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ASPECTOS QUÍMICOS Y MOLECULARES DEL PROCESO DE PRODUCCIÓN DE MEZCAL

Tesis que presenta

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Para obtener el grado de

Doctora en Ciencias en Biología Molecular

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Resumen

En el presente trabajo se realizó la identificación y cuantificación de compuestos volátiles presentes en mezcales comerciales producidos en la región mezcalera del país. Se investigó el efecto de las condiciones iniciales de concentración de azúcares y temperatura en el proceso de fermentación; se analizó el comportamiento del potencial redox y finalmente se llevó a cabo la identificación molecular de la microbiota presente al inicio de la fermentación.

El análisis de los compuestos volátiles en los mezcales se efectuó mediante cromatografía de gases (GC) y espectrometría de masas (MS). Los destilados obtenidos a partir de *Agave salmiana*, producidos en la región del altiplano entre Zacatecas y San Luis Potosí, mostraron la presencia de una gran variedad de compuestos volátiles como alcoholes, ácidos orgánicos, esteres, furanos, cetonas y aldehídos. También se detectó la presencia de terpenos como limoneno, α-terpineol y α-terpineno. Únicamente los mezcales con gusano presentaron compuestos insaturados como el 6, 9-pentadecadieno-1-ol, 3-hexen-1-ol, 1-8 nonadieno y 1-dodecino. Compuestos como limoneno y el pentilbutanoato fueron identificados por primera vez, en mezcales producidos a partir de *Agave salmiana*.

El estudio de bebidas alcohólicas como son la raicilla, sisal, tequila, mezcal, bacanora y sotol, producidos en diversas regiones del país, reveló la presencia de 105 compuestos volátiles diferentes. De los cuales once compuestos se clasificaron como mayoritarios y el resto se clasificaron como minoritarios. El análisis multivariable permitió clasificar los destilados a partir la concentración de sus compuestos. También se identificaron 17 compuestos minoritarios específicos, en los destilados de *Agave*, tales como el azuleno, cinnamol, butirolactona y la piridina entre otros, que podrían ser los responsables de la firma o huella de cada bebida. Finalmente se observó que el proceso de producción y la materia prima juegan un papel muy importante en el perfil sensorial de la bebida.

La optimización de las variables de operación, concentración inicial de azúcares y temperatura, permitió maximizar la producción de etanol, el rendimiento y la productividad. Esto se realizó mediante la metodología de superficie de respuesta (RSM), la cual permitió optimizar simultáneamente las dos variables de operación. La máxima producción de etanol se obtuvo a 28°C y 105 g/L para el caso del rendimiento 28°C y 77g/L y por último para la productividad fue de 34.6°C y 90g/L. Se encontró que la producción de etanol está asociada al crecimiento microbiano, mientras que el crecimiento celular presentó una cinética de inhibición por sustrato. El comportamiento del potencial redox es inversamente proporcional al crecimiento microbiano y a la producción de etanol. La cuantificación por GC de los compuestos volálites mayoritarios en fermentaciones realizadas a diferentes condiciones iniciales, mostraron que la temperatura influye en la producción de alcoholes superiores y por lo tanto en la calidad de la bebida.

Se emplearon técnicas microbiológicas de cultivo en placa para aislar bacterias y levaduras presentes al inicio de la fermentación. La identificación se realizó amplificando una región del DNA ribosomal, 16S rDNA en el caso de bacterias e ITS (Internal Transcriber Spacer) para las levaduras, los productos obtenidos se clonaron y secuenciaron. Los resultados mostraron que existen al menos 3 géneros distintos de levaduras, *Pichia fermentans, Clavispora lusitaniae* y *Kluyveromyves marxianus*. En el caso de las bacterias se identificó al género *Lactobacillus* como la población dominante. Se identificó y aisló *L.kefiri, L.farraginis, L.plantarum*, *Weissella cibaria* y *W. paramesenteroides*. También se identificó a *Zymomonas mobilis.*

PALABRAS CLAVE: Compuestos volátiles, RSM, 16S rDNA, ITS

Abstract

In this work the identification and quantification of the volatile compounds of the commercial mezcals, produced in the mezcal region of the country is performed. Moreover, the effects of the initial substrate concentration and the temperature in the fermentation process were investigated. Finally, the molecular identification of the microbiota present at the beginning of the fermentation was carried out.

The volatile compounds analysis of the mezcals was carried out using gas chromatography (GC) and mass spectrometry (MS). The distillation product of the A*gave salmiana* juices, produced in the altiplano region between Zacatecas and San Luis Potosi, shown a great variety of volatile compounds such as alcohols, organic acids, esters, furans, ketoses and aldehydes. In addition, the presence of terpenes (limonene, α-terpinol and α-terpinene) was detected. Only the mezcals with worms shown unsatured compounds like the 6,9-pentadecadien-1-ol, 3-hexen-1-ol, 1,8-nonodiene and 1-dodecine. We mentioned here that compounds such as limonene and pentylbutanoate were identified for first time, in mezcals produced from *Agave salmiana*.

The study of alcoholic beverages such as raicilla, sisal, tequila, mezcal, bacanora, sotol and pulque produced in different region of the country reveled the presence of the 105 different volatile compounds. Eleven of them were classified as major compounds and the others were classified as minor compounds. The multivariate analysis allows us to establish a compositions-based classification for the distillated products. Then seventeen minor compounds, specific of the beverages, were identified providing a better fingerprinting. Finally, it is shown that the process production and the raw material play an important role in the sensorial profile of the beverage.

The optimization of the operation variables such as initial sugar concentration and temperature was achieved maximizing of the ethanol concentration, yield and productivity. This was performed trough the application of the surface response methodology (RSM), optimizing at the same time the operation variables. The maximum ethanol production was obtained at 28 °C and 90 g/L. It was found that ethanol production is associated to the biomass growth, whereas the cell-growth showed a substrate inhibition behavior. The time evolution of the redox potential was inversely proportional to the microbial growth and to the ethanol production. The GC volatile compounds quantification of fermentations performed at different initial conditions shown an influence of the temperature in the production of higher alcohols and therefore in the quality of the beverage.

To insolate the different yeasts and bacteria, present at the beginning of the fermentation, microbiological techniques, such as grown on plates, were employed. The identification was performed trough the amplification of the 16S for bacteria and the Internal Transcriber Spacer (ITS) for the yeasts; then, the PCR products were cloned and sequenced. The results have shown the existence of at least 3 different yeasts, *Pichia fermentans, Clavispora lusitaniae* and *Kluyveromyves marxianus*. Regarding to the bacteria the *Lactobacillus* was identified as the dominant specie, including *L.kefiri, L.farraginis* and *L.plantarum.* In addition, the *Weissella cibaria* and *W. paramesenteroides* and *Zymomonas mobilis*.

KEY WORDS: Volatile compounds, RSM, 16S rDNA, ITS rDNA

I. INTRODUCCIÓN

La producción de bebidas tradicionales fermentadas y destiladas, constituye un área de creciente interés. La calidad y producción en condiciones controladas dependen del conocimiento y control de la microbiota presente y de las condiciones de operación. Para obtener una idea detallada de las interacciones entre las comunidades microbianas durante el proceso fermentativo, es necesario identificarlas y establecer el papel que juegan en el producto terminado. El mezcal es la bebida que representa a México frente al mundo y paradójicamente ha sido objeto de estudio hasta hace unos pocos años.

Actualmente, en casi todos los estados de la República Mexicana se producen, con métodos tradicionales, bebidas alcohólicas a partir de la fermentación de las azúcares del *Agave*. El nombre que se usa para referirse a ellas varía de una región a otra. Actualmente la norma Mexicana de bebidas alcohólicas (NOM-070, 1994) establece que únicamente las bebidas producidas en los estados de Durango, Zacatecas, San Luis Potosí, Guerrero y Oaxaca pueden ser denominadas mezcal. En el proceso de producción están involucradas las cinco etapas siguientes: recolección de las piñas, cocción, molienda, fermentación y destilación. En este trabajo se seleccionó la etapa fermentativa como fase de estudio; debido a que en ésta se produce la mayor concentración de compuestos volátiles. Entre los aspectos que se investigarán destacan, la optimización de las variables de operación y la identificación de la microbiota mediante técnicas de biología molecular. Debido, a que el perfil sensorial juega un papel fundamental en la aceptación de la bebida, por parte del consumidor, adicionalmente se propuso como objetivo identificar los compuestos volátiles en bebidas alcohólicas de *Agave.*

1.1 *Antecedentes*

A mediados del siglo XVIII el naturalista sueco Carlos de Linneo establece al *Agave* (del vocablo grecolatino *agavus*) como el nombre científico del maguey. En México ésta planta era conocida y utilizada en la época prehispánica. El maguey llamado *metl* o *mexcalmetl*, en lengua náhuatl, se aprovechaba de forma integral. De sus jugos se preparaban bebidas fermentadas con fines rituales y medicinales. Las hojas se empleaban para la producción de fibras, papel y clavos. La palabra mezcal también tiene su origen en vocablos de la lengua náhuatl. Mezcal deriva de *mexcalli* donde metl o *meztl* significa maguey e *ixcalli* cocer. Es decir, maguey cocido. El mezcal se obtiene de la fermentación y destilación de los jugos de *Agave*. Entre las plantas que destacan como materia prima utilizada para su producción se encuentra el *Agave salmiana, A. angustifolia* y *A. potatorum* entre otras (NOM-070).

