and Rapidase (RAP). The values are the means of two replicates ± SD.

When the residual sugars were evaluated in the SHF and SSF processes at 48 h, a correlation between lower ethanol yields of the diffuser ABR source (Table 5) and residual sugars were observed. In the products of enzymatic hydrolysis by Rapidase using ABR from the autoclave and masonry oven processes, independent of the yeast strains or fermentation process, we did not detect residual glucose after SSF. In the rest of the experiments, the residual glucose was from 2 to 4 g/L. In some conditions, the two *K. marxianus* yeasts consumed the xylose and arabinose completely while in all the conditions

tested with the *S. cerevisiae* (Ethanol Red), these sugars are present. One of the main compounds that affect the fermentation times of compounds is of the furfural family. The concentration of furfural was evaluated in the fermentation processes as described above. Although the higher concentrations of furfural were less than 0.3 g/L, a direct relation between the presences of furfural, residual glucose and ethanol yield (Tables 5 and 6) was observed. In both the SHF and SSF processes, whose bagasse were produced from the diffuser treatment, higher values of furfural (0.28 g/L) were detected.

Discussion

Tequila production utilizes *Agave tequilana* Weber (blue agave), in which 40% (on wet weight) corresponds to the agave bagasse residue (ABR).[25] The potential use of the ABR for ethanol production has been considered in previous works; [3,4,5,6] however, in this process, the use of a thermotolerant yeast had not been considered, even when thermotolerant yeasts have been used to produce ethanol from other lignocellulosic materials. [26, 27, 28] Thermotolerant yeasts can reduce the production costs of biomass conversion to ethanol. [11, 29, 30] In bioethanol production from ABR, the process of simultaneous saccharification and fermentation (SSF) had also not been considered, which also has advantages. [31, 32, 33] Yang et al.[28] using a thermotolerant *K. marxianus* yeast through SSF, which shows a greater ethanol production than the *S. cerevisiae* angel yeast, showing the potential of bioethanol production through SSF using a thermotolerant yeast. In this work, ABR recollected from three tequila distilleries with different agave pines process were used. In these distilleries, the agave pines were autoclaved or were processed using a masonry oven or diffuser, and the processes are explained by Cedeño and Alvarez-Jacobs[1], and Casas.[24] On one hand, in the solid fractions of the ABR, the percent of

cellulose and hemicellulose were similar, even after thermo-acid treatment (Table 1a-b), whereas the percentage of hemicellulose was reduced, generating an increased cellulose percentage. This effect that had been reported when using H_2SO_4 and temperature.[34] On the other hand, according to our results (Figures 1-2), we suggested that the treatment by which the agave pines are processed in the tequila distillery could affect the concentration, as well as the kind of sugars and organic acids released by the thermo-acid treatment of the ABR sources. Jönsson and Martín[35] reported a minor lignin content in ABR, which is a reason why this compound was not investigated in this work. The theoretical percent of sugar released by the thermo-acid treatment used (Table 1d) was significantly less than that reported by Saucedo-Luna et al.[4], which used similar conditions. This result probably was due to the fact that our work did not invest energy in reducing the particle size, and we used all the ABR fibers. Due the tendency of the ABR to reduce their particle size, this required a high-energy investment or additional equipment that could generate increased costs in bioethanol production. As we observed in our results and in agreement with Woiciechowski et al.[36] for the use of industrial residual lignocellulosic biomass such as ABR, it is important that industrial processes vary the conditions of the residual biomass and affect the final yield of bioethanol production.

Commercial enzyme preparations have been used to convert lignocellulosic biomass to fermentable sugars.[3] Previously, Salcedo-Luna et al.[4] reported an increase in sugars released after thermo-acid treatment by an enzymatic hydrolysis, which released 73.6% of fermentable sugars from the ABR used. In this work, which used commercial cellulase complexes CTec2, HTec2 and Rapidase, we reported previously high enzymatic hydrolysis of lignocellulosic biomass.[37, 38] In contrast with the results obtained from the thermo-acid treatment where the sugars released were approximately 3 g/L, the use of cellulase

complexes generated an increase until 16 g/L of sugar was released (Figure 3a). In some samples, the amount of sugar detected by the DNS method and HPLC were different at approximately 3 g/L, due to the DNS method measuring only reduced sugars and HPLC measuring all sugars. Therefore, a slight disagreement could be expected.[39] After thermo-acid treatment of the ABR diffuser, the product showed lesser organic acid content (Figure 2b), which could contribute towards the high sugar release by enzymatic hydrolysis of this ABR (Table 3). In contrast, in the beginning of enzymatic hydrolysis of the ABR from the autoclave and masonry oven, organic acids were present (Figure 2b). This is probably the reason why the percent of reducing sugars released by enzymatic hydrolysis was less (Table 3), which could be because organic acids can reduce the activity of cellulase complexes.[40] High temperatures and long reaction times favor organic acid production.[6] This effect could generate the acids observed after 48 h of enzymatic hydrolysis of ABR from the diffuser source (Figure 3b).

Due to the viscosity of lignocellulosic materials for fermentation processes particularly in an SSF process, the biomass content is suggested to be limited to 10% (w/v).[41] This is a reason why one might do separate hydrolysis and fermentation (SHF) or SSF, which used 10% w/v of ABR. Moreover, bioethanol and compounds such as organic acids, volatile compounds and glycerol are generated in fermentations processes. In our results, a glycerol production up to 10 g/L by the SLP1 yeast was observed through SHF (Table 4). Glycerol has an essential role as a compatible solute during osmoregulation in yeasts.[42] This compound is synthesized by glycerol-3 phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp). Each enzyme has two isoenzymes, the constitutive and the osmotically induced.[43] The glycerol accumulation in the flasks could originate by organic acids, whose compounds could generate an acid-stress and, in consequence, induce synthesis of

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osmoprotectants, such as glycerol.

Through SSF, SLP1 yeasts produced higher ethanol concentration and conversion yields at 48 h after yeast inoculation. The thermotolerant *K. marxianus* CECT10875 yeast was used in three different fermentations processes, which demonstrated that SSF was the best fermentation process, since it could reduce end-product inhibition and avoid enzyme deactivation.[44] In our results, these factors, together with the intrinsic thermotolerance of the SLP1 yeast, probably contributed to the higher ethanol yield obtained by SSF using the SLP1 yeast. Although the SLP1 yeast showed better results than the OFF1 and Ethanol Red yeasts, the higher ethanol concentration generated in this work is less than that reported by Caspeta et al.[6] who reported a production of 64 g/L of ethanol from ABR after 9 h of Superstar yeast inoculation. Moreover, it is important to mention that they used a concentration of 20% w/w organosolv-pretreated solid ABR and attained enzymatic hydrolysis at 50°C, while sugar fermentations were performed at 37°C. In another work using ABR, 41 g/L of fermentable sugars were released by a thermo-acid and enzymatic hydrolysis. Through fermentation with the *Pichia caribbica* UM-5 yeast, Saucedo-Luna et al.[4] obtained a production of 18.3 g/L of ethanol with a total conversion yield of 0.18 g/g of dry ABR. Although the higher ethanol production by the thermotolerant SLP1 yeast is less than that reported by Saucedo-Luna et al.[4] and Caspeta et al.[6], the potential conversion of ABR to fuel ethanol by the SLP1 yeast could be optimized through the addition of nutrients, temperature and pH regulation, solid-liquid separations, and controlling others parameters that affected the ethanol yield in this work.

The metabolic state of yeasts used in fermentation processes has an important function in the ethanol production yield.[45] This metabolic state could be affected by the formation of toxic compounds,[46, 47] such as furfural. The furfural in the fermentation medium can cause

cell growth inhibition, DNA damage and inhibition of glycolysis enzymes, decreasing ethanol productivity.[48] Moreover, and the ABR diffuser showed higher sugar release by enzymatic hydrolysis (Table 3). Independent of the yeast strain used, lower ethanol yields were obtained with this hydrolysis (diffuser ABR), which was approximately 50% or even only of 26%, showing higher residual sugars. The less ethanol yield and higher residuals sugar could be the result of the higher furfural concentrations observed in these samples, where the values were 0.28 g/L. Although the furfural concentrations detected in this work are low, the toxic effect of this compound in the presence of organic acids could be increased.[49] Furfural functions as a thiol-reactive electrophile, reducing the level of cellular reduced glutathione, which causes an accumulation of reactive oxygen species.[50] Though the generation of yeast with a higher capability of detoxification and toleration to oxidative stress had been suggested,[51] these are possible traits of the SLP1 yeast strain. The importance of yeast resistance to inhibitor compounds produced in bioethanol fermentation had been reported.[52] In this respect, yeast strains naturally exposed to stress conditions, such as those isolated from spontaneous ethanol processing, such as the SLP1 yeast, in addition to their thermotolerance, could make ethanol production possible, similar to the commercial yeast Ethanol Red. In the same way as furfural, the presence of another compound, such as organic acids or volatiles compounds produced by yeast metabolism, could negatively affect the metabolic state of the yeast, reducing the total ethanol production yield. However, these compounds are not usually evaluated through the fermentation of lignocellulosic biomass. Lastly, we suggested to deep in the study of these compounds.

The data presented in this work show the importance of the processing of agave pines are because these processes can influence the proportion of sugars released and inhibitory yeast

compounds produced by the ABR. According to our results, we suggest to focus the work on ethanol production from ABR to SSF with selected thermotolerant yeasts strains, with an effort toward robust stress resistance and high ethanol production.

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Capítulo 5:

Organic compounds generated in bioethanol production from agave bagasse

Este capítulo ha sido sometido para su revisión como:

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Organic compounds generated in bioethanol production from agave bagasse

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Abstract:	In bioethanol production, lignocellulosic residue fermentation generates byproducts, such as organic compounds (OCs). OCs have been thoroughly studied in the wine and beer industry, but little is known about OCs in the bioethanol industry, even though they affect the physiological state of yeasts and are considered to be economically desirable in the chemical industry. In this study, we evaluated the production of OCs in bioethanol production processes of different agave bagasse residues (ABR): i) separate hydrolysis and ii) fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Fermentation was conducted using the Kluyveromyces marxianus SLP1, and OFF1 strains, and Saccharomyces cerevisiae Ethanol Red yeast. The OCs that were majority detected included ethyl acetate, methanol, 1-propanol, isobutanol, butanol, isoamyl-alcohol, ethyl-lactate, furfuryl-alcohol, phenyl-acetate, and 2-phenyl ethanol. High content of OCs was found in the SSF process when K. marxianus OFF1 and SLP1 yeasts were used. This study provided information regarding the types and concentrations of OCs produced by fermentation of lignocellulosic Agave bagasse residues, which could lead to using bioethanol byproducts as potential sources of economically desirable compounds.

Title:

Organic compounds generated in bioethanol production from agave bagasse

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Abstract

In bioethanol production, lignocellulosic residue fermentation generates byproducts, such as organic compounds (OCs). OCs have been thoroughly studied in the wine and beer industry, but little is known about OCs in the bioethanol industry, even though they affect the physiological state of yeasts and are considered to be economically desirable in the chemical industry. In this study, we evaluated the production of OCs in bioethanol production processes of different agave bagasse residues (ABR): i) separate hydrolysis and ii) fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Fermentation was conducted using the *Kluyveromyces marxianus* SLP1, and OFF1 strains, and *Saccharomyces cerevisiae* Ethanol Red yeast. The OCs that were majority detected included ethyl acetate, methanol, 1-propanol, isobutanol, butanol, isoamyl-alcohol, ethyl-lactate, furfuryl-alcohol, phenyl-acetate, and 2-phenyl ethanol. High content of OCs was found in the SSF process when *K. marxianus* OFF1 and SLP1 yeasts were used. This study provided information regarding the types and concentrations of OCs produced by fermentation of lignocellulosic Agave bagasse residues, which could lead to using bioethanol byproducts as potential sources of economically desirable compounds.