La producción del mezcal esta regulada por la Norma Oficial Mexicana NOM 070. Ésta norma se refiere a denominación de origen del mezcal. Establece las características y especificaciones que se deben cumplir para su producción y/o comercialización. El mezcal está definido como una “bebida alcohólica regional obtenida por destilación y rectificación de mostos preparados directa y originalmente con los azúcares extraídos de las cabezas maduras de los *Agaves*, antes mencionadas, previamente hidrolizadas o cocidas, y sometidas a fermentación alcohólica con levaduras, cultivadas o no” (NOM 070). Las características que presenta son: líquido de olor y sabor suigéneris de acuerdo a su tipo, incoloro o ligeramente amarillento cuando es reposado o añejado (NOM 070).

El empleo de levaduras en el proceso fermentativo para la obtención de bebidas alcohólicas genera compuestos como alcoholes, esteres, ácidos orgánicos, sulfuros orgánicos y compuestos de carbonilo. Los alcoholes superiores

y sus esteres son los compuestos que más influyen en el perfil organoléptico en las bebidas alcohólicas (Ter Schure et al., 1998). En tequila existen alrededor de 175 compuestos que pertenecen a las familias de los acetales, ácidos, alcoholes, aldehídos, ésteres, furanos, cetonas, fenoles, piracinas, compuestos con azufre y terpenos. Entre los más importantes responsables del olor se encuentran: isovaraldehído, alcohol isoamílico, β-damascenona, 2-feniletanol y vainillina (Benn y Peppard, 1996). López (1999), reportó que los compuestos volátiles mayoritarios presentes en tequila son el etanol, alcohol isoamílico, feniletanol y los ácidos acético, decanoico y dodecanoico. Otro grupo de compuestos presentes en en el tequila son los terpenos, los cuales provienen de la planta. Peña-Alvarez (2004) analizó los terpenos presentes en plantas de *Agave salmiana, A. angustifolia* y *A. tequilana*. De un total de 32 terpenos identificados, 21 de ellos se encontraron exclusivamente en el *A. tequilana.* Los terpenos como el linalool, geraniol, p-cimene, limonene, β-trans-ocimene y trans-nerolidol se encontraron en los tres tipos de *Agave* analizados. La presencia de esteres en tequila pueden ser resultado del metabolismo de las levaduras o producto de la esterificación durante el añejamiento. La esterificación se lleva a cabo en presencia de altas concentraciones de etanol y ácidos grasos. En plantas de *Agave salmiana, A. angustifolia* y *A. tequilana* se idenficaron ácidos grasos de cadena larga (Peña-Alvarez et al., 2004). Posteriormente, Vallejo-Cordoba et al., (2004) propone que la cuantificación de los esteres puede ser una alternativa para la clasificación de los diferentes tipos de tequila.

1.2 *El mezcal y su aroma*

Según Abbott (1999) los atributos que más condicionan la aceptabilidad del alimento por parte del consumidor son los relacionados con la calidad sensorial u organoléptica, que incluye la apariencia, la textura, el aroma y el gusto. En este sentido, uno de los rasgos organolépticos más complejos y determinantes de la calidad sensorial es el aroma del alimento, que se puede definir como la sensación

global producida por los compuestos que interaccionan con las terminaciones nerviosas sensitivas del gusto, del olfato y la visión (Goff y Klee, 2006). El aroma está compuesto por centenares de compuestos volátiles que pertenecen a distintas familias químicas y que se encuentran en muy variable concentración (Ruiz y Martínez, 1997). La elevada producción y la necesidad de encontrar alimentos aromáticamente estandarizados, requieren herramientas analíticas eficientes para la caracterización y algoritmos que permitan el control automático de la producción. Cabe mencionar que el umbral de percepción de las sustancias que condicionan el aroma puede variar desde µg/l a mg/l, pero no necesariamente por encontrarse en mayor concentración su incidencia será mayor (Riu, 2005). En este sentido, el impacto sensorial esta relacionado con las presencia de compuestos volátiles. Así pues, una de las principales variables a medir es la fracción aromática.

El aroma puede provenir de la planta (*Agave)*, y es llamado *aroma primario* que incluye dos subcategorías: el *varietal* (compuestos volátiles libres presentes en la planta que dependen de la variedad utilizada y sus características) y el *prefermentativo* (aromas que se liberan de su combinación con otras sustancias llamadas precursores, debido a la actividad enzimática provocada por la tecnología aplicada). El *aroma secundario* proviene de los compuestos producidos por los microorganismos que se desarrollan durante la primera etapa de la fermentación. Estos compuestos constituyen el principal aroma. Finalmente el *aroma terciario* o *post-fermentativo* es el que se forma durante el añejamiento. Este último se desarrolla mediante reacciones químicas y/o bioquímicas (esterificación) a partir de compuestos volátiles producidos en las etapas anteriores (Riu, 2005).

El desarrollo de técnicas cromatograficas y equipos para la detección de compuestos han permitido identificar la composición de bebidas alcohólicas como el mezcal (De León-Rodríguez et al., 2006). La Cromatografía de Gases es una técnica de separación dinámica que se basa en la interacción de distintos analitos con dos medios: la fase estacionaria y la fase móvil. Como resultado de las interacciones cada analito migra a distinta velocidad a través de la fase

estacionaria. La distribución de los analitos entre la fase móvil y la estacionaria depende básicamente de la temperatura de la columna (Harris, 1992). Los espectrómetros de masas se utilizan ampliamente en combinación con la cromatografía de gases. La espectrometría de masas proporciona el peso molecular, información acerca de la fórmula molecular, empleando una cantidad muy pequeña de muestra. La detección del analito se realiza mediante su rompimiento con electrones de alta energía; los fragmentos producidos son medidos y con esta información se reconstruye la molécula (Wade, 1993). Cuando el objetivo es analizar compuestos que se encuentran en bajas concentraciones en la muestra se puede hacer uso de La microextracción en fase sólida. Esta técnica fue desarrollada por Arthur C.L. (1990) y su principal ventaja es que es económica ya que no requiere solventes (Kataoka, 2000).

1.3 *En busca de las condiciones óptimas para la producción de mezcal*

Durante el proceso fermentativo es muy importante conocer qué ocurre con los consorcios de levaduras y bacterias. Los trabajos pioneros de Jacob Monod y J. B. S. Haldane han servido como un punto de inicio de importantes modelos matemáticos que dan cuenta de diferentes aspectos del crecimiento microbiano en monocultivos por lote, lote-alimentado y continuos. Estos trabajos suponen cultivos puros. Existe una vasta cantidad de artículos sobre modelos matemáticos de crecimiento microbiano (Nielsen et al., 2003). Sin embargo, en el ámbito de cultivos mixtos la literatura se reduce considerablemente. En el modelado de la mayoría de estos sistemas biológicos se asume de manera implícita que son de naturaleza continua, aplicando las ecuaciones diferenciales como la herramienta para su modelado. Las variables analizadas son denominadas estados, y son propiedades tales como la concentración de microorganismos, concentración del producto, y concentración de sustrato; y en algunos casos concentración interna de enzimas. Sin embargo, existen limitaciones para medir estos estados en tiempo real. De ahí que una solución a éste problema es el desarrollo de modelos

basados en ecuaciones diferenciales ordinarias que contemplen entre sus estados, variables que sean fácilmente monitoreables en tiempo real. Dichos modelos permitirían el desarrollo de controladores que hagan frente a las perturbaciones inherentes a estos sistemas. Durante la fermentación se llevan a cabo reacciones de óxido-reducción, las cuales están estrechamente relacionadas al crecimiento microbiano (Berovič, 2003). La oxidación es la pérdida de electrones mientras que la reducción es la ganancia de electrones. Cuando una sustancia se oxida es decir libera electrones, simultáneamente otra se reduce o capta los electrones liberados. Así que la medida del potencial redox permite determinar el grado de reducción o de oxidación de un sistema. Esta medida sugiere la posibilidad de conocer la actividad microbiana a través de las interacciones químicas de sus productos. El potencial redox es una herramienta mediante la cual es posible monitorear en línea, siendo de gran utilidad para entender la dinámica de las fermentaciones.

Un aspecto de gran utilidad para la industria mezcalera es conocer las condiciones óptimas de operación del sistema o determinar la región espacial de los factores en la que se satisfacen las condiciones de operación. Esto se logra maximizando las variables de interés, entre las que destacan, la productividad, rendimiento y la producción de etanol. Para lograr tal objetivo se puede hacer uso de técnicas de optimización como la metodología de superficie de respuesta. La RSM, por sus siglas en inglés, es un conjunto de técnicas matemáticas y estadísticas útiles para modelar y analizar problemas en los cuales una respuesta de interés es influida por varias variables, y el objetivo es optimizar esa respuesta.

1.4 *Identificación molecular de microorganismos en alimentos fermentados*

El estudio ecológico de los alimentos fermentados permite conocer la dinámica y composición de la microbiota presente. Su producción y calidad dependen de su conocimiento y control. (Díaz y Watcher, 2003; Torija Martínez, 2002; Querol et al., 1992; Vasdinyei y Deák, 2003). Los alimentos fermentados,

especialmente los que se producen de forma tradicional, se obtienen mediante fermentaciones naturales, es decir, no se añaden inóculos sino que actúan los microorganismos naturales presentes en el alimento.

Las fermentaciones espontáneas no son el producto de la acción de una única especie, durante éstas se llevan a cabo procesos bioquímicos muy complejos, en los que intervienen e interaccionan levaduras, bacterias y otros microorganismos (Torija Martínez, 2002). Existen argumentos a favor y en contra de las fermentaciones espontáneas. El principal argumento a favor indica que en estas fermentaciones se consiguen características organolépticas típicas de la zona que no estarían presentes si se utilizara un inóculo de cepas foráneas. Sin embargo la calidad del producto puede ser muy variable. La composición cualitativa y cuantitativa de las microbiota presente a lo largo de la fermentación del mosto puede depender principalmente de los siguientes factores: región de donde es originaria la materia prima, procedimiento de producción, tipo de bebida a ser producida, concentración inicial de la microbiota, temperatura, pH, concentración de SO_2 y de etanol, (Torija *et al*., 2001; Granchi *et al*., 1999). El empleo de inóculos con poblaciones mixtas y/o inóculos secuenciales, constituye una herramienta importante para estandarizar el producto y preservar aquellas características deseables. Seleccionar cepas de una determinada región parece ser la solución para asegurar un producto estandarizado preservando las características organolépticas que distinguen a la zona de producción.

Durante el último siglo se dependió del aislamiento y cultivo de los microorganismos para su identificación (Díaz y Watcher, 2003; Escalante et al., 2004). Las levaduras se identificaban y clasificaban por medio de sus características morfológicas y fisiológicas (Las Heras-Velazquez et al., 2003; Orberá, 2004). Estos métodos tienen las desventajas de ser complejos, laboriosos y requieren mucho tiempo (Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Las Heras-Velazquez et al., 2003; Deák, 1998). Además, algunas veces proporcionan identificaciones dudosas, porque dependen de las condiciones del cultivo. Dichos

factores limitan la comprensión de la diversidad microbiana, ya que ahora se sabe que más del 90% de los microorganismos en los ambientes naturales no pueden ser cultivados empleando las técnicas microbiológicas tradicionales (Amann, 1995; Borneman et al., 1996). La dificultad de obtener cultivos puros de algunos microorganismos que dependen de la actividad de otros microorganismos, la pérdida de su capacidad de reproducción, manteniendo su actividad metabólica, la programación genética de las células para adaptarse a las condiciones adversas (como agotamiento de nutrientes, temperatura baja u otros tipos de estrés) y generar formas de latencia (Nyström, 2003) producen como resultado una visión deformada de la diversidad microbiana en un ambiente natural. El empleo de métodos convencionales para la identificación de microorganismos involucrados en procesos fermentativos resulta ser insuficiente (Deák, 1998). Una alternativa para la determinación de la microbiota en cualquier alimento fermentado es aislar los microorganismos y tipificarlos mediante técnicas de biología molecular, otra es utilizar métodos que no dependan del cultivo, en los que se extraen ácidos nucleicos directamente del alimento.

El RFLP (análisis de polimorfismos de longitud de fragmentos de restricción) es una técnica comúnmente utilizada para la clasificación de microorganismos, basada en el DNA. Por medio de ésta técnica es posible distinguir microorganismos a nivel de especie (Penderson, 1986; Granchi et al., 1999; Orberá, 2004). Está técnica consiste en la extracción del DNA (el genoma completo) de cada cepa, posteriormente éste se digiere con enzimas de restricción (endonucleasas) y los fragmentos obtenidos, de diferentes tamaños, se separan y visualizan por electroforesis en geles de agarosa. Los patrones de bandas obtenidos tienen buena reproducibilidad pero por lo general son muy complejos (Díaz y Watcher, 2003). Una variante de la técnica conocida como RFLP-PCR se utiliza ampliamente para la identificación de levaduras de interés enológico, en fermentaciones espontáneas. La variante en ésta técnica consiste en amplificar fragmentos específicos de DNA por PCR para después ser tratados con enzimas de restricción, endonucleasas, obteniéndose patrones específicos. Las diferencias

en las secuencias nucleotídicas de las diferentes especies dan lugar a fragmentos de distintos tamaños que son examinados por electroforesis. En el ribosoma de las levaduras se encuentran regiones (18S, 5.8S y 28S) extraordinariamente conservadas entre especies e incluso entre géneros, familias y órdenes diferentes. Entre las regiones del 18S, 5.8S y 28S se encuentran las secuencias conocidas como ITS (Internal Transcribed Spacers, secuencias internas transcritas). Éstas regiones muestran una elevada variación longitudinal en las levaduras, es decir, son zonas muy poco conservadas (Carro, 2000; Orberá, 2004). Las secuencias altamente conservadas del rDNA que flanquean las regiones ITS1 e ITS2 permiten diseñar oligos universales para levaduras. Ver Figura 1**.**

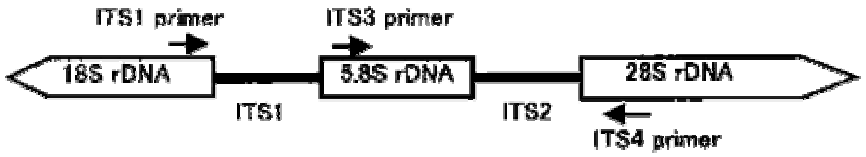

Figura 1. Representación esquemática de los genes ribosomales en hongos (Fujita et al., 2001).

White et al. (1990), estableció las condiciones óptimas para amplificar la región del ITS. La amplificación de ésta región permitió identificar un total de 132 levaduras pertenecientes a 25 géneros diferentes. El objetivo de Esteve-Zarzoso (1999), fue generar una base de datos para una fácil y rápida identificación de levaduras. En muchos casos el tamaño del producto de PCR y los patrones de restricción obtenidos con las endonucleasas *CfoI, HaeIII* y *HinfI* produjeron un patrón único para cada especie. Las Heras-Velazquez (2003), encontró 9 géneros de levaduras diferentes en la fermentación espontánea de jugo de naranja, a partir de 100 levaduras aisladas. Las levaduras aisladas mostraron diferente tamaño en el producto de PCR amplificado en un rango entre 370bp y 880bp. Los productos de PCR fueron digeridos con tres enzimas de restricción: *CfoI, HaeIII* y *HinfI* mostrando patrones únicos para cada uno de los géneros. En suma, está técnica representa una alternativa para la identificación de levaduras de interés enológico en fermentaciones espontáneas de jugos de frutas (uva, naranja y manzana) y

producción de bebidas alcohólicas. (Sabate et al., 2002; Las Heras-Velazquez et al, 2003; Garrett y Dobson, 2004).