Key words: Agave bagasse; Bioethanol; By-products; Fermentation; Organic compounds

Introduction

Yeasts are used to produce chemicals, pharmaceuticals, and other products, such as bioethanol (Wang et al. 2001; Johnson 2013; Liu et al. 2013; Carlquist et al. 2015). Bioethanol production using lignocellulosic wastes is considered to be a promising process (Tesfaw and Assefa 2014). The agave bagasse residue is a lignocellulosic waste that has potential for bioethanol production (Hernández-Salas et al. 2009; Saucedo-Luna et al. 2011; Perez-Pimienta et al. 2013; Caspeta et al. 2014). However, few studies have been conducted on the other organic compounds produced in the fermentation process of lignocellulosic residue. On the one hand, OCs are toxic for sugar yeast fermentation, which leads to decreased ethanol yield (Urit et al. 2013; Morrissey et al. 2015), but on the other hand, OCs have multiple industrial applications, such as food additives, pharmaceutical, and cosmetic excipients (Carlquist et al. 2015). In yeast fermentation, OCs, such as esters, aldehydes, ketones, carbonyls, furans, and terpenes, are

produced (Carlquist et al. 2015). The OCs production is affected by pH, temperature, carbon source, and yeast strain (Molina et al. 2009; Olaniran et al. 2011; Gethins et al. 2015). The *Kluyveromyces marxianus* genus has high potential for industrial production of OCs as volatiles compounds (Morrissey et al. 2015) as well as fast growth rate (Groeneveld et al. 2009) and GRAS status.

The aim of this study was to determine which OCs types are obtained through fermentation of hydrolyzed ABR by using different methods in the process, such as separate hydrolysis fermentation and simultaneous saccharification and fermentation stages, using the native yeast strains of *K. marxianus* SLP1 and OFF1 and comparing the results to a common used industrial yeast, *S. cerevisiae* (Ethanol Red). To the best of our knowledge, this is the first study to report on the organic compounds produced in the fermentation of lignocellulosic ABR as a source of desirable chemicals obtained of bioethanol process, suggesting it as a potential way to produce economically desirable OCs.

Materials and Methods

Yeast strains

Yeast strains were obtained from the culture collection of the CIATEJ (*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, México*) (Gschaedler et al. 2004) and from the ATCC (American Type Culture Collection, Rockville, MD, USA). The *K. marxianus* yeasts strains (SLP1 and OFF1) were isolated at handcraft mezcal distilleries in the Mexican State of San Luis Potosi and Guerrero, respectively. The Ethanol Red yeast was acquired from the ATCC.

Agave bagasse residue and saccharification process

The agave bagasse residues, which were classified as masonry oven, autoclave and diffuser, were obtained from the distilleries "Casa de Piedra", "Gonzalez-Gonzalez", and "La Madrileña", respectively. The three distilleries are located in Jalisco, Mexico. The tequila industries have different pine agave treatments, as described by Cedeño-Cruz (2003) and Casas (2006). The ABR received a thermo-acid treatment that led to higher sugar release under conditions that were previously determined (Table 1). Enzymatic hydrolysis was conducted using the commercial cellulases complexes CTec2, HTec2, and Rapidase (Novo enzymes) at 1.5 g of enzyme/g ABR (dry weight).

Fermentation processes

The enzymatic hydrolysates of ABR were fermented through separate hydrolysis and fermentation or simultaneous saccharification and fermentation using the *K. marxianus* yeasts SLP1 and OFF1 or the *S. cerevisiae* Ethanol Red. Then, 1 X 10^7 cells/ml suspensions were added to the enzyme mix or to enzymatic hydrolysates of ABR after 48 h, and then were added to SSF or SHF, respectively. Fermentation was performed at 40°C and 100 rpm. Samples were taken at 24 h and 48 h after yeast inoculation. The OCs were detected by gas chromatography as mentioned below.

Analysis of organic compounds

The OCs determination was carried out, as described previously (Flores et al. 2013). Briefly, after 48 h of fermentation, the OCs were quantified using a Hewlett–Packard 6890 gas chromatograph (Palo Alto, CA, USA) with a flame ionization detector (FID) equipped with an HP-Innowax PEG column (60 m, 0.320 mm). The initial column temperature was 45°C, then ramped up at 10°C/min to 160°C, followed by a 20°C/min ramp to 220°C, maintained over 4 min. Injector and detector temperatures were maintained at 250°C. The injection system includes a headspace sample (Hewlett–Packard 7694E). The preparation program and injection sample started with the vial temperature at 80°C the loop temperature at 110°C. and the transfer line temperature at 115°C. The cycle time of the headspace and gas chromatograph was 40 min with vial and an equilibrium time of 5 min, pressurization time of 0.2 min, a filling loop time of 0.2 min, a loop equilibrium time of 0.5 min, and an injection time and agitation time of 1 min. The OCs measured in this study were ethyl-acetate, methanol, ethyl-butyrate, 1-propanol, isobutanol, isoamyl-acetate, butanol, isoamyl-alcohol, ethyl-hexanoate, ethyl-lactate, ethyl-octanoate, ethyl-decanoate, furfuryl-alcohol, phenyl-acetate, and 2-phenylethanol. The external standards that were used for each compound were purchased from Sigma Aldrich.

Yeast cell growth

Counting cells in a Neubauer chamber determined cell number by a sample taken at 24 h and 48 h after yeast fermentation. The counting was conducted according to the methods of Strober (1997).

Results

Total organic compounds produced

The *K. marxianus* OFF1 produced a higher number of total organic compounds (TOCs) at higher concentrations than the other yeasts. TOCs concentrations ranged from 11 to 374 mg/L, and SLP1 cells generated the highest concentration (Table 2). Although the higher TOCs concentrations were produced through SHF (Table 2), more OCs were generated in the SSF process. The compounds ethyl acetate, 1-propanol, isobutanol, butanol and isoamyl-alcohol were detected in the SSF of autoclave source ABR, whereas in the SHF of the same ABR no OCs were detected. In general, a smaller number of OCs as well as TOCs concentrations were detected in the fermentation of masonry oven ABR compared to fermentation of autoclave and diffuser ABR (Table 2). The phenyl-acetate and furfuryl-alcohol were not detected in the fermentation of diffuser ABR, while the rest of the samples had variable concentrations approximately 15 and 50 mg/L, respectively. The ethyl-acetate concentration was most influenced by the type of ABR kind, and when the autoclave ABR was used, ethyl acetate was detected only in the SSF process. For the masonry oven ABR, the presence of ethyl acetate was variable, whereas it was present in all samples generated with the diffuser ABR. Other OCs present in all of the samples included methanol and 2-phenylethanol (Table 3).

Higher concentrations of OCs detected

Ten OCs with concentrations smaller than 300 mg/L were detected (Table 3). The OC with the highest concentration was ethyl acetate (249 mg/L), followed by 2-phenylethanol (75.31 mg/L). The *K. marxianus* yeasts SLP1 and OFF1 produced a higher number of OCs and higher concentrations of seven out of the ten compounds that were detected. The methanol, butanol and ethyl-lactate were the OCs produced in higher concentrations by the *S. cerevisiae* Ethanol Red yeast. While *K. marxianus* cells generated 70% of the higher OC concentrations, 60% were generated through SSF.

The compounds found in higher concentrations in SHF included methanol, ethyl-lactate, furfuryl-alcohol and 2-phenylethanol. The enzymatic complex showed a significant influence on higher concentrations and 60% of the higher OCs concentrations were generated from hydrolysates by the Rapidase enzyme (Table 3).

Yeast growth

In eleven of the eighteen different fermentation conditions that were generated, the SLP1 yeast increased their cell number at 48 h with respect to 24 h of fermentation. In the seven rest conditions, they preserved their cell numbers at 48 h. In contrast, the *K. marxianus* OFF1 yeast showed a reduction in cell number from 24 h to 48 h of fermentation in six conditions. The industrial *S. cerevisiae* yeast (Ethanol Red) showed a reduction in cell number in three samples, preserved its cell number in ten conditions, and increased the cell number in five conditions out of the eighteen that were compared at the 48-h time point after 24 h of fermentation (Table 4).

Discussion

The utilization of lignocellulosic wastes as a substrate in bioprocesses has increased in recent years due to their renewable capacity, low price, and abundance (Girio et al. 2010; Isikgor and Becer 2015). In Mexico, lignocellulosic agave bagasse is produced in the tequila industry as waste product at a rate of 360 thousand dry tons (Cedeño-Cruz 2003; Tequila Regulatory Council 2010). The agave bagasse residue (ABR) has been reported as a promising lignocellulosic biomass for the production of fermentable sugars (Saucedo-Luna et al. 2011) and bioethanol (Hernández-Salas et al. 2009; Saucedo-Luna et al. 2011; Perez-Pimienta et al. 2013; Caspeta et al. 2014; Barrera et al. 2016). However, there has been little study of bioethanol byproducts from ABR.

Organic compounds (OCs) are produced in secondary yeast metabolism and can be considered bioethanol contaminants (Styarini et al. 2013), which could be toxic to yeast (Rossouw et al. 2009; Urit et al. 2013; Morrissey et al. 2015). Moreover, these compounds have possible industrial uses with high commercial potential in the food, cosmetics, detergent and pharmaceutical industries (Carlquist et al. 2015). *K. marxianus* yeasts produced some of the best organic volatiles compounds (Morrissey et al. 2015), and using these yeasts to produce these compounds through cassava bagasse fermentation is very feasible (Medeiros et al. 2001). As well as cassava bagasse, sugar cane bagasse has been used for organic volatiles compounds production (Bramorski et al. 1998; Soares et al. 2000), and ABR had similar results.

In this work, we studied the OCs produced as bioethanol byproducts from ABR fermentation using two *K. marxianus* yeasts (SLP1 and OFF1) and the industry yeast *S. cerevisiae* (Ethanol Red). Ethanol production from lignocellulosic biomass, such as ABR, includes four main steps: pre-treatment,

saccharification, fermentation and distillation (Tomas-Pejo et al. 2009). This study focused on the OCs produced in the fermentation step using the ABR after saccharification.

In our results, we detected a smaller number of OCs than the number reported by López-Alvarez (2012) when the *K. marxianus* yeast UMPe-1 was used to ferment agave. This effect arose because the agave must have higher sugar concentration than the ABR (Hernández-Salas et al. 2009), and a major sugar concentration can result in a higher concentration of OCs (Ali et al. 2010).

Tequila industries processed the agave pines by autoclave, masonry oven or diffuser, as described by Cedeño-Cruz (2003), and Casas (2006). We observed that the agave pine process could affect the TOCs concentrations as well as the type of OCs that were produced (Table 2). This effect could be the result of the type and concentration of sugars in the ABR after the agave pine was processed.

The compound ethyl-acetate is a solvent with many industrial applications. *K. marxianus* yeasts have some of the highest potential for producing ethyl-acetate at an industrial scale (Löser et al. 2014). In this study, the highest ethyl-acetate concentration was 249 mg/L, which was smaller than the concentration reported for use a *K. marxianus* growth inhibitor (17 g/L) (Urit et al. 2013). Additionally, copper limitation can increase ethyl-acetate synthesis in *Kluyveromyces* yeasts (Löser et al. 2015).

Yeasts are considered the most promising producers of 2-phenylethanol, which is one of the more commercially used organic volatiles compounds due to its rose-like aroma (Wittmann et al. 2002), and it was the second most abundant compound (Table 3). Although *Kluyveromyces* strains are considered to be good producers of this compound (Kim et al. 2014), their resistance to this compound is lower compared to *S. cerevisiae* (Etschmann and Schrader 2002). A concentration of 2 g/L of 2-phenylethanol is toxic to *K. marxianus* yeasts, and ethanol generates a synergistic interaction that amplifies its cytotoxicity (Wang et al. 2011). The higher 2-phenylethanol concentration produced in this work was 75 mg/L, which was similar to the amount produced using a molasses-based medium and *K. marxianus* CBS 600 (89 mg/L) in the study by Etschmann et al. (2003). The levels of this compound could be increased through addition of exogenous L-phenylalanine (Etschmann et al. 2002; Etschmann et al. 2005) or through solid-phase *in situ* product removal (Gao and Daugulis, 2009).

The concentrations of higher alcohols detected in the ABR fermentations ranged from 3.69 to 34.81 mg/L. The 1-propanol and isobutanol were detected in an agave tequila fermentation using the same *K. marxianus* yeasts used in our study (Arellano et al. 2012). While isobutanol can be used for production of bio-based product packaging (Peters), butanol is considered a fuel additive (Lee et al. 2008). The butanol

concentration (20 mg/L) detected in the "Tequila Blanco" beverage obtained using the *K. marxianus* UMPe-1 (López-Alvarez et al. 2012) was higher than the amount detected using ABR (3.69 mg/L). With the exception of butanol, the *K. marxianus* yeasts in this study led to higher production of alcohols compared to the *S. cerevisiae* yeast, which agrees with the results reported by López-Alvarez et al. (2012). Although amino acid availability influenced higher alcohol production, the uptake and assimilation of these substrates determined the final concentration (Carlquist et al. 2015). According to our results, the uptake and amino acid assimilation could be better in the *K. marxianus* yeasts (SLP1 and OFF1) compared to the *S. cerevisiae* yeast (Ethanol Red).