La mayoría de las bacterias provenientes de ambientes naturales no pueden ser cultivadas en condiciones de laboratorio con las tecnologías actuales (Borneman et al., 1996; Dojka et al., 1998; Escalante et al., 2001; Díaz y Watcher, 2003). El uso de PCR para amplificar un gen común en todos los microorganismos permite la identificación de organismos antes desconocidos. Principalmente, microorganismos que no crecen en condiciones de laboratorio. Los genes comúnmente amplificados para este propósito codifican pequeñas subunidades del DNA ribosomal, SSU "small ribosomal subunit" (Borneman et al., 1996). Los estudios taxonómicos y filogenéticos se realizan con las secuencias del 16S rRNA en procariotes y 18S rRNA en eucariotas. Rivas (2004), reportó la existencia de dos secuencias altamente conservadas en el ribosoma de todos los organismos. Aprovechando esa característica propone el uso del mismo par de oligos llamado "Universal Amplified Ribosomal Region" (UARR), para la amplificación de todos los organismos; obteniendo bandas de 495 bp en procariotes y 508 bp en eucariotas. El UARR contiene los dominios V6, V7 y V8 del SSU rRNA en ambos procariotes y eucariotas.

El estudio de la diversidad microbiana y el análisis de la estructura de las comunidades reveló que la diversidad microbiana es mucho mayor de lo que se había considerado (Díaz y Watcher, 2003). Cabe mencionar una serie de limitaciones que presentan las técnicas moleculares para la identificación de microorganismos. Los métodos de extracción de ácidos nucleicos de las muestras no aseguran la lisis de todos los microorganismos presentes y la recuperación de los ácidos nucleicos intactos. Es importante purificar los ácidos nucleicos intactos para eliminar sustancias que puedan inhibir la reacción de PCR o la acción de enzimas de restricción. La reacción de PCR puede introducir errores, ya que pueden ocurrir amplificaciones preferenciales, debido a la reasociación del DNA

que se usa como templado y que evita la unión de los oligos (Díaz y Watcher, 2003).

Los avances recientes en los métodos para el análisis de comunidades microbianas hacen posible complementar a los métodos tradicionales para obtener información sobre aspectos importantes de la ecología microbiana de alimentos fermentados: diversidad, estructura y función. La selección adecuada de cultivos iniciadores, en los casos que se considere conveniente, así como el monitoreo y control de la fermentación asegurarán productos de mayor calidad.

OBJETIVO GENERAL

Estudiar los aspectos químicos y moleculares del proceso de producción del mezcal.

OBJETIVOS PARTICULARES

1. Identificar mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS) los compuestos volátiles mayoritarios y minoritarios, presentes en mezcales comerciales (joven, añejo y reposado con y sin gusano) producidos de *Agave salmiana.*

2. Analizar los compuestos volátiles mayoritarios por GC y minoritarios previamente concentrados mediante SPME-GC-MS, presentes en bebidas destiladas obtenidas a partir de diferentes plantas de *Agave.*

3. Determinar las condiciones de temperatura y concentración inicial de azúcares que permitan la optimización de la fermentación de mezcal producido a partir de *Agave salmiana*.

4. Aislar e identificar por medio de técnicas moleculares tales como: amplificación de la región 16SrDNA para el caso de las bacterias y la región de ITS para las levaduras los microorganismos que participan en la fermentación del mezcal.

CAPÍTULO I

3. IDENTIFICACIÓN DE COMPUESTOS VOLÁTILES EN BEBIDAS ALCOHÓLICAS DE AGAVE

En este capítulo se presentan los resultados obtenidos del análisis cualitativo y cuantitativo de compuestos volátiles presentes en distintos destilados de *Agave;* mediante el uso de Cromatografía de Gases (GC) y Espectrometría de Masas (MS). Las muestras se sometieron a un tratamiento previo por microextracción en fase sólida (SPME), para concentrar los componentes volátiles. El capítulo está formado por los siguientes artículos:

De León-Rodríguez, A. González-Hernández, L. Barba de la Rosa, Ana P. Escalante-Minakata, P. and López, M.G. 2006. Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana*. Journal of Agriculture and Food Chemistry. 54, 1337-1341.

Antonio De León-Rodríguez, Pilar Escalante-Minakata, María I. Jiménez-García, Leandro G. Ordoñez-Acevedo, José L. Flores-Flores and Ana P. Barba de la Rosa. Characterization of volatile compounds from ethnic Agave alcoholic beverages by gas chromatography-mass spectrometry. Submitted to Food Technology and Biotechnology. (Aceptado, en prensa)

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Characterization of Volatile Compounds of Mezcal, an Ethnic Alcoholic Beverage Obtained from *Agave salmiana*

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Commercial mezcals (white, white with worm, rested, rested with worm, and aged) produced from *Agave salmiana* were analyzed by solid-phase microextraction−gas chromatography−mass spectrometry (SPME−GC−MS). Thirty-seven compounds were identified, and nine of them were classified as major compounds of mezcal (MCM). Saturated alcohols, ethyl acetate, ethyl 2-hydroxypropanoate, and acetic acid form the MCM group. Minor compounds of mezcal group include other alcohols, aldehydes, ketones, large chain ethyl esters, organic acids, furans, terpenes, alkenes, and alkynes. Most of the compounds found in mezcals in this study are similar to those present in tequilas and other alcoholic beverages. However, mezcals contain unique compounds such as limonene and pentyl butanoate, which can be used as markers for the authenticity of mezcal produced from *A. salmiana.*

KEYWORDS: Mezcal; *Agave*; tequila; ethanol; SPME; terpenes; alcohols

INTRODUCTION

Mezcal is a Mexican alcoholic beverage obtained by artisan fermentation and distillation from *Agave* syrup. Species of *Agave* plants such as *A. salmiana*, *A. angustifolia*, and *A. potatorum* are used as raw materials (*1*). However, only the wild-type *A. salmiana* is used for the production of mezcal in the Mexican altiplano, whereas *A. angustifolia* and *A. potatorum* are used in the south of Mexico (*1*, *2*). Mezcals are beverages related to tequila (produced from *A. tequilana*). A full description of both processes and differences between mezcal and tequila has been published elsewhere (*2*−*4*).

Mezcals have been classified into three types based on the aging process after distillation. White mezcal is bottled just after distillation; rested mezcal is aged from 2 to 6 months in oak casks, whereas aged mezcal must be aged up to 12 months (*1*). Only the white and rested mezcals are conditioned with one to four larvae of *Agave* worms. Thus, mezcal is currently named "the worm's beverage".

The organoleptic properties and the bouquet of alcoholic beverages such as tequila, wine, and others are determined by the composition of alcohols mixture, esters, and other compounds. Principally, alcohols with three or more carbons and ethyl esters are the major agents responsible for the aroma (*5*−*10*). Microorganisms produce these compounds during the fermentation, although there are others coming from the raw material such as terpenes and large chain fatty acids (*6*). There exist some reports describing the components of tequilas (*7*−*10*); however, there is little information on the mezcal composition produced from *A. salmiana*, and only few reports of mezcal from *A. potatorum* and *A. angustifolia* are available (*11*, *12*).

The techniques reported for analysis of alcoholic beverages include the concentration of components by micro distillation, liquid−liquid extraction, and solid-phase microextraction (SPME) (*8*−*11*). SPME is a current sampling technique recommended for the analysis of alcoholic beverages because it is solvent-free and sample handling is minimized (*10*−*15*). The goal of this work was to characterize the volatile compounds of mezcals obtained from *A. salmiana* through GC and SPME−GC−MS techniques.

MATERIALS AND METHODS

Chemicals and Mezcal Samples. All chemicals used as standards with purity up to 99.0% GC grade were purchased from Sigma-Aldrich (St. Louis, MO). Sixteen mezcal brands were analyzed: four white mezcals, three white with worm, three rested, three rested with worm, and three aged mezcals. They were kindly provided by the Association of Mezcal Producers of San Luis Potosi State, Mexico.

Major Compounds of Mezcal. The major compounds of mezcal (MCM) are the substances with concentration larger than 10 mg/L, and they are detected and quantified by GC using direct injection of the raw samples. For the quantitation of MCM, 1 mL of 2-pentanol at

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3.2 Characterization of volatile compounds from ethnic Agave alcoholic beverages by gas chromatography-mass spectrometry.

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Running title: Composition of alcoholic beverages from *Agave*
Submitted to: Food Technol and Biotechnol.

Abstract

Ethnic *Agave* alcoholic beverages such as raicilla, sisal, tequila, mezcal, bacanora, sotol and pulque were analyzed by gas chromatography and solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS). One hundred-five compounds were identified, and eleven of them were classified as major compounds and the rest were classified as minor compounds. Seventeen minor compounds were unique for specific type of beverage and they could be used as authenticity markers. Cluster analysis (CA) shown that *Agave* alcoholic beverages may be distinguished by multivariate analysis of major compounds, however, the analysis of minor compounds provides a best fingerprinting.

KEYWORDS: ethanol, pulque, spirits, SPME, terpenes, alcohols

Introduction

Many countries have the origin denomination and exclusive trademarks for local alcoholic beverages such as Scotland for whisky (Aylott et al., 1994), France for cognac (Ledauphin et al., 2006), Mexico for tequila and others (Aguilar-Cisneros et al., 2002). Mexico has several native plants of *Agave* genus and some of them are used for the production of ethnic alcoholic beverages (Gentry, 1982). For example, Pulque is a non-distilled alcoholic beverage with 4-6 %v/v of alcohol grade and it is produced by fermentation of *Agave* sap (aguamiel) obtained mainly from *A. mapisaga*. Distilled beverages with high alcohol grade (30-45%) are produced by fermentation and distilling of sugars obtained from cocked *Agave* plants. The names of ethnic alcoholic beverages are designed depending both the *Agave* species used as raw material and the geographic region of Mexico where they are cultivated. Thereby, tequila is produced from *A. tequilana* Wever var*. azul*, raicilla from *A. maximiliana*, sisal from *A. fourcroydes* and sotol from *Dasylirion wheeleri*. *A. angustifolia* Haw is used to produce bacanora in the North and mezcal in the South of Mexico. Mezcal is also produced from *A. salmiana, A. potatorum* and *A. durangensis* (Figure 1).

Volatile compounds determine the organoleptic properties and the bouquet of alcoholic beverages. Then the flavor is a combination of both aroma and taste, being the aroma the perception of volatiles through mouth and olfactory system (Van Ruth, 2001). Composition of tequila (López, 1999), mezcal (De León-Rodríguez et al., 2006), sotol and bacanora (Lachenmeier et al., 2006) has been reported previously. However, exhaustive studies on volatile compounds by gas chromatography (CG) coupled to mass spectrometry (MS) for other *Agave* alcoholic beverages such as sisal, raicilla, pulque and mezcals from different plants.

The solid-phase microextraction (SPME) is a sampling technique currently recommended for the CG-MS analysis of alcoholic beverages since it is solvent-

free and minimizes sample handling (Jelen et al., 1998; Vallejo-Córdoba et al., 2004). The aim of this work was an extensive characterization of the volatile compounds of different ethnic alcoholic beverages produced from *Agave* plants by GC and SPME-GC-MS techniques.

Fig. 1. Geographical location of *Agave* plants used for production of *Agave* alcoholic beverages.

Materials and Methods

Chemicals and mezcal samples

Ethanol, methanol, propanol, 2-butanol, 2-methyl-propanol, 2-methyl-1-butanol, acetic acid, ethyl acetate, and ethyl 2-hydroxypropanoate with purity up 99.0% GC grade were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Certificated beverages were purchased in prestigious beverage stores, whereas Pulques were purchased in bulk.

Gas chromatography

The quantitation of major compounds present in a concentration larger than 10 mg/L was performed in a gas chromatograph 6890N (Agilent technologies, Wilmington, DW) provided with an auto-sampler 7863 (Agilent technologies, Wilmington, DW) and a capillary column HP-Innowax (30 m x 0.25 mm i.d., 0.25 µm film thickness; Agilent technologies, Wilmington, DW). Helium was used as carrier gas at a flow rate of 1.5 mL/min and the setting temperatures for the injector and flame ionization detector (FID, Agilent technologies, Wilmington, DW) were 220 and 250 °C, respectively. The operative conditions and details on the calibration curves have been described elsewhere (De León-Rodríguez et al., 2006). Samples were analyzed by triplicate and the average concentration of each compound was used for our results.

Headspace SPME and gas chromatography-mass spectrometry

The minor compounds are the substances detected after sample concentration by SPME followed by GC-MS analysis. The SPME operation was carried out as described by De León et al. (7) using separately a SPME orange fiber of 65 µm Carbowax/Divinylbenzene (CW/DVB) and a SPME black fiber of 65 µm (Carboxen/Polydimethylsiloxane; CAR/PDMS).The SPME fibers were immediately inserted in the GC injector in split-less mode for 1 min at 180 °C. The GC-MS analyses were carried out in a gas chromatograph 6890N (Agilent technologies,

Wilmington, DW) coupled to a HP 5973N mass selective detector (Agilent technologies, Wilmington, DW) and using a DB-WAX column (30 m x 0.32 mm, 0.5 µm thickness; Agilent technologies, Wilmington, DW). The chromatographic conditions were 40 °C for 3 min, increased at 3 °C/min to 120 °C, 6 °C/min to 200 °C and maintained at this temperature for a final time of 60 min. Helium was used as carrier gas at a flow rate of 1.0 mL/min and the injector and detector temperatures were 180 and 230 °C, respectively. The MS ionization potential was 70 eV, transfer line temperature of 230 °C, and scan mode (50-700 m/z). The compounds were tentatively identified by comparing their mass spectra with those obtained in the NIST library from the MS database.

Cluster analysis

The Cluster analysis (CA) for the quantitative major compounds was carried out by the Nearest Neighbor (Single Linkage) clustering method using Statgraphic Plus software V.5 (Statistical Graphic Corp). The CA for minor compounds was carried out with the UPGMA (with Jaccard coefficient) clustering method using NTSysPc V. 3.21 (*11*).

Results and Discussion

Analysis of major compounds

The chromatograms for *Agave* alcoholic beverage are shown in Figure 2 and the compounds and their respective concentrations are summarized in Table 1. The alcohols detected were methanol, ethanol, propanol, 2-butanol, 2-methyl-propanol, 2-phenylethanol and the mixture of 2-methyl-1-butanol and 3-methyl-1-butanol. Since these isomers can be solved only on specific chromatographic conditions, they are reported together as 2-(3)-methyl-1-butanol (López, 1999; De León-Rodríguez, 2006). Acetic acid, ethyl acetate, and ethyl 2-hydroxypropanoate were also found in all alcoholic beverages. Important differences in the major compounds concentration of each type of beverage were observed. The

compounds marked as non-detectable had concentration lower than 10 mg/L and they were referred as minor compounds (described below). The major compounds are produced by the alcoholic fermentation (Berovič et al., 2003; Szamelan et al., 2005). The mixture of 2-(3)-methyl-1-butanol has a fruit aroma, and its presence is desired in alcoholic beverages (Berovič et al., 2003; Szamelan et al., 2005)). In our study, bacanora, mezcal (from *A. durangensis*) and tequila were the beverages with the highest amount of 2-(3)-methyl-l-butanol containing up to 900 mg/L, while sotol had the lower concentration (83 mg/L). Propanol has a light unpleasant aroma and its presence should be as low as possible. Mezcal from *A. salmiana* had the highest concentration with 708 mg/L, while raicilla had only 41 mg/L. For Pulque, the concentrations of 2-(3)-methyl-1-butanol and propanol were 65 mg/L and non detectable, respectively. The differences on the concentration of major compounds between the alcoholic beverages could be attributed to the microorganism community during fermentation phase and distilling conditions as well.

Figure 3 shows the cluster analysis (CA) for the *Agave* alcoholic beverages using the major compounds. The primary cluster included all distilled alcoholic beverages, whereas pulque was separated due to the low concentration of ethanol. Mezcals were grouped in a subset, because they are regulated by the same rules as described by the Mexican Ministry of Commerce and Industry (NOM-070). A subsequent branch included sisal and the last grouped bacanora and tequila. CA showed that *Agave* alcoholic beverages could be distinguished by multivariate analysis of major compounds. Cardoso et al. (Cardoso et al., 2004) reported that cachaça and rum (two alcoholic beverages produced from sugar cane from Brazil and Caribbean countries, respectively) could be distinguished by CA based in the concentration of higher alcohols (propanol, isobutanol and isopentanol) and metals. The content of higher alcohols has been used to certify Irish whiskey authenticity (González-Arjona et al., 1999). The analysis of major compounds has been proposed as aging indicator of mezcal from *A. salmiana* (De León-Rodríguez,

2006). The concentration of alcohols, esters and fatty acids led classify and differentiate the origin of a variety of wines (Etiévant et al., 1989).