Ethyl-lactate, which has been used as a solvent and "building block" to produce degradable plastic polymers (Zlokazov and Veselovsky 2000), was not found in the fermentation with SLP1 yeast, while the Ethanol Red yeast produced 2 mg/L and the OFF1 yeast produced 60 mg/L. This final concentration was similar to the amount reported by Arellano et al. (2012) during mezcal fermentation using the same yeast strain (OFF1). When López-Alvarez et al. (2012) compared the production of this compound between the *K. marxianus* UMPe-1 and the *S. cerevisiae* baker's Pan1 yeast, the *S. cerevisiae* yeast produced seven times more ethyl-lactate than the *K. marxianus* yeast, which did not correlate with the results obtained in this study because the production by the *K. marxianus* OFF1 was higher than the amount produced by the *S. cerevisiae* yeast Ethanol Red. Due to this difference, we suggested that the production of ethyl-lactate could depend heavily on the yeast strain that is used, even if the yeasts are in the same genus.

While the *K. marxianus* SLP1 yeast showed more adaptability and maintain their cell number after 48 h of fermentation, the yeast strain with less adaptability was OFF1, which had a reduction in cell number after SHF and higher concentrations of furfuryl-alcohol were detected (Table 4). Although the concentrations of OCs detected were smaller than the toxic values of the yeast, the OCs mix could exert a synergic toxic effect with other stress conditions, such as pH and temperature (Belloch et al. 2008; Wang et al. 2011). Yeast robustness and physiological fitness is very important for an efficient fermentation process (Carlquist et al. 2015). A promising approach for adapting yeasts to produce OCs could be investigating membrane fatty acids because the membrane is one of the first targets of OCs (Mannazzu et al. 2008).

In this study, we found that ABR fermentation could be a source of OCs. Additionally, immobilized cells of *S. cerevisiae* produced a higher amount of OCs compared cells in suspension (Lalou et al. 2013), and Rossi et al. (2009) obtained a higher overall concentration of OCs when different carbon and nitrogen

sources were mixed. Therefore, OCs production as bioethanol byproducts from ABR could be improved through the use of similar techniques. In addition, continuously removing OCs from ABR fermentation is an option to reduce their toxic effect and increase bioethanol production.

According with the results of this study, acetate esters and higher alcohols are generated in bioethanol production from ABR, and higher concentrations were obtained through SSF by *K. marxianus* yeasts. Although bioethanol production from ABR is as potential source of byproducts that are economically desirable, improving the production process should be considered.

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Table 1 Conditions for the agave bagasse residue thermo-acid treatment.

ABR	Autoclave	Masonry oven	Diffuser
H_2SO_4 (%)	3	1	1
Temperature (°C)	110	130	110
Time (min)	40	30	10

ABR, Agave Bagasse Residue.

Table 2 Total organic compounds (TOCs) in separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) of agave bagasse residue (ABR).

TOCs (mg/L)																		
Yeast	SLP1						OFF1						Ethanol Red					
Enzyme	C		H		RAP		C		H		RAP		C		H		RAP	
Process	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF	SHF	SSF
a)	214	71	150	113	122	175	134	66	135	95	106	184	150	43	136	33	106	203
b)	137	110	158	150	176	34	260	56	61	218	139	196	216	125	168	218	136	11
c)	122	99	158	125	188	374	43	75	112	205	94	237	150	107	216	38	176	36

ABR hydrolysates from an autoclave (a), masonry oven (b) and a diffuser (c). The fermentations were carried out in flasks heated at 40°C and shaking at 100 rpm. Enzyme: C (CTec2), H (HTec2) and RAP (Rapidaze). Samples were taken after 48 h of fermentation using 1.5 g of enzyme/g ABR (dry weight).

Table 3 Higher concentrations of the organic compounds (OCs) were detected.

OCs	Concentration (mg/L)	Yeast	Process	ABR	Enzyme
Ethyl-acetate	249,38	S	SSF	D	RAP
Methanol	45,97	ER	SHF	D	RAP
1-propanol	12,56	O	SSF	A	RAP
Isobutanol	24,52	O	SSF	A	RAP
Butanol	3,69	ER	SSF	A	RAP
Isoamyl-alcohol	34,81	O	SSF	A	RAP
Ethyl-lactate	65,17	ER	SHF	M	C
Furfuryl-alcohol	57,05	O	SHF	A	C
Phenyl acetate	17,61	S	SSF	M	C
2-phenyl ethanol	75,31	S	SHF	M	C

Yeasts: SLP1 (S), OFF1 (O) and Ethanol Red (ER). Processes: Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Agave bagasse residue (ABR): A (autoclave); M (masonry oven); D (diffuser). Enzymatic complex: CTec2 (C) and Rapidaze (RAP).

Table 4 Number of yeast cells (1 X 10^6 cells/ml)

Yeast	SLP1																	
Enzyme	C						H						RAP					
ABR	A		M		D		A		M		D		A		M		D	
Time (h)	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
SHF	266±15	336±49	18±7	24±11	153±23	171±58	203±55	392±67	22±9	47±23	57±23	125±56	57±27	117±65	21±11	29±16	48±26	305±40
SSF	103±26	150±21	89±18	94±43	139±26	202±26	94±24	137±21	53±14	59±6	53±20	322±56	89±19	121±36	36±16	41±7	49±11	133±19
Yeast	OFF1																	
SHF	62±17	79±26	18±8	59±11	98±25	176±35	69±28	29±11	72±17	54±16	77±15	93±21	89±24	89±53	41±25	32±11	62±18	162±44
SSF	75±32	45±25	141±45	57±31	94±20	81±30	126±35	50±17	63±24	100±33	70±14	157±36	167±38	265±74	81±41	81±24	70±26	189±56
Yeast	Ethanol Red																	
SHF	462±80	227±80	33±10	15±5	383±77	54±19	54±39	99±50	41±11	52±25	71±29	180±45	62±24	236±54	22±7	42±11	152±40	348±67
SSF	244±51	286±79	33±5	51±18	160±61	91±46	57±21	69±24	41±7	52±17	92±41	97±54	142±57	268±75	42±12	62±11	66±30	149±56

The cells were counted after 24 h and 48 h of separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). Agave bagasse residue (ABR): A (autoclave); M (masonry oven); D (diffuser). Enzymatic complex: CTec2 (C), HTec2 (H) and Rapidaze (RAP).

Capítulo 6:

Levaduras Termotolerantes: Aplicaciones Industriales, Estrés Oxidativo y Respuesta Antioxidante.

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Levaduras Termotolerantes: Aplicaciones Industriales, Estrés Oxidativo y Respuesta Antioxidante

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Resumen

Se presenta una revisión sobre características y aplicaciones de levaduras termotolerantes, enfocada a aplicaciones industriales y cambios fisiológicos que experimentan las levaduras, con especial énfasis en el estrés oxidativo y la respuesta antioxidante. Las levaduras tienen una amplia aplicación en la biotecnología y son los microorganismos más utilizados en la investigación médica y en la industria. Uno de los usos tradicionales de las levaduras es la fermentación, mediante la cual se pueden obtener bebidas, alimentos y proteínas, entre otros productos. Sin embargo, en este proceso como en otros en los que son utilizadas las levaduras, se generan altas temperaturas que provocan cambios fisiológicos, por lo que se afecta su crecimiento y viabilidad. Lo anterior, ha generado interés en el estudio y desarrollo de levaduras capaces de crecer a temperaturas elevadas (termo tolerantes). Por lo tanto, es de interés caracterizar cepas de levaduras que se utilicen en procesos de fermentación sometidos a altas temperaturas.

Palabras claves: levaduras termotolerantes; estrés oxidativo; respuesta antioxidante; biotecnología

Thermotolerant Yeast: Industrial Applications, Oxidative Stress and Antioxidant Response

Abstract

A review on characteristics and applications of thermo tolerant yeast, focused on industrial applications and physiological changes that experienced by yeasts, with particular emphasis on oxidative stress and antioxidant response, is presented. Yeasts are widely applied in biotechnology and are the microorganisms most used in medical research and industrial processes. One of the traditional uses of yeast is fermentation, by which beverage, foods and proteins, among others products, can be obtained. However, in this process like in others that use yeasts, high temperatures are generated, causing physiological changes, affecting their growth and viability. This has generated an interest in studying and formulating yeasts capable of growing at elevated temperatures (thermo tolerant yeast). Therefore, it is important to characterize yeast strains to be used in fermentation processes at high temperatures.

INTRODUCCIÓN

Desde la antigüedad, las levaduras se han reconocido como protagonistas en la producción de alimentos y bebidas mediante la fermentación. Actualmente, son utilizadas en diferentes áreas de la biotecnología, en la tabla 1 se mencionan ejemplos. Aunado a que son un modelo de estudio de células eucariontes las levaduras son los microorganismos más importantes en la biotecnología (Johnson, 2013). Al igual que todos los microorganismos, las levaduras presentan características específicas de acuerdo a la temperatura a la que son cultivadas. Recientemente, se ha generado un creciente interés en las levaduras capaces de crecer a temperaturas elevadas, ya que presentan ventajas en distintos procesos industriales con respecto a las levaduras que no tienen esta cualidad (Koedrit *et al.*, 2008). Las levaduras resistentes a altas temperaturas son denominadas levaduras termotolerantes, sin embargo, no existe un valor absoluto de temperatura, ya que los límites a partir de los cuales se consideran levaduras termotolerantes varían en la literatura. El primer intento por definir levadura termotolerante fue realizado por Arthur y Watson (1976). McCracken y Gong (1982), definen estas levaduras como aquellas con una temperatura de crecimiento máxima de 37 a 45°C. Koedrith *et al.* (2008), definieron las levaduras termotolerantes como aquellas capaces de crecer a temperaturas ≥ 40°C.

Las altas temperaturas generadas durante el uso industrial de levaduras elevan la susceptibilidad de estos microorganismos hacia compuestos inhibitorios del crecimiento (Abdel-Banat *et al.*, 2010), e intensifican el efecto inhibitorio del etanol, por lo que las levaduras termotolerantes podrían presentar una mayor resistencia a estas condiciones. Kwon *et al.* (2013), reportaron que la cepa termotolerante *Isshatchenkia orientalis* presentó una tolerancia de hasta 96.7 gL^{-1} de etanol, siendo el mayor nivel de tolerancia observado en una cepa de levadura crecida a temperaturas de 40 a 45°C. Las ventajas generales del uso de estas levaduras en los procesos industriales se mencionan en la tabla 2. A continuación, se describen aplicaciones y ejemplos de levaduras termotolerantes en procesos industriales donde presentan ventajas, con respecto a las levaduras que no tienen esta característica, así como una recopilación de los principales cambios fisiológicos que experimentan estas levaduras, enfocado al estrés oxidativo y respuesta antioxidante.

Tabla 1. Industria y usos de las levaduras (Tomado y modificado de Johnson, 2013).

Fermentaciones tradicionales	Producción de cerveza, vino, sake y salsa de soya
Alimentaria	Producción de enzimas, saborizantes, pigmentos, aminoácidos y ácidos orgánicos
Biocatalisis	Estudio de farmacéuticos e intermediarios químicos
Biotecnología ambiental	Aplicación en biorremediación y degradación de contaminantes
Biocontrol	Protección de cultivos, alimentos y probióticos
Producción de proteínas heterólogas	Producción de proteínas farmacéuticas, enzimas, hormonas, vacunas y toxinas
Investigación en biología	Estudio de biología molecular y celular, genómica, vías de ingeniería y mecanismos de sistemas biológicos
Investigación biomédica	Descubrimiento, resistencia y metabolismo de drogas, así como elucidación de mecanismos de enfermedades

Tabla 2. Ventajas del uso de levaduras termotolerantes en procesos industriales (Fernández *et al.*, 2008).

Reducción de contaminación y costos de enfriamiento.
Mayor viabilidad, actividad metabólica y velocidad de fermentación.
Mayor mantenimiento de condiciones anaerobias al disminuir la solubilidad del O_2.
Disminución de la viscosidad del medio de fermentación.
Reducción en la formación de subproductos indeseables debido a lisis celular.

Producción de biomasa a partir de levaduras termotolerantes

La producción de biomasa de levaduras, es un proceso económicamente importante y una industria creciente en los últimos años, debido a un aumento en la demanda para las prácticas de la industria vinícola, procesos de manufactura de pan y como complemento en la dieta. La biomasa obtenida de levaduras tiene otras aplicaciones como suplemento proteico en alimentación animal, en la fabricación de ingredientes funcionales y para resaltar el sabor de alimentos procesados (Lee, 1996). El proceso industrial para la producción de biomasa de levaduras, consiste en la propagación de las levaduras de un cultivo en agar a su propagación

en bioreactores, incrementando el volumen en cada fase de propagación (Di-Serio *et al.*, 2001). Una transformación eficiente de azúcares a biomasa requiere que la producción de metabolitos como etanol y acetaldehído sea minimizada, desviando su metabolismo celular hacia el metabolismo oxidativo, aumentando el rendimiento de la energía en forma de ATP y con esto, la formación de biomasa (Van *et al.*, 1998). Con el objetivo de conservar la biomasa, las levaduras son expuestas a altas temperaturas en un proceso de deshidratación, por ejemplo, la levadura residual de la industria cervecera, la cual después de ser sometida al proceso de deshidratación, es vendida como alimento para animales (Ferreira *et al.*, 2010). Ghorbani *et al.* (2008), expusieron la biomasa de levadura a altas temperaturas con el objetivo de incrementar la bioabsorción de Cd (cadmio), observando un aumento de hasta 200%. Sin embargo, tanto en este proceso como en la deshidratación para la conservación de biomasa, las moléculas de agua son removidas y las temperaturas incrementan. Lo que afecta la viabilidad y vitalidad de las células (Matthews y Webb, 1991).

La deshidratación genera arresto del ciclo celular y daño a membranas y proteínas (Singh *et al.*, 2005). Por lo anterior, la tolerancia de las levaduras hacia la temperatura es crítica en el mantenimiento de sus componentes celulares y su vitalidad después del proceso de deshidratación. Uno de los principales factores limitantes en la generación de biomasa de levadura, es el alto costo de las fuentes de carbono, por lo que el uso de subproductos agroindustriales es ideal (Zheng *et al.*, 2005). Dichos subproductos deben recibir un tratamiento previo, siendo la exposición a altas temperaturas uno de estos tratamientos. Escalante *et al.* (1990), analizaron la producción de biomasa por la levadura resistente a temperatura *Hansenula polymorpha* en un proceso lignocelulósico realizado a 45 °C, observando que es posible obtener mejores resultados al llevarlo a cabo en altas temperaturas. La cepa termotolerante de *Kluyveromyces marxianus* CBS 712, fue utilizada para la producción de biomasa a partir de suero de leche, un desecho agroindustrial. En los resultados Zoppellari y Bardi, (2013), sugieren el uso de esta cepa para la obtención de biomasa a partir de este desecho, lo que podría reducir los costos de eliminación de efluentes.

Utilización de levaduras termotolerantes como agentes prebióticos y probióticos

Los prebióticos son ingredientes de alimentos no digestibles que estimulan el crecimiento de bacterias ácido láctico y bifidógenos en el tracto gastrointestinal. Ciertas especies de levaduras, se han utilizado como agentes prebióticos y probióticos para la prevención o el tratamiento de diversos padecimientos intestinales, nutricionales y trastornos toxicológicos (Mosiehi-Jenabian *et al.*, 2010). Los oligosacáridos de la pared celular de las levaduras han demostrado ser un prebiótico de alto valor. Estudios en el uso de las levaduras como probióticos, están relacionados con la generación de levaduras recombinantes con mutaciones en los genes *SRB1/PSA*1 y *PKC1*, genes involucrados con la formación de mananos y glucanos de la pared celular. La mutación de estos genes sensibiliza a la levadura a las condiciones del estomago, permitiendo que estas levaduras puedan ser utilizadas como transportadores no solo de probióticos si no además de vacunas terapéuticas (Omara *et al.*, 2010). Algunas levaduras utilizadas con propiedades próbioticas son cepas de *Kluyveromyces* (Hun *et al.*, 2013) y *Saccharomyces* (Knoll *et al.*, 2007). La levadura *S. cerevisiae* es un producto natural de la industria de la cerveza que contiene diferentes compuestos como β-glucanos, oligosacáridos y ácidos nucleicos. Se ha observado que estos compuestos son capaces de estimular la respuesta inmune (Ortuño *et al.*, 2002).

Por lo anterior *S. cerevisiae* es utilizada como probiótico en humanos con aplicación oral (Omara *et al.*, 2010). Grobiotec™AE, es un prebiótico comercial formado de mezclas de autolisados parciales de levadura de cerveza y productos de fermentación. Celmanax™ formulado con la pared celular de células de levaduras, actúa como anti adhesivo para la toxina "Shiga" producida por la cepa de *Escherichia coli* O157:H7, así como micotoxinas. Celmanax™ también mejora la producción de leche en bovinos y la eficiencia en la conservación de alimentos lácteos (Baines *et al.*, 2011). La levadura *S. boulardii* actúa como un transportador liberando enzimas, proteínas y factores tróficos durante su tránsito interintestinal, mejorando las defensas inmunológicas del huésped, la digestión y la absorción de nutrientes (Czerucka *et al.*, 2000). *S. boulardii* también presenta actividad benéfica en la inflamación intestinal mediante la supresión de la activación del NF-KB e inhibiendo la expresión de genes de citocinas proinflamatorias (Dalmasso *et al.*, 2006). En el sobrenadante de esta levadura se identificó una molécula estable a temperatura con propiedades anti-inflamatorias denominada "factor anti-inflamatorio de *Saccharomyces*" o "AIF" por sus siglas en ingles (Sougioultzis *et al.*, 2006). Uno de los principales retos para la producción de esta cepa a nivel industrial, es su alta sensibilidad a temperaturas en el proceso de secado. Enshasy (2012), generó en las células de esta levadura tolerancia a la temperatura del proceso de secado, mediante períodos cortos de exposición a altas temperaturas, para posteriormente realizar el secado mediante pulverización, obteniendo mejores resultados. Otra levadura termotolerante la cual se ha sugerido para ser utilizada como probiótico fue aislada de hojas de banana por Koedrith *et al.* (2008).

Levaduras termotolerantes en la producción de proteínas recombinantes

Las levaduras han sido utilizadas desde principios de 1980 para la producción a gran escala de proteínas intracelulares y extracelulares de humanos, animales y plantas (Romanos, 1995). Los sistemas de expresión de proteínas recombinantes basados en levaduras, han demostrado ser una fuente eficiente y económica de proteínas tanto de interés industrial como académico (Mattanovich *et al.*, 2012), convirtiéndose en una de las alternativas más utilizadas para la producción a gran escala. Las levaduras como sistemas de producción de proteínas recombinantes presentan las ventajas de los organismos unicelulares (práctica manipulación genética y rápido crecimiento), así como modificaciones postraduccionales eucariotas. Lo anterior, aunado al mejoramiento de los sistemas de expresión en levaduras, así como la metodología de hibridación, permite un aumento del uso de las levaduras en la producción de proteínas. Algunas de las modificaciones postraduccionales eucariotas que pueden realizar las levaduras, son procesamiento proteolítico, plegamiento, formación de puentes disulfuro y glicosilación (Eckart y Bussineau, 1996). Con respecto a modelos de expresión de proteínas en organismos eucariotas más complejos como células de ovarios de hámster chino y líneas celulares infectadas de baculovirus, las levaduras son más económicas, generalmente presentan mayores rendimientos, no contienen pirógenos y son menos demandantes en términos de tiempo y esfuerzo (Cregg *et al.*, 2000).

Algunas de las desventajas de las levaduras en la producción de proteínas heterólogas, es su incapacidad para realizar ciertas modificaciones postraduccionales como prolil-hidroxilación y amidación (Cregg y Higgins, 1995). La levadura *S. cerevisiae* es una levadura ampliamente utilizada para la producción de proteínas recombinantes de interés industrial o medicinal (Mattanovich *et al.*, 2012). Algunos ejemplos de estas proteínas son la insulina humana, vacunas para virus de hepatitis y del virus del papiloma humano (Martínez *et al.*, 2012). Existen diferentes factores que afectan la producción de proteínas heterólogas en levaduras, algunos de estos son el punto isoeléctrico y la formación de complejos. García-Fruitós *et al.* (2011) reportaron que la temperatura de crecimiento de las levaduras, es otro factor que afecta el plegado correcto de las proteínas recombinantes, sin embargo, el mecanismo por el cual ocurre esto, aún se desconoce. Recientemente, Zhong *et al.* (2014), reportaron que la capacidad de plegamiento del retículo endoplasmático (RE) y la viabilidad de la cepa GS115 de *Pichia pastoris*, son mantenidas a 20°C, permitiendo altas producciones de la proteína recombinante interleucina-10 de humano (rhIL-10), mientras que a 30°C el RE de la levadura sufre de estrés mediante la retención de G3-pro-rhIL10 en un estado inmaduro, dañando la capacidad de plegamiento del RE, disminuyendo la viabilidad celular y producción de la proteína rhIL-10.

Como alternativa a este problema, se ha sugerido que las levaduras termotolerantes podrían producir proteínas termotolerantes, además de poseer ventajas sobre proteínas producidas por otros microorganismos termotolerantes no eucariontes (Takashima *et al.*, 2009). Rodríguez *et al.* (2000), realizaron un análisis centrado en las diferencias de la proteína recombinante fitasa obtenida en *P. pastoris*, comparada con la proteína obtenida de otros sistemas de expresión, donde se observó que la proteína obtenida de esta levadura, presentó una mayor termotolerancia, conservando el 57 y 40 % de estabilidad a temperaturas de 65 y 90°C, respectivamente, lo cual es importante, ya que la termotolerancia de la proteína fitasa, es una de las características más deseadas. La levadura *Hansenula polimorpha* (*P. angusta*), es una levadura reconocida como termotolerante con capacidad de crecer hasta 49°C. Esta levadura es ampliamente utilizada para la búsqueda de proteínas termotolerantes (Celik y Calik, 2012). Otra levadura reconocida como termotolerante, aunque menos utilizada para la producción de proteínas heterólogas es *Arxula adeninivorans* (Wartmann *et al.*, 1995). Aunque existen diversos sistemas de expresión de proteínas heterólogas en levaduras metilotróficas, Promdonkoy *et al.* (2014), sugirieron que levaduras metilotróficas termotolerantes o termofílicas, pueden ser modelos más adecuados para procesos de producción industrial. El impacto de una gama de temperaturas en la producción de diversas proteínas heterólogas, se ha explorado mediante el uso de la levadura *K. marxianus*, demostrando ser un sistema práctico para investigar el impacto de la temperatura sobre la eficiencia de la producción de proteínas en levaduras (Raimondi *et al.*, 2013). La levadura *K. marxianus* es relativamente poco estudiada para la expresión de proteínas heterólogas, en contraste con *S. cerevisiae* y *K. lactis* (Hang *et al.*, 2003). Una mayor comprensión de la fisiología y respuesta a la temperatura en levaduras productoras de proteínas heterólogas, podría permitir la obtención de mayores rendimientos y calidad en la producción de proteínas.

Producción de etanol por levaduras termotolerantes

El incremento en el uso del etanol por diferentes industrias como fuente de energía, solventes industriales, agentes de limpieza y preservativos, ha incrementado la producción de este alcohol (Brooks, 2008). La producción de etanol puede ser químicamente o a través de la fermentación de carbohidratos, principalmente, por levaduras vía glucólisis en condiciones anaerobias. La producción de etanol mediante la fermentación es un proceso exotérmico, por lo que a medida que la fermentación progresa, el calor generado eleva la temperatura del medio de fermentación (Kumar *et al.*, 2013), además, debido a la corta duración de la fermentación, la refrigeración no siempre es lo suficientemente eficaz en la eliminación de calor, por lo que la

temperatura puede alcanzar hasta 40°C (Andrietta *et al.*, 2002). West y Kennedy, 2014, entre otros, realizaron experimentos con el fin de seleccionar cepas termotolerantes para su uso industrial en la fermentación. Algunas de las ventajas de las levaduras termotolerantes en el proceso de fermentación para la obtención de etanol, son: una mayor tolerancia a compuestos presentes en el medio, ahorro de energía a través de la reducción de costos de refrigeración, mayores tasas de fermentación, separación continua de etanol, reducción del riesgo de contaminación, disminución de la energía requerida para la agitación del medio de fermentación, mayor presión de vapor y aumento de la solubilidad de compuestos orgánicos (Murata *et al.*, 2015).