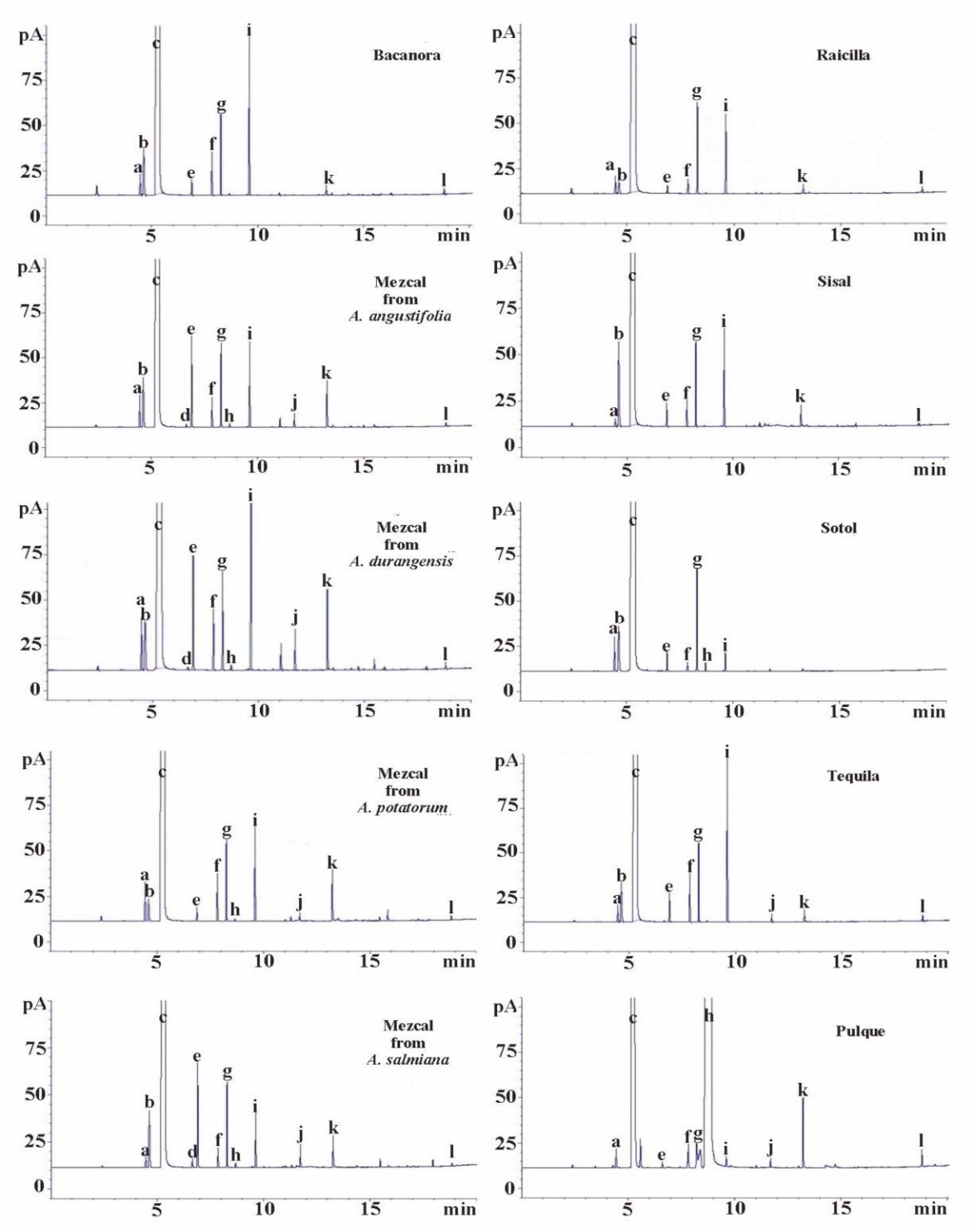

Fig.2. Chromatograms of *Agave* alcoholic beverages analyzed by GC-FID.

Table 1. Concentration of major compounds (mg/L) found in alcoholic beverages produced from different species of *Agave* plants.

Rt[a] (min)	Compound	Bacanora	Mezcal from *A. angustifolia*	Mezcal from *A. durangensis*	Mezcal from *A. potatorum*	Mezcal from *A. salmiana*	Raicilla	Sisal	Sotol	Tequila	Pulque
4.46	Ethyl acetate	284 ±5	395 ±3	474 ±6	492 ±1	170 ±0	166±1	100 ±2	323 ±2	221 ±2	270 ±5
4.63	Methanol	1065 ±2	1024 ±6	942±3	472 ±1	1162 ±4	216.9±3	1826 ±11	805 ±8	880 ±7	ND
5.30	Ethanol	339000	307700	370000	290000	307000	315000	307000	315000	362000	39000
6.66	2-Butanol	ND	21 ±0	17±0	ND	59 ±0	ND	ND	ND	ND	47 ±1
6.91	n-Propanol	111±0	612 ±2	580±1	97 ±0	708 ±2	41±1	163 ±1	99 ±1	208 ±8	ND
7.87	2 Methyl-propanol	294 ±2	201±1	307±0	321 ±0	126 ±0	83±1	172 ±0	50 ±0	340 ±1	218 ±4
8.64	n-butanol	ND	21 ±1	22±3	13 ±2	14 ±1	ND	ND	35 ±1	ND	ND
9.62	2-(3-)-Methyl-1-butanol	926 ±1	472 ±0	1064±2	589±1	307±1	350±3	545 ±1	83 ±0	1068 ±10	65 ± 1
11.72	Ethyl 2-hydroxypropanoate	ND	124 ±2	265±3	73 ±1	189 ±2	ND	ND	ND	77 ±1	85 ±2
13.26	Acetic acid	207 ±2	939 ±6	1192±25	1042 ±29	638 ±8	160±6	441 ±55	ND	214 ±47	1530 ±19
18.82	2-Phenylethanol	23 ±0	14 ±1	18±0	17 ±0	10 ±2	16±1	9 ±0	ND	23 ±0.5	64 ± 0
-	Higher alcohols[b]	1354 ±1	1339 ± 4	2007±1	1037 ±2	1220 ±5	490±4	888 ±2	267 ±1	1638 ±13	394 ±4

[a]Rt=Retention time in the HP-Innowax column, [b]Sum of alcohol with three or more carbons. ND=non detectable. Data represent the average ± standard deviation as described in material and methods.

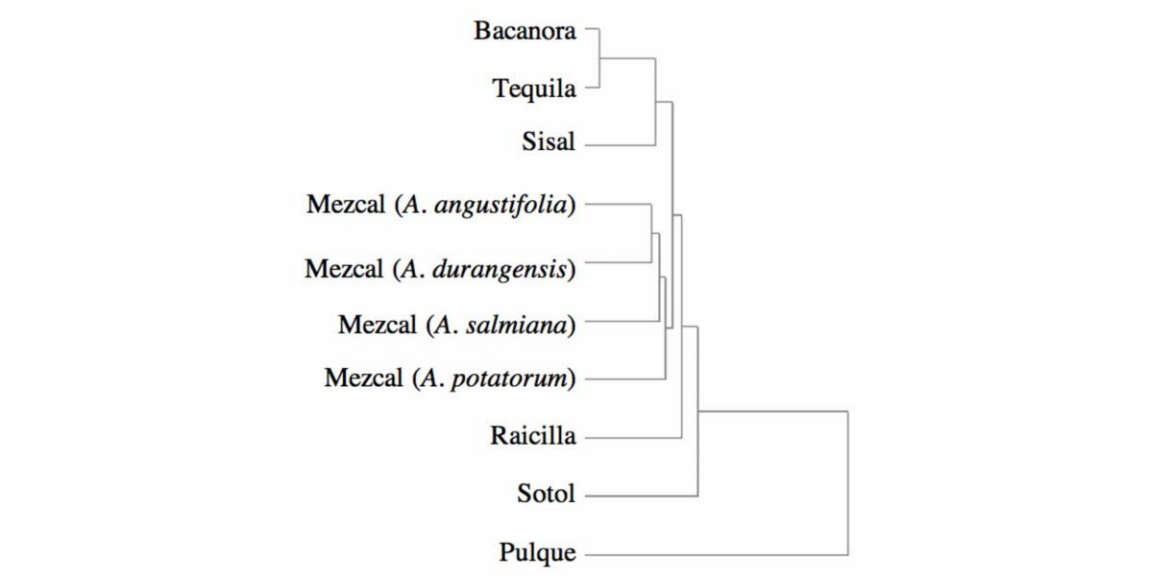

Fig. 3. Dendogram of cluster analysis for alcoholic beverages produced from different species of *Agave* plants.

Analysis of minor compounds by SPME-GC-MS

The SPME-GC-MS analysis for tequila using orange and black fibers is shown in Fig. 4. Compounds such as 3-furaldehyde (Rt=21.20), ethanone-1-2-furanyl (Rt=22.86) and others showed a higher relative abundance with the black fiber than using the orange fiber, due to the Since high peaks let a better comparison with the MS database, we used both fibers polarity of the matrix. Thus, we used both fibers for a full characterization of volatile compounds in this study. The compounds identified in *Agave* alcoholic beverages are summarized in Table 2. Nineeeeteen eight compounds were detected by the SPME-CG-MS technique. However, it is possible that other compounds wich are present at lower concentration, were not detected in this work. Almost all minor compounds previously reported for tequila, mezcal, sotol and bacanora (López, 1999; De León-Rodríguez et al., 2006; Lachenmeir et al., 2006) were detected here, but we detected new compounds such as azulene, cinnamol, butyrolactone, pyridine and others. The minor compounds detected include chemical groups such as

aldehydes, ketones**,** alcohols, organic acids, fatty acid ethyl esters, furans, terpenes, napthalenes and alkenes. Despite of the low concentration of minor compounds, their presence is relevant since they harmonically synergize to produce the characteristic flavor and aroma for each type of beverage. Some minor compounds are produced during fermentation by microbial catabolism (Szamelan et al., 2005; Janssens et al., 1992), whereas others become from the raw material such as terpenes, napthalenes, hydrocarbures and large chain fatty acids (Vallejo-Córdoba et al., 2001), thus they could be used as authenticity markers (López, 1999). Nineteen compounds were detected in all beverages and 17 compounds were unique for each type of beverage (Table 2) as follows: 3-methylthio-1-propanol and nonanoic acid for pulque, 2-butyl furan (Sisal), 3-methyl-cyclopentanone and benzofuran (mezcal from *A. durangensis*), 4-methoxy-benzaldehyde, (mezcal from *A. potarorum*), cinnamol (bacanora), 5-methyl-2-(1-methylethyl)-cyclohexanol (sotol), 1-methyl-4-(1-methylethenyl)-cyclohexene and geranyl ethyl ether (raicilla), heptanoic acid (mezcal from *A. salmiana*), naphtalene derivatives (mezcal from *A. angustifolia*), 2-methoxy-4-methyl-phenol, and 2-methyl-5-(1-methylethyl)-phenol (tequila). Perhaps other beverages contain the compounds referred here as unique, but their concentrations were too low to be detected. Nonanoic acid ethyl ester, 2-methyl-naphthalene and 2-acetyl-furan were proposed as authenticity markers for mezcal (from *A. angustifolia*), sotol and tequila, respectively (López y Guevara-Yáñez, 2001). However, we have found these substances in other type of alcoholic beverages analyzed here. Therefore, our work updates the database of components that could be used as authenticity markers. The proposition of a compound as authenticity marker is not simple; it is more reliable a fingerprinting based on the presence and relative abundance of several compounds than solely in one of them.

The CA for the *Agave* alcoholic beverages based on the presence/absence analysis of minor compounds is shown in Fig. 5. The main cluster included distilled and non-distilled beverages obtained from *Agave* plants, while sotol was separated. *Agave* and *Dasylirion* genus belong to the *Agavaceae* and *Nolinaceae*

families, respectively. Despite they are close related phylogenetically, they have significant structural and biochemical differences and therefore their secondary metabolites differ (Gentry, 1982). Then the CA using the minor compounds depends mainly on the raw material used for the production of each alcoholic beverage. Further work is needed, but it appears that the best signature for *Agave* alcoholic beverages could be based on minor compounds.

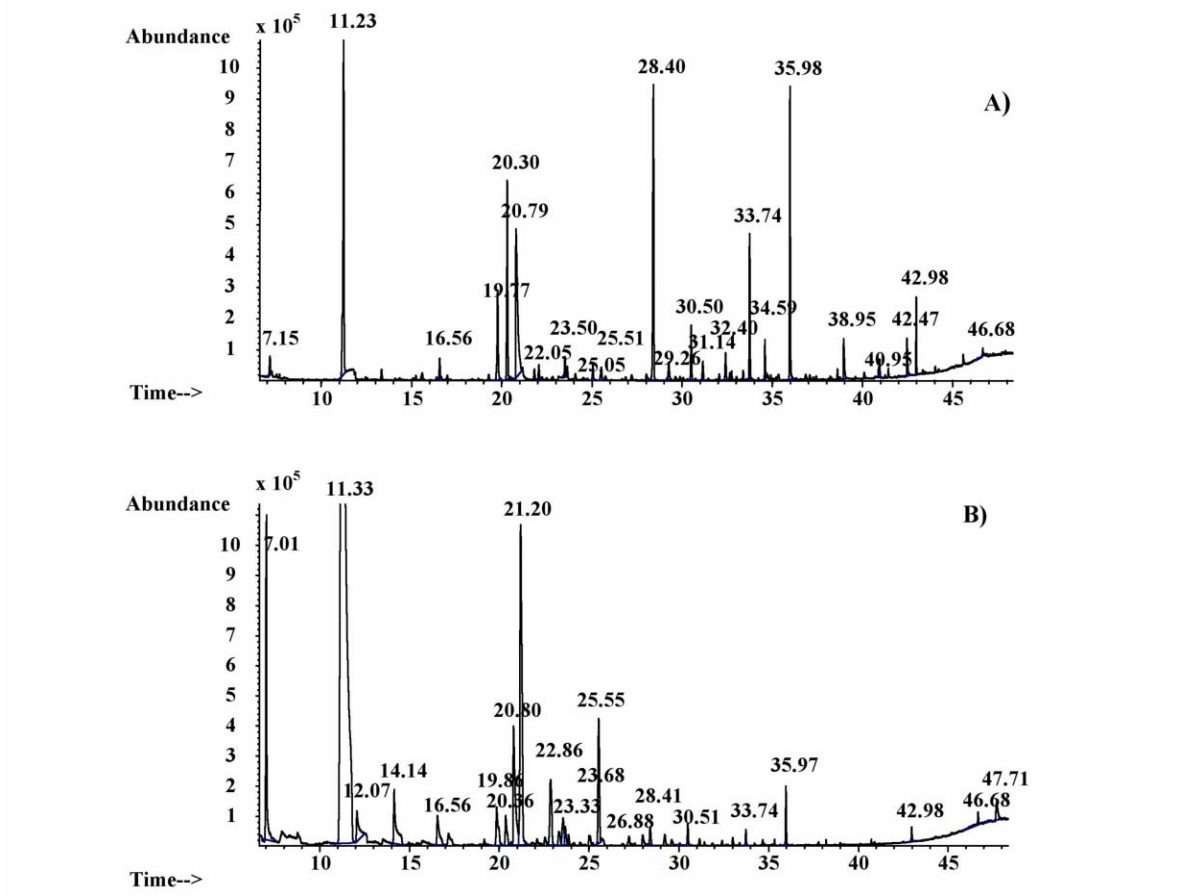

Fig. 4. Comparison of chromatograms obtained for tequila by SPME-GC-MS using different fibers. A) Orange fiber (Carbowax/Divinylbenzene; CW/DVB). B) Black fiber (Carboxen/Polydimethylsiloxane; CAR/PDMS).

Table 2. Compounds detected in *Agave* alcoholic beverages by SPME-GC-MS.