Por lo anterior, la utilización de cepas de levaduras termotolerantes podría mejorar la eficiencia de la producción de etanol llevando a cabo la fermentación a temperaturas mayores a 40°C (Abreu-Cavalheiro y Monteiro, 2013; Caspeta *et al.*, 2014). La levadura termotolerante *H. polymorpha* es una de las levaduras más estudiadas y la secuencia del genoma completa de la cepa NCY495 es de libre acceso. Se ha observado que cepas de *H. polymorpha* presentan la capacidad de fermentar a altas temperaturas (45 a 50°C) glucosa, celobiosa y xilosa a etanol. Kurylenko *et al.* (2014), generaron una cepa mutante de *H. polymorpha* obteniendo rendimientos de hasta 9.8 g/L de etanol, durante la fermentación de xilosa a 45°C. La producción de etanol a partir de materias primas renovables (bioetanol) representa el proceso de fermentación industrial con mayor crecimiento, con una producción de 85.2 billones de litros de etanol en el 2012 (Renewable fuels association, 2012). La producción de bioetanol es ampliamente investigada como una fuente de combustibles renovables (Vázquez y Dacosta, 2007), debido a que presenta diversas ventajas respecto a los combustibles obtenidos de fósiles. La mayoría de los procesos de obtención de bioetanol a partir de lignocelulosas, comienzan con una hidrólisis termoquímica de la parte hemicelulósica, seguido de una hidrólisis enzimática de la parte celulósica y una fermentación a base de levadura de los azúcares resultantes (Bothast y Schlicher, 2005).

La sacarificación y fermentación simultánea (SFS) es un proceso óptimo para la producción de etanol a partir de fuentes lignocelulósicas. De acuerdo a West y Kennedy (2014), existe una necesidad de realizar investigaciones para el aislamiento de cepas de levaduras termotolerantes que puedan realizar el proceso de SFS a temperaturas mayores de 40°C. La SFS aumenta los rendimientos de etanol al minimizar la inhibición por producto y eliminar la necesidad de bioreactores separados para ambos procesos (Luo *et al.*, 2008). Uno de los principales inconvenientes del proceso de SFS, son las condiciones de funcionamiento, ya que la hidrólisis enzimática por celulasas tiene una temperatura óptima de 45 a 50°C, pero la mayoría de las levaduras etanologénicas tienen una temperatura óptima entre 30 y 37°C (Taylor *et al.*, 2009). Debido a lo anterior, es que diversos estudios se han centrado en las cepas etanologénicas termotolerantes capaces de realizar la SFS de lignocelulosas (Araque *et al.*, 2008). En la tabla 3, se mencionan estudios en los cuales se utilizaron levaduras termotolerantes y residuos agroindustriales en procesos de SFS para la obtención de etanol, lo que demuestra el potencial de la combinación de estos tres elementos. Castro y Roberto (2014), reportaron que la cepa de *K. marxianus* NRRL Y-6860, presenta potencial para la producción de etanol mediante la SFS de sustratos celulósicos, ya que presentó un rendimiento de 0.44 g/g en la producción de etanol a 45°C. Moreno *et al.* (2013), observaron que al remover los fenoles de lignina mediante un tratamiento con lacasa y utilizando la cepa termotolerante de *K. marxianus* CECT 10875, se obtienen rendimientos similares en la producción de etanol, que al utilizar una cepa industrial de *S. cerevisiae* (Fermentis Ethanol Red®, France).

Tabla 3. Producción de bioetanol usando residuos agroindustriales y levaduras termotolerantes en procesos de SFS.

Hojas de maleza *Antigonum leptopus*	*K. fragilis* NCIM3358	Hari *et al.* (2001).
Alcachofa de Jerusalén (*Helianthus tuberosus*)	*K. marxianus* (PT-1) y *S. cerevisae* (JZ1C)	Hu *et al.* (2012).
Rastrojo de maíz	*S. cerevisae* DQ1	Chu *et al.* (2012).
Subproducto del proceso de extracción del jugo de zanahoria	*K. marxianus* K21	Chi-Yang *et al.* (2013).

De acuerdo a Souza *et al.* (2012), el uso de levaduras termotolerantes y una fase pre-sacarificación antes de realizar la SFS, son claves en el incremento del rendimiento en la producción de bioetanol. Este proceso es conocido como pre-sacarificación y sacarificación y fermentación simultánea (PSFS). Además del proceso de PSFS, se ha investigado el uso de diferentes reactores y condiciones de crecimiento con levaduras termotolerantes para la producción de bioetanol (Lin *et al.*, 2013). La inhibición del crecimiento de las levaduras termotolerantes en el proceso de SFS, debido a los productos de la fermentación de la materia prima lignocelulósica, ha sido estudiada por Wallace-Salinas y Gorwa-Grauslund (2013), desarrollando una cepa clasificada como ISO12, la cual además de la termotolerancia presentó una resistencia a los inhibidores mencionados.

Principales especies de levaduras termotolerantes

Las especies de levaduras con más reportes acerca de termotolerancia son *S. cerevisae* y *K. marxianus*, siendo estas últimas las que han mostrado mayor termotolerancia (Nonklang *et al.*, 2008; Suryawati *et al.*, 2008). Las levaduras de *K. marxianus* son capaces de crecer a temperaturas de hasta 52 °C (Koedrith *et al.*, 2008), y son una de las especies de levaduras termotolerantes más conocidas y utilizadas. Debido a su alta capacidad de producción de biomasa, etanol, proteínas heterólogas, además de la amplia gama de fuentes de carbono que pueden metabolizar, hacen que esta especie sea atractiva para aplicaciones industriales (Fonseca *et al.*, 2008). Otras de las principales especies de levaduras con termotolerancia, además de las mencionadas, son *H. polymorpha* (Escalante *et al.*, 1990), *P. pastoris* (Rodriguez *et al.*, 2000) y *K. fragilis* (Hari *et al.*, 2001).

Mecanismos de respuesta fisiológicos de las levaduras a altas temperaturas

La termotolerancia en las levaduras de *S. cerevisiae* puede ser inducida por la exposición a períodos cortos a condiciones estresantes, no letales, tales como bajo pH, alta osmolaridad y concentraciones de etanol, así como temperaturas ≥ a 37°C (Piper, (1993)). Los efectos letales del estrés por altas temperaturas en levaduras, es el resultado de daño a proteínas, membranas y otras estructuras celulares (Webster y Watson, 1993). Cuando las levaduras se exponen a temperaturas elevadas, exhiben una rápida respuesta molecular, lo que se conoce como la respuesta de choque térmico. La respuesta de choque térmico en *S. cerevisiae* es una de las respuestas más caracterizadas a nivel molecular en células eucariontes. En esta respuesta, se ha observado la expresión de diferentes proteínas, conocidas como proteínas de choque térmico (Hsp's). Mientras que la síntesis de la mayoría de proteínas disminuye a altas temperaturas, la concentración de Hsp's aumenta (Parsell y Lindquist, 1994). La proteína Hsp104 está involucrada en los cambios estructurales en proteínas y complejos proteicos (Cashikar *et al.*, 2005). La proteína Hsp104 de *S. cerevisiae* restaura la estructura de proteínas desnaturalizadas usando energía en forma de ATP y un sistema de chaperonas Hsp70p/Hsp40p (Weibezahn *et al.*, 2004). La expresión de la Hsp104 en *S. cerevisiae* es baja en condiciones normales de temperatura, sin embargo, a altas temperaturas aumenta significativamente su concentración. La expresión del gen de la proteína Hsp104 provee a *S. cerevisiae* de termotolerancia (Lindquist y Kim, 1996). Además de la síntesis transitoria de Hsp's, la respuesta a altas temperaturas, consiste en cambios en la composición lipídica de las membranas (Benschoter e Ingram, 1986).

En una investigación reciente, se demostró que cepas de *S. cerevisiae* termotolerantes aisladas mediante evolución adaptativa a temperaturas de 40°C o mayores, modificaron la composición de esteroles de sus membranas de ergosterol a fecosterol. Lo anterior, causado por una mutación en el gen de la desaturasa esterol C-5 y a un incremento en la expresión de genes relacionados con la biosíntesis de esteroles. Se ha sugerido que la alteración en la composición de esteroles de las cepas adaptadas, resulta en una fluidez óptima de las membranas permitiendo que estas levaduras sobrevivan (Caspeta *et al.*, 2014). Sotamura *et al.* (2013), generaron la cepa termotolerante YK60-1, a partir de la cepa de *S. cerevisiae* MT8-1, mediante la exposición a temperaturas con aumento progresivo, e investigaron mediante análisis de microarreglos de ADN el mecanismo mediante el cual, la cepa MT8-1 adquirió termotolerancia. En sus resultados, observaron la inducción de genes de respuesta a estrés, tales como los de Hsp y biosíntesis de trehalosa. Mediante análisis de metaboloma, mostraron que la cepa YK60-1 acumuló mayor concentración de trehalosa.

Originalmente, se creía que la trehalosa funcionaba como un carbohidrato de almacenamiento en las levaduras, pero se ha observado que puede ser un protector de la tensión relacionada con la termotolerancia inducida. La trehalosa es un carbohidrato protector que se acumula después de la exposición a altas temperaturas (Mahmud *et al.*, 2010; An *et al.*, 2011). Virgilio *et al.* (1994), proporcionaron evidencia genética de que la síntesis de trehalosa inducida por altas temperaturas es un factor importante para la inducción de la termotolerancia. La sobre expresión del gen TPS1 que convierte la glucosa 6-fosfato y glucosa-UDP en trehalosa 6-fosfato, genera un aumento en la concentración de trehalosa, por lo que TPS1 está involucrado en la tolerancia a la temperatura (An *et al.*, 2011). Recientemente, Petitjean *et al.* (2015), utilizando una combinación de mutantes de levaduras en el gen Tps1 y cepas de levaduras capaces de asimilar trehalosa de una fuente externa, proporcionaron evidencia de que la trehalosa no protege las células, identificando la proteína Tsp1 con una función clave para la resistencia a la temperatura, posiblemente a través del mantenimiento de los niveles de ATP. Otros factores fisiológicos implicados en la termotolerancia, incluyen el agua celular no congelable (Komatsu *et al.*, 1991), la detención del ciclo celular en la fase G0 (Plesset *et al.*, 1987), la fosforilación de proteínas independiente de AMPc y de la ATPasa unida a membrana (Coote *et al.*, 1994). La tolerancia del metabolismo de las levaduras a alta temperatura es controlada por diferentes genes, los cuales están relacionados con la síntesis o acumulación de metabolitos específicos para la protección de las células en altas temperaturas, sin embargo, en cuanto a la genómica que podría estar relacionada con la termotolerancia de las levaduras, poco se conoce (Abreu-Cavalheiro y Monteiro (2013)).

En un trabajo realizado por Krsmanović y Kölling (2004), se describió una cepa de levadura con la capacidad de crecer a altas temperaturas, la cual presenta un alelo conocido como RSP5. RSP5 tiene una función en el transporte celular y degradación de proteínas. Levaduras mutadas en RSP5 perdieron la habilidad para acumular conjugados de ubiquinona, después de ser expuestas a choque térmico, por lo que se sugirió que RSP5 está relacionado con la sensibilidad a la temperatura. En las levaduras en las que se aumentó los niveles de la transcripción del gen RSP5-C, se observó un aumento en la ubiquitinación de proteínas, así como en la tolerancia a la temperatura (Shahsavarani *et al.*, 2011). En otro estudio, en el que se clonó el gen Hsp100 de *Pleurotus sajor-caju* en una levadura, se observó un aumento en la sobrevivencia de las levaduras expuestas a 50°C (Lee *et al.*, 2006). Kim *et al.* (2011), reportaron que la inhibición de los genes SSK2, PPG1 y PAM1, generan una reducción en la tolerancia a la temperatura. Más reciente, Caspeta *et al.* (2014), secuenciaron el genoma de 7 cepas de levaduras termotolerantes generadas mediante evolución adaptativa de la cepa CEN.PK113-7D, la cual, fue expuesta a temperatura de 39°C hasta por 90 días. La secuenciación del genoma de las levaduras reveló un total de 30 variaciones de nucleótidos simples en 18 genes. La mayoría de las variaciones fueron detectadas en genes que afectan la composición y estructura de la membrana, respiración, replicación y reparación del ADN. Además de lo mencionado, se ha reportado que el estrés por temperatura induce una hiperpolarización o desporalización (Balogh *et al.*, 2005) mitocondrial, causando probablemente, una subsecuente producción de especies reactivas de oxígeno (ERO) (Perl *et al.*, 2004) y estrés oxidativo.

Estrés oxidativo en levaduras generado por altas temperaturas

El estrés oxidativo generado por la exposición a altas temperaturas, es uno de los principales factores en la muerte de *S. cerevisiae* (Davidson y Schiestl, 2001). Las ERO generadas a altas temperaturas, alteran las membranas celulares, proteínas y ADN, y causan finalmente, la muerte celular (Storz y Imlay, 1999). Sugiyama *et al.* (2000), observaron que independiente del tipo de metabolismo de las levaduras (oxidativo o fermentativo), se presenta un incremento en los niveles de ERO conforme aumenta la temperatura. El estrés por temperatura puede inducir diferentes genes antioxidantes en levaduras, mientras que el H_2O_2 induce diferentes Hsp's. Lo anterior muestra una relación entre el estrés por temperatura y el estrés oxidativo, por lo cual, se ha sugerido que las Hsp's y los sistemas antioxidantes, contribuyen a la termotolerancia intrínseca de las levaduras (Morano *et al.*, 2012). Rikhvanov *et al.* (2001), observaron que la exposición de levaduras a 45°C estimula su respiración, lo cual podría incrementar la generación de ERO y eventualmente causar la muerte celular, mientras que la represión de su respiración celular permite una mayor tolerancia a temperaturas de 45°C.

Mutantes de levaduras con deleción en genes para enzimas antioxidantes como catalasa, superóxido dismutasa y tiorredoxina peroxidasa, son más sensibles al efecto letal de altas temperaturas, mientras que la sobreexpresión de los genes de la catalasa y la superóxido dismutasa, causa un aumento en su termotolerancia (Davidson y Schiestl, 2001). Caspeta *et al.* (2014), reportaron que cepas de levaduras termotolerantes generadas mediante exposición a 39°C, fueron incapaces de metabolizar fuentes de carbono no fermentables y no mostraron cambio diáuxico a 40 y 30°C. Estas cepas acumularon mutaciones en los genes ATP2 o ATP3, los cuales son esenciales para el crecimiento en fuentes de carbono no fermentables. A lo anterior, sugieren que el fenotipo óptimo de levaduras termotolerantes podría no ser a través de la evolución, mientras exista una respiración oxidativa totalmente funcional, posiblemente, debido a que esto generaría una mayor concentración de ERO induciendo estrés oxidativo.

Recientemente, Cao *et al.* (2013), reportaron una asociación en el incremento de la producción de intermediarios de especies reactivas de oxígeno (IERO), con la vía de la lanzadera de GABA, cuando las levaduras fueron expuestas a choque térmico. Esta vía contiene las enzimas glutamato descarboxilasa (GAD), GABA aminotransferasa (GAT) y succinato semialdehído deshidrogenasa (SSADH). En levaduras, esta vía está relacionada con la formación de succinato a partir de α-cetoglutarato, la cual provee carbono en la cadena respiratoria en la mitocondria. En su trabajo, Cao *et al.* (2013), concluyen que el daño en las células de levadura producido por la exposición a altas temperaturas, es prevenido por la función de las enzimas de la lanzadera GABA, a través de un mecanismo que se relaciona con el abatimiento de la acumulación de IERO durante el estrés térmico. Se ha observado esta relación, entre la producción de ERO y la termotolerancia, ya que en nuestro grupo de investigación, se determinó la concentración de ERO generadas en tres cepas de levaduras, las cuales fueron aisladas de zonas productoras de mezcal en México, de los estados de Oaxaca, Guerrero y San Luis Potosí. Estos lugares son típicos de tener variaciones en la temperatura, sobretodo, altas temperaturas (Arellano-Plaza *et al.*, 2013).

CONCLUSIONES

Entre las principales caracteristicas que tienen las levaduras termotolerantes, se pueden mencionar las siguientes:

i) La termotolerancia de las levaduras es una propiedad importante en aplicaciones industriales.

ii) La respuesta antioxidante es un factor importante en la termotolerancia, sin embargo, es necesario ampliar el conocimiento del efecto de la temperatura en esta respuesta, con posibles implicaciones en el uso industrial de las levaduras.

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8. Discusión general

En las levaduras mesófilas (aquellas que no sobreviven a temperaturas ≥40°C) se ha observado que las especies reactivas de oxígeno (ERO) y sistemas antioxidantes están relacionados con el daño generado por altas temperaturas, así como con el mantenimiento del estado redox celular (Davidson *et al*., 1996; Davidson y Schiestl, 2001), el cual afecta la producción de metabolitos como el etanol (Almeida *et al*., 2011). Por lo anterior en este trabajo se estudió el estado de los sistemas antioxidantes y la cadena transportadora de electrones mitocondrial (CTEm) (principal fuente de ERO), así como la capacidad fermentativa de una levadura con óptimo crecimiento a 40°C (termotolerante). Los géneros de levaduras *Kluyveromyces* y *Saccharomyces,* son algunos de los que presentan un mayor número de especies de levaduras termotolerantes (Boyle *et al.,* 1997; Lark *et al.,* 1997; Bollók *et al.,* 2000). Previamente Arellano-Plaza *et al*. (2013) reportaron que las cepas de *S. cerevisiae* MC4 y *K. marxianus* SLP1 y OFF1 presentan tolerancia a estrés oxidativo inducido por H_2O_2 y menadiona, mientras que Flores *et al*. (2013) observaron que estas levaduras presentan potencial para la producción de etanol. Por lo cual en este trabajo se utilizarón estas cepas (*K. marxianus* OFF1 y SLP1, y *S. cerevisiae* MC4).

En los resultados obtenidos se observó que la cepa *K. marxianus* SLP1 presentó una sobrevivencia igual a 40°C que la presentada a 30°C, además de un menor efecto deletéreo por el aumento de temperatura en sus parámetros de crecimiento, comparado con el resto de las levaduras utilizadas en este trabajo (Capítulo 1). Por lo que se clasificó a *K. marxianus* SLP1 como una levadura termotolerante, de acuerdo a la definición de Koedrit *et al*. (2008).

La mayor sobrevivencia de *K. marxianus* SLP1 correlacionó con una menor producción de ERO. Kim *et al*. (2013) reportaron resultados similares utilizando la levadura termotolerante *S. cerevisiae* KNU5377. La menor concentración de ERO puede ser una consecuencia del aumento de la actividad de la catalasa, como se observó en *K. marxianus* SLP1 (Capítulo 1). Adicionalmente, esta levadura mostró una mayor proporción de ácidos grasos saturados en su membrana citoplasmática, característica reportada en levaduras termotolerantes (Le *et al*., 2013). La modificación de la proporción de ácidos grasos de la

membrana de *K. marxianus* SLP1 evidenció una relación inversa entre el grado de poli-insaturaciones de los ácidos grasos adicionados y la termotolerancia de la levadura, mientras que la relación entre el grado de poli-insaturaciones de los ácidos grasos adicionados y la lipoperoxidación en la levadura termotolerante fue directa (Capítulo 2). Estos resultados coinciden con los reportados para la levadura *S. cerevisiae* mediante la adición de un agente inductor de estrés oxidativo (Cortés-Rojo *et al.*, 2009).

De acuerdo a los resultados obtenidos de los parámetros evaluados en la levadura termotolerante *K. marxianus* SLP1 se propone el modelo mostrado en la figura 13.

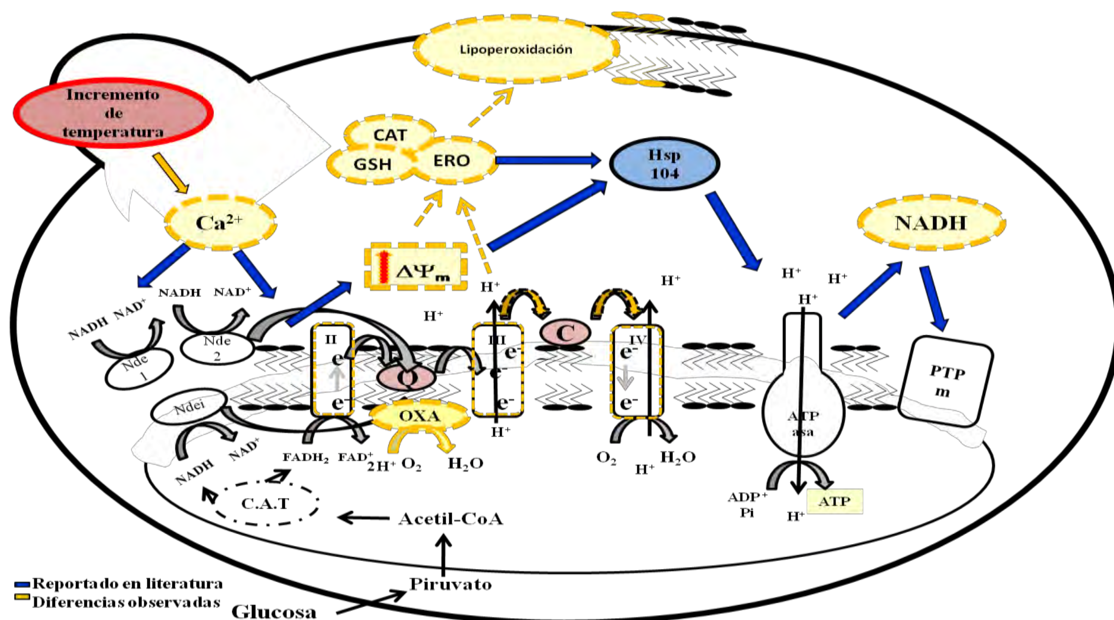


Figura 13. Efecto de un incremento de temperatura en la levadura *K. marxianus* SLP1. Parámetros determinados en color amarillo; en color azul los parámteros reportados en la literatura. El incremento de temperatura indujo un aumento en la concentración de calcio citoplasmático (Ca^{2+} Cyt) y ERO, así como una mayor actividad de los sistemas antioxidantes, y una disminución en la actividad de los complejos de la CTEm. Deshidrogenasa externa (Nde) e interna (Ndi); Ciclo de los ácidos tricarboxilicos (C.A.T.); Potencial mitocondrial (ΔΨm); Especies reactivas de oxígeno (ERO); Glutatión reducido (GSH); Catalasa (CAT); Proteína de choque térmico 104 (Hsp104); Poro de transición de permeabilidad mitocondrial (PTPm).

La inducción de la actividad de una oxidasa alterna que reduzca el oxígeno se ha reportado en levaduras en condiciones no favorables de crecimiento (Umbach *et al*., 2005). La actividad de esta enzima contribuye a evitar la sobre reducción de la CTEm, disminuyendo así la producción de ERO cuando la CTEm no funciona correctamente (Guerrero-Castillo *et al*., 2012). Lo anterior explica la activación de esta enzima en *K. marxianus* SLP1 cuando fue expuesta a 40°C y se disminuyó la actividad de los complejos de su CTEm (Capítulo 3). La inhibición parcial o total de la actividad de los complejos de la CTEm es sugerida como indispensable para la generación de levaduras termotolerantes (Caspeta *et al*., 2014b), ya que esto evitaría una sobreproducción de ERO que dañaran biomoléculas disminuyendo la tolerancia de las levaduras a la temperatura. Aunque tal fenómeno se sugirío previamente, este trabajo es el primero en determinar que efectivamente los complejos de la CTEm de una levadura termotolerante presentan menor actividad (Capítulo 3). La reducción en la actividad de los complejos de la CTEm por el incremento de temperatura, se propone que es debido al daño generado en los componentes termolábiles de la CTEm, como la ubiquinona, el citocromo c y las proteínas con centros Fe-S (Davidson *et al*., 2001). Por lo que en próximos trabajos se podría analizar el estado de estos componentes en la CTEm de *K. marxianus* SLP1.

Al observar una reducción de la actividad de los complejos de la CTEm y el mantenimiento del potencial, se evalúo la concentración de Ca^{2+} Cyt, ya que un aumento en las concentraciones de este ión con su posterior ingreso a la mitocondria, es un mecanismo generador de potencial mitocondrial (Ueom *et al*., 2003). Se observó que al exponer a *K. marxianus* SLP1 a un incremento de temperatura efectivamente hay un aumento en sus concentraciones de Ca^{2+} Cyt, lo cual podría contribuir a mantener el potencial mitocondrial en *K. marxianus* SLP1.

Aún con los mecanismos (inhibición de la actividad de los complejos de la CTEm y activación de una oxidasa alterna) que contribuyen a mantener los niveles basales de ERO en *K. marxianus* SLP1, en esta levadura se observó un aumento de ERO, el cual fue significativamente menor con respecto a la levadura no termotolerante OFF1 (Capítulo 1). El aumento de las ERO puede ser a través de la fuga de electrones de la CTEm,

específicamente del complejo III (Cortés-Rojo *et al*., 2009). Un incremento de la concentración de ERO puede inducir la síntesis de proteínas que protejan la célula del estrés; sin embargo, si la concentración de ERO es mayor se puede inducir la muerte celular (Rhoads *et al*., 2006; Kreslavsky *et al*., 2012). El mantenimiento de las ERO en concentraciones no letales podría ser además de los mecanismos mencionados debido a la mayor concentración de GSH y al incremento de la actividad de la catalasa (Capítulo 1). Estos sistemas antioxidantes (GSH y catalasa), así como la relativa alta proporción de ácidos grasos saturados presentes en la membrana citoplasmática de *K. marxianus* SLP1 (Capítulo 2), contribuyen al menor grado de lipoperoxidación observado. Por otro lado, el mantenimiento de ERO a niveles no letales podría estar induciendo la síntesis de la proteína de choque térmico 104 (Hsp104), la cual mantiene la estructura de la ATP sintasa y la integridad mitocondrial (Ueom *et al*., 2003), contribuyendo a que no existan cambios significativos en la concentración del equivalente reducido NADH (Capítulo 3) y por ende, el mantenimiento del equilibrio en el estado redox celular. El mantenimiento del estado redox y los bajos niveles de lipoperoxidación en *K. marxianus* SLP1 propician la sobrevivencia de esta levadura a 40°C.

Con los resultados anteriores, en este trabajo se sugiere que los niveles de etanol producidos por *K. marxianus* SLP1 (10.36 g/L), que fueron similares a los generados por la levadura industrial *S. cerevisiae* Ethanol Red® (10.23 g/L) (Capítulo 4), se logran mediante el mantenimiento del estado redox celular de *K. marxianus* SLP1, al no existir cambios en la concentración GSH así como del NADH, a la temperatura a la cual se llevó la fermentación (40°C). Mientras que el GSH pudo contribuir al mantenimiento del estado celular reducido, necesario para la actividad de diferentes enzimas (Ostergaard *et al*., 2004), el NADH pudo ser utilizado para reducir el acetaldehído en etanol, al ser oxidado a través de la alcohol deshidrogenasa. De los compuestos orgánicos producidos por *K. marxianus* SLP1 en la fermentación a 40°C (Capítulo 5), destaca por su concentración el 2-fenil etanol (75 mg/L), el cual es un compuesto de gran interés para diferentes industrias (Wittmann *et al*., 2002). Las mayores concentraciones de 2-fenil etanol producidas mediante la fermentación de biomasa lignocelulósica son de 89 mg/L, por *K. marxianus* CBS600 (Etschmann *et al*., 2003). Aunque las concentraciones obtenidas de 2-fenil etanol así como

de etanol, mediante la fermentación a 40°C con *K. marxianus* SLP1 son menores que los reportados por Caspeta *et al*. (2014a) y Etschmann *et al*. (2003), es posible que mediante el aumento de la temperatura en la fermentación se obtengan mayores rendimientos, ya que *K. marxianus* SLP1 mostró capacidad adaptativa a las condiciones de fermentación, aumentando su número de células en 11 de las 18 condiciones de fermentación generadas (Capítulo 5). Una capacidad adaptativa a las condiciones de fermentación es una de las características más buscadas en levaduras para la producción de bioetanol (Caspeta *et al*., 2015), debido a que en estos procesos se generan una gran cantidad de compuestos, como furfural y ácido acético, que inducen estrés oxidativo en las levaduras. Como se observó en este trabajo, ya que *K. marxianus* SLP1 presenta sistemas antioxidantes más activos que otras levaduras, es posible que pueda mantener su viabilidad y con ello generar mejores rendimientos en la producción de compuestos orgánicos, en condiciones estresantes como las generadas en la fermentación de diferentes tipos de biomasa lignocelulósica.

9. Resumen de resultados

La cepa *K. marxianus* SLP1 es una levadura termotolerante con mayor actividad de sistemas antioxidantes, menor producción de ERO y lipoperoxidación, a 40°C.

La adición de ácidos grasos poli-insaturados decrece la termotolerancia de *K. marxianus* SLP1 incrementando el grado de lipoperoxidación, mientras que la adición del ácido araquídico genera una tendencia a incrementar su termotolerancia.

La termotolerancia de la levadura *K. marxianus* SLP1 es influenciada por un aumento de la actividad de sus sistemas antioxidantes y disminución de la función de su CTEm, que contribuyen en el manteniendo su estado redox.

La levadura termotolerante *K. marxianus* SLP1 presenta potencial para la producción de bioetanol y 2-fenil etanol, a través de la fermentación del bagazo de agave.

10. Conclusión

La levadura *K. marxianus* SLP1 presenta termotolerancia y potencial para ser usada en procesos de fermentación a 40°C, ya que ante un incremento de temperatura aumenta la actividad de sus sistemas antioxidantes y disminuye la función de su CTEm, manteniendo su estado redox y capacidad fermentativa.

11. Trabajos complementarios

11.1. Artículo de divulgación

Jorge A. Mejia-Barajas y Alfredo Saavedra-Molina. 2014. "Conociendo las levaduras". Saber más, Revista de Divulgación de la Universidad Michoacana de San Nicolás de Hidalgo. Año 3/ Enero-Febrero, No. 13. 7-9

CONOCIENDO LAS LEVADURAS

Jorge Arturo Mejía Barajas
y Alfredo Saavedra Molina

Las levaduras son microorganismos con los que comúnmente estamos en contacto, encontrándolas en plantas, animales e insectos, sin embargo, no es del conocimiento de la mayoría, que estos microorganismos también se encuentran en superficies como las cáscaras de frutas y nuestra propia piel.

Las levaduras impactan diferentes sectores comerciales que incluyen alimentos, bebidas, farmacéuticos y enzimas industriales. En general, las levaduras tienen efectos benéficos en la vida humana. A pesar de esto, nuestro conocimiento de las levaduras parece estar limitado a identificarlas como una clase de hongo, utilizado en la producción de vino, cerveza y pan, por lo que es evidente, que siendo uno de los microorganismos de mayor importancia industrial, modelo de estudio para enfermedades como Alzheimer, Parkinson y cáncer, y con el que estamos en constante contacto, es necesario que conozcamos más información de este microorganismo.

Imagen tomada de Yeast Biotechnology: Diversity and Applications

Importancia de las levaduras en diferentes áreas.

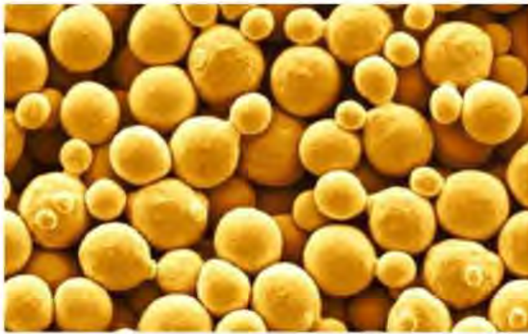
Las levaduras, efectivamente, son hongos unicelulares, con tamaños de 3 a 40 micrómetros, por lo que no es posible verlas a simple vista, solamente en conjunto formando agregados. Su tiempo de reproducción varía entre especies y es de 2 a 3 horas en las condiciones de crecimiento más favorables. Se conocen alrededor de 500 especies de levaduras y fueron identificadas por primera ocasión por su capacidad de fermentación por Louis Pasteur en 1857. El término levadura proviene del latín "levare", que significa levantar, ya que produce dióxido de carbono que causa la expansión de las proteínas del gluten en la harina y hace que se expanda la masa.

La especie más conocida y utilizada es *Saccharomyces cerevisiae*, su nombre significa levadura comedora de azúcar, entre otros significados similares. De acuerdo a diferentes autores, *S. cerevisiae* fue seleccionada como modelo de estudio de laboratorio desde 1930, debido a que conserva procesos esenciales que son llevados a cabo por las células en humanos, por lo que las observaciones de los efectos causados por enfermedades como Alzheimer, se pueden extrapolar a humanos.

Saccharomyces cerevisae.

Como la mayoría conocemos, *S. cerevisiae* también se emplea en la fermentación del pan, cerveza, vino y producción de alcohol. Esta levadura es la más resistente a etanol, resistiendo hasta 14% de etanol en su medio. Con esta característica no es de sorprender que sea la levadura más utilizada en la producción de vinos. Se han aislado cepas de levaduras *no-Saccharomyces* como *Candida* y *Kluyveromyces* con capacidades de resistencia a etanol similares a S. cerevisiae.

Candida albicans.



Kluyveromyces lactius.

Las cepas *no-Saccharomyces* tienen la capacidad de producir y secretar enzimas como esterasas, glucosidasas y proteasas, que interactúan con compuestos de las fermentaciones para producir aromas característicos que varían para cada vino. Dentro de las levaduras clasificadas como *no-Saccharomyces, K. marxianus* se ha considerado prometedora para la producción de proteínas eucariotas con fines terapéuticos.

Las levaduras presentan ventajas en la producción de proteinas debido a su rápido crecimiento y modificaciones postransduccionales. La producción de proteínas con fines terapéuticos en levaduras, ha sido estudiada desde 1980, con la producción de proinsulina, sin embargo, actualmente es un área con reciente auge, debido al incremento en la demanda de las proteínas terapéuticas. Otras de las proteínas producidas mediante levaduras son la insulina y el factor de crecimiento epidérmico.

Además de la producción de proteínas, las levaduras en su estructura celular presentan una alta concentración de aminoácidos, péptidos, carbohidratos y sales, que pueden ser extraídos mediante la disolución en agua, previa digestión por enzimas. A esta disolución se le conoce como extracto de levaduras, estos extractos presentan propiedades antioxidantes y son utilizados como saborizantes en sopas, salsas, preparaciones de carnes, condimentos y como suplementos alimenticios en la comida de algunos animales como aves y cerdos.

Con el objetivo de conferir a las levaduras, nuevas o deseadas propiedades para diferentes aplicaciones, es que las levaduras son de los principales microorganismos más modificados mediante ingeniería genética, aunque la mayoría de estas investigaciones, hasta el momento, son mantenidas como tales, ya que estas levaduras aún no están autorizadas para ser utilizadas en la producción de productos para consumo humano, por asociaciones como GRAS (Generally Recognized as Safe) y QPS (Qualified Presumption of Safety) en los Estados Unidos y en la Unión Europea, respectivamente.

Para la obtención de levaduras con mejores y nuevas propiedades, el aislamiento de levaduras de ecosistemas con condiciones ambientales extremas, son una alternativa, ya que se ha observado que existe una relación en las características metabólicas de las levaduras, con respecto a la historia evolutiva de sus sustratos, por lo que el aislamiento y caracterización de nuevas cepas de levaduras de estas zonas, es prometedor tanto para centros de investigación como para industrias.

Imagen tomada de http://biospringer-na.com/products/springer

Extracto de levadura.

Como observamos, la mayoría de las aplicaciones de las levaduras presentan un potencial benéfico, aunque si alguna vez escuchó que tuviera cuidado con las levaduras, es verdad, ya que existen levaduras como *Candida glabrata*, la cual comúnmente se encuentra como comensal en las mucosas de individuos sanos, pero puede invadir tejidos más profundos y causar enfermedades cuando el sistema inmunológico del hospedero se encuentra atenuado.

Ahora que conocemos un poco más acerca de las levaduras, es que podemos darnos cuenta de la transcendencia de estos microorganismos en nuestro entorno, así como su función en la investigación científica y tecnológica para la producción y mejoramiento de productos tan cotidianos como el vino, cerveza y pan, o tan complejos como proteínas terapéuticas e indeseables como enfermedades.

Para Saber Más:

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M. en C. Jorge Arturo Mejía Barajas. Estudiante del Programa Institucional de Doctorado en Ciencias Biológicas, Laboratorio de Bioquímica, Instituto de Investigaciones Químico-Biológicas. Universidad Michoacana de San Nicolás de Hidalgo. Dr. Alfredo Saavedra Molina. Profesor Investigador. Laboratorio de Bioquímica, Instituto de Investigaciones Químico-Biológicas. Universidad Michoacana de San Nicolás de Hidalgo.

11.2. Coasesoría de tesis de licenciatura:

Efecto de la temperatura sobre la actividad de la ATPasa en las cepas de levaduras OFF1, SLP1 y MC4, aisladas de hábitats extremos. Presentada por José Alberto Martínez Mora en la Facultad de Químico Farmacobiología, de la Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán. Enero, 2015.

UNIVERSIDAD MICHOACANA DE
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FACULTAD DE QUÍMICO
FARMACOBIOLOGÍA

INSTITUTO DE INVESTIGACIONES
QUÍMICO BIOLÓGICAS

"EFECTO DE LA TEMPERATURA SOBRE LA ACTIVIDAD DE LA
ATPasa EN LAS CEPAS DE LEVADURAS OFF1, SLP1 Y MC4
AISLADAS DE HÁBITATS EXTREMOS"

TESIS QUE PRESENTA:

p.Q.F.B.: JOSÉ ALBERTO MARTÍNEZ MORA

PARA OBTENER EL TÍTULO DE:

QUÍMICO FARMACOBIÓLOGO

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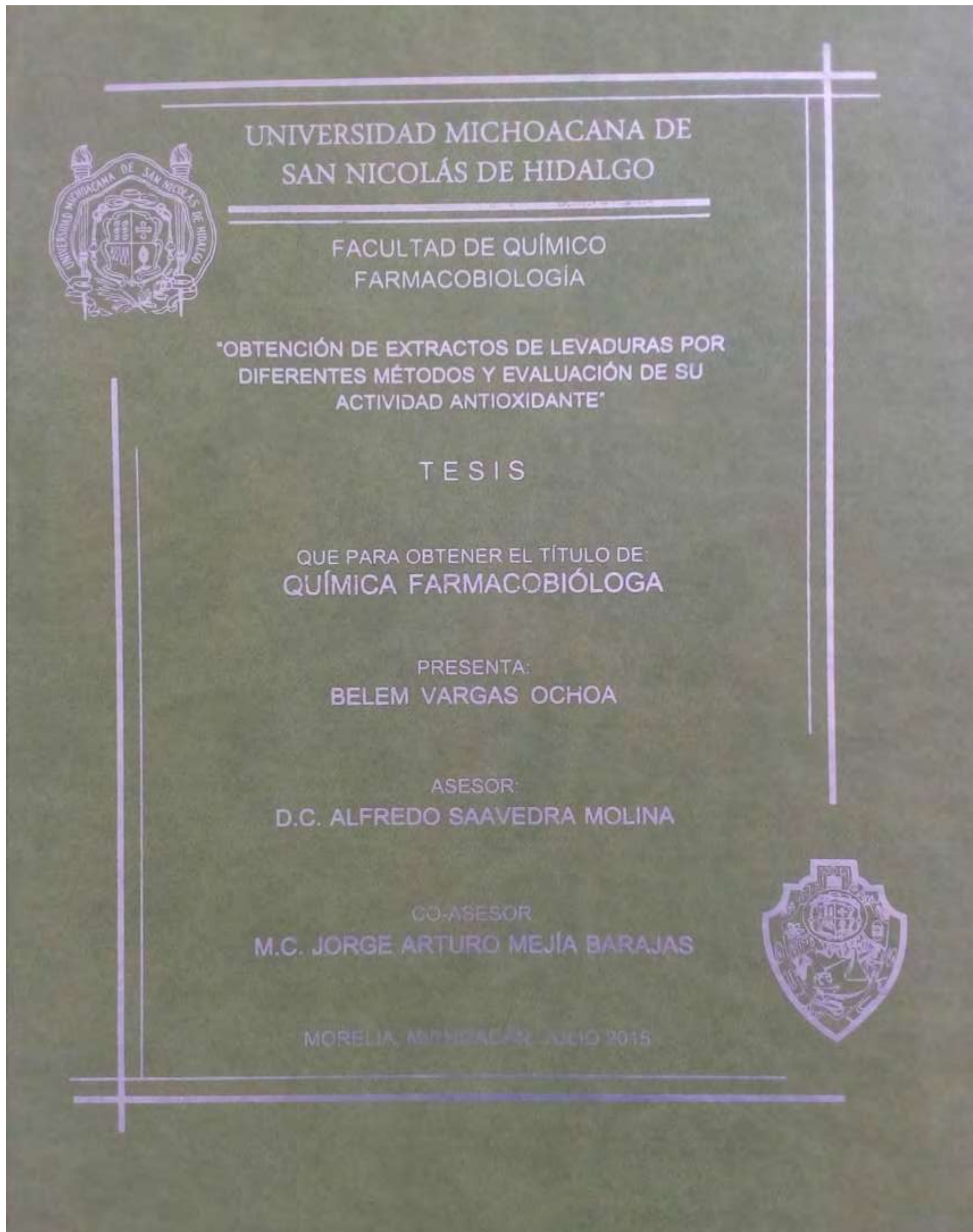
Maestro en Ciencias en Biología Experimental:
JORGE ARTURO MEJÍA BARAJAS

MORELIA MICHOACÁN, ENERO DE 2015

Obtención de extractos de levaduras por diferentes métodos y evaluación de su actividad antioxidante. Presentada por Belem Vargas Ochoa en la Facultad de Químico Farmacobiología, de la Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán. Julio, 2015.



Actividad antioxidante del aceite de la semilla *Eryngium carlinae*, “in vitro” y en *Saccharomyces cerevisiae*, como modelo biológico. Presentado por Adriana Lizbeth Rodríguez Lino en la Universidad Tecnológica de Morelia, Morelia, Michoacán. Agosto, 2015.

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UNIVERSIDAD TECNOLÓGICA DE MORELIA
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DIRECCIÓN DE BIOTECNOLOGÍA

“ACTIVIDAD ANTIOXIDANTE DEL ACEITE DE LA SEMILLA *Eryngium carlinae*, “*in vitro*” Y EN *Saccharomyces cerevisiae* COMO MODELO BIOLÓGICO”

REPORTE TÉCNICO PRESENTADO PARA OBTENER EL TITULO DE TÉCNICO SUPERIOR UNIVERSITARIO EN QUÍMICO ÁREA BIOTECNOLOGÍA

PRESENTA

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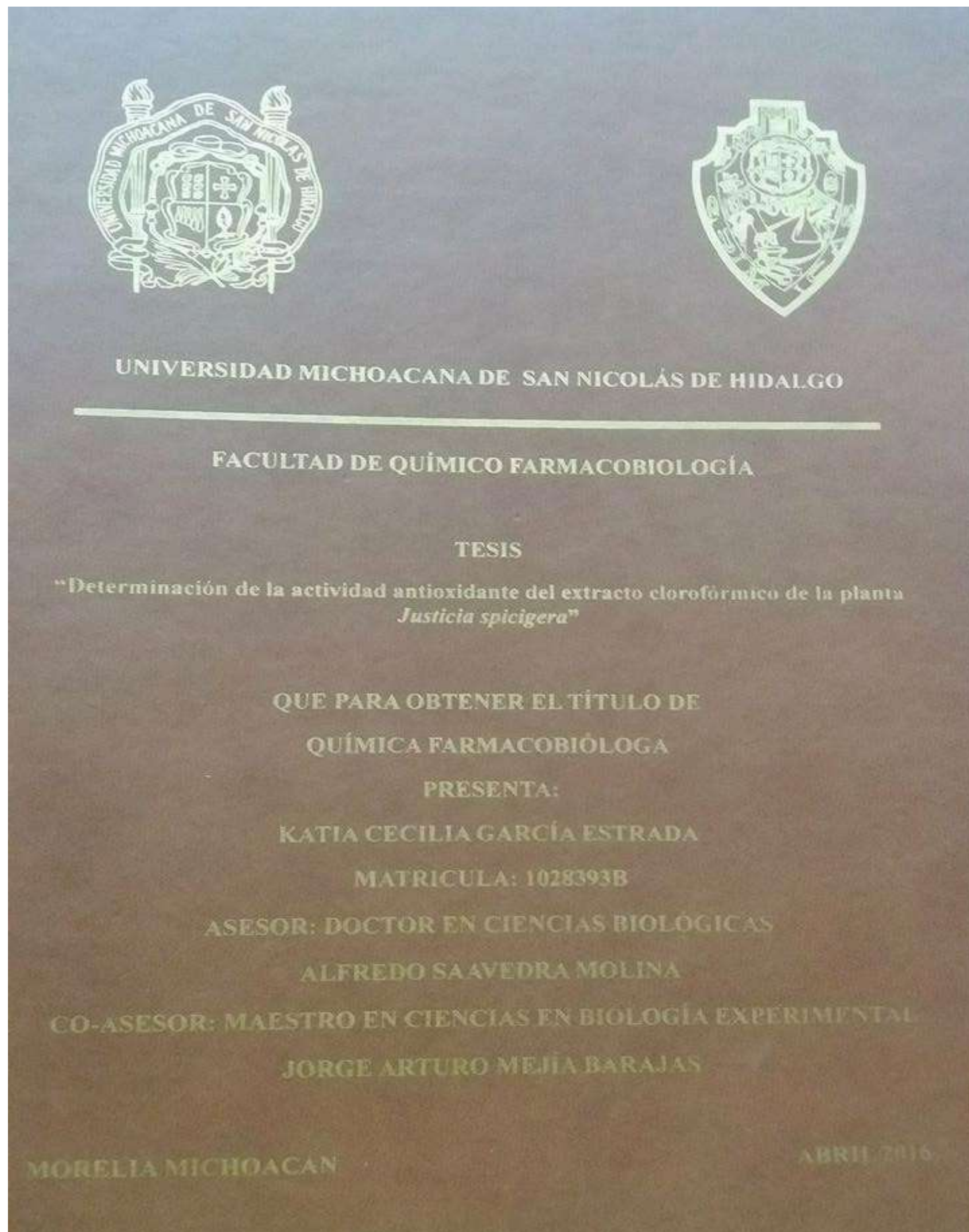
M.C. DAVID GARCÍA HERNÁNDEZ.

MORELIA, MICHOACÁN AGOSTO, 2015

UTM
Universidad Tecnológica de Morelia

Análisis de la actividad antioxidante del extracto hexánico de la inflorescencia de *Eryngium carlinae*, "in vitro" y en *Saccharomyces cerevisiae*, como modelo biológico. Presentada por Donovan Javier Peña Montes en en la Facultad de Químico Farmacobiología, de la Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán. Enero, 2016.

Determinación de la actividad antioxidante del extracto clorofórmico de la planta *Justicia spicigera*. Presentada por Katia Cecilia García Estrada en la Facultad de Químico Farmacobiología, de la Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán. Marzo, 2016.



UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

FACULTAD DE QUÍMICO FARMACOBIOLOGÍA

TESIS

"Determinación de la actividad antioxidante del extracto clorofórmico de la planta *Justicia spicigera*"

QUE PARA OBTENER EL TÍTULO DE

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JORGE ARTURO MEJÍA BARAJAS

MORELIA MICHOACAN ABRIL 2016

Estudio de la producción de bioetanol mediante diferentes procesos de fermentación utilizando una levadura termotolerante. Presentada por Ariel Joaquín Ramíreaz Uribe en la Facultad de Químico Farmacobiología, de la Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán. Febrero, 2017.

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