Rt[a]	Compound	Bac	Ang	Dur	Pot	Sal	Rai	Sis	Sot	Teq	Pul
7.02	2-Methyl-1-propanol*	1	1	1	1	1	1	1	1	1	1
7.89	3-Methyl-1-butanol acetate	1	1	1	1	0	1	1	0	1	1
7.97	3-Methyl-2-hexanol	0	0	0	0	0	0	0	1	0	1
8.34	Pentanoic acid ethyl ester	0	1	1	1	1	1	0	0	0	1
8.74	1-Butanol	0	1	1	1	1	1	1	1	0	1
9.76	Cyclopentanone	0	1	1	1	1	1	1	1	0	0
9.89	1-Methyl-4-(1-methylethenyl)-cyclohexene†	0	0	0	0	0	1	0	0	0	0
10.00	Pyridine	1	0	0	1	1	0	1	0	0	1
10.30	Limonene	0	1	0	0	1	1	1	0	0	1
10.75	Eucalyptol	0	0	0	1	0	1	0	0	0	0
10.89	3-Methyl-cyclopentanone†	0	0	1	0	0	0	0	0	0	0
10.92	3-methyl-cyclopentanone*	0	1	0	1	0	0	0	0	0	0
11.18	2-(3-)-Methyl-1-butanol*	1	1	1	1	1	1	1	1	1	1
11.19	1-Pentanol	1	0	0	1	1	1	0	1	0	0
11.27	3-Methyl-1-butanol formate	0	0	1	0	0	0	1	0	0	1
11.88	Hexanoic acid ethyl ester*	1	1	1	1	1	1	1	1	1	1
12.46	Cinnamol†	1	0	0	0	0	0	0	0	0	0
13.41	1,3,5-Trimethyl-benzene*	1	1	1	1	1	1	1	1	1	1
13.91	3-Hydroxy-2-butanone	0	1	1	0	0	0	0	0	0	1
14.12	2-Butyl furan†	0	0	0	0	0	0	1	0	0	0
14.27	3-Hexen-1-ol	0	0	0	0	1	0	1	0	0	0
14.48	1-Methyl-4-propyl-benzene	0	1	1	1	1	1	1	1	0	1
15.23	1-Methyl-2-(1-methylethyl)-benzene*	1	1	1	1	1	1	1	1	1	1
16.08	Heptanoic acid ethyl ester	0	1	1	1	1	1	0	0	0	1
16.56	2-Hydroxy-propanoic acid ethyl ester	0	1	1	1	1	1	1	1	1	1
16.69	1-Ethyl-2,4-dimethyl-benzene	1	1	0	0	1	0	1	1	1	0
17.16	1-Hexanol	0	1	1	1	1	1	1	1	1	1
18.30	2-Nonanone	0	1	1	1	0	0	0	0	0	1
19.28	1,2,3,5-Tetramethyl-benzene	0	0	1	0	0	0	1	1	1	0
19.37	1,2,3,4-Tetramethyl-5-methylene-1,3-cyclopentadiene	1	0	0	0	1	0	0	0	0	0
19.86	1,3-bis(1,1-dimethylethyl)-benzene*	1	1	1	1	1	1	1	1	1	1
20.29	Octanoic acid ethyl ester*	1	1	1	1	1	1	1	1	1	1
20.55	*p*-Menth-1-en-8-ol	0	1	1	0	0	0	0	0	0	1
20.65	5-Ethenyl-tetrahydro-5-trimethyl-2-furanmethanol	1	1	1	0	0	1	1	0	0	0
20.66	Acetic acid*	1	1	1	1	1	1	1	1	1	1
21.14	Furfural*	1	1	1	1	1	1	1	1	1	1
21.20	3-Furaldehyde	0	1	0	0	0	0	0	0	1	0
21.37	1-Heptanol	0	0	0	1	0	1	0	0	0	1
21.87	1,2,3,4-Tetramethyl-benzene*	1	1	1	1	1	1	1	1	1	1
22.59	Benzofuran†	0	0	1	0	0	0	0	0	0	0
22.86	1-(2-Furanyl)-ethanone*	1	1	1	1	1	1	1	1	1	1
23.34	Benzaldehyde*	1	1	1	1	1	1	1	1	1	1
23.42	Geranyl ethyl ether †	0	0	0	0	0	1	0	0	0	0
23.52	3-Methyl-6-(1-methylethylidene)-cyclohexene*	1	1	1	1	1	1	1	1	1	1
24.07	5-(Dimethylamino)-benzofurazan	0	0	1	0	0	1	0	0	1	0
24.41	Nonanoic acid ethyl ester	0	1	1	0	0	0	0	0	0	1
24.50	Propanoic acid	0	1	1	1	1	1	0	0	0	0
24.78	2,3-Butanediol	0	0	0	0	0	1	0	0	0	1
25.04	3,7-Dimethyl-1,6-octadien-3-ol	1	1	1	1	1	1	1	0	0	1
25.50	5-Methyl-2-furancarboxaldehyde*	1	1	1	1	1	1	1	1	1	1
25.65	2-Methyl-propanoic acid	0	1	1	1	1	0	0	0	1	0
26.06	2-Methyl-benzofuran	1	1	1	0	0	1	1	0	0	0

Continuation of the Table 2

Rt[a]	Compound	Bac	Ang	Dur	Pot	Sal	Rai	Sis	Sot	Teq	Pul
26.68	Decanoic acid methyl ester	0	1	0	0	0	0	1	0	0	0
26.85	4-Methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	1	1	1	1	1	1	1	0	1	1
27.14	2-Acetyl-furan	0	1	1	0	0	1	0	1	1	0
27.41	Butyrolactone	0	0	0	0	0	0	0	1	0	1
27.54	2-Furancarboxylic acid ethyl ester	1	1	0	1	0	1	1	1	0	1
27.93	Butanoic acid	1	1	1	1	1	1	0	1	1	1
28.38	Decanoic acid ethyl ester*	1	1	1	1	1	1	1	1	1	1
28.44	5-Methyl-2-(1-methylethyl)-cyclohexanol†	0	0	0	0	0	0	0	1	0	0
29.14	*p*-Allyl-anisole	0	1	0	1	0	1	0	0	0	1
29.20	2-Furanmethanol	1	0	1	0	1	0	1	0	0	0
29.52	3-Methyl-butanoic acid	0	1	1	1	0	0	0	0	0	0
29.63	3-Methyl-pentanoic acid	0	0	0	0	1	0	1	0	0	0
29.87	Butanedioic acid, diethyl ester	0	1	0	0	0	0	0	0	1	1
30.50	4-Trimethyl-3-cyclohexene-1-methanol*	1	1	1	1	1	1	1	1	1	1
31.09	3-(Methylthio)-1-propanol†	0	0	0	0	0	0	0	0	0	1
31.13	Naphthalene	1	1	1	1	1	1	1	1	1	0
31.14	Azulene	0	0	0	0	1	0	0	0	1	1
31.86	Pentanoic acid	0	1	1	1	1	1	0	0	0	0
31.95	1,2,3,5,6,8-hexahydro-4,7-dimethyl-1-(1-methylethyl)-Naphthalene†	0	1	0	0	0	0	0	0	0	0
32.04	1-Methoxy-4-(1-propenyl)-benzene	0	0	0	1	0	1	0	0	0	1
32.41	Methyl salicylate	1	1	0	1	1	0	1	1	1	0
32.73	3,7-Dimethyl-6-octen-1-ol	0	1	0	0	1	1	1	0	1	1
33.38	2,3-Dihydro-1-H-inden-1-ol	1	0	0	1	0	0	0	0	0	0
33.38	3,5-Dimethyl-benzaldehyde	0	0	1	0	1	1	1	1	1	1
33.74	Acetic acid 2-phenylethyl ester	1	1	1	1	1	1	1	0	1	1
33.83	1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	0	0	0	0	1	0	0	1	0	0
33.96	1,2,3,4-Tetrahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene†	0	1	0	0	0	0	0	0	0	0
34.22	2-Methyl-naphthalene	1	1	1	0	1	1	0	1	0	0
34.57	Dodecanoic acid ethyl ester	1	1	1	0	0	1	1	1	1	0
34.61	Hexanoic acid	1	1	1	1	1	0	0	1	1	1
34.75	2-Methoxy-phenol	1	0	1	0	0	0	1	0	0	0
35.20	Benzyl alcohol	1	1	0	1	1	0	1	0	0	0
35.35	Benzenepropanoic acid ethyl ester	0	1	1	0	0	0	0	0	0	0
35.96	Phenylethyl alcohol*	1	1	1	1	1	1	1	1	1	1
36.37	1-Ethyl-naphthalene†	0	1	0	0	0	0	0	0	0	0
36.85	2-Methoxy-4-methyl-phenol†	0	0	0	0	0	0	0	0	1	0
36.98	Heptanoic acid†	0	0	0	0	1	0	0	0	0	0
37.44	2,6-Dimethyl-naphthalene	0	1	1	0	0	1	0	0	1	0
37.78	Phenol*	1	1	1	1	1	1	1	1	1	1
38.11	4-Methoxy-benzaldehyde†	0	0	0	1	0	0	0	0	0	0
38.75	Octanoic acid	1	1	1	1	1	1	1	0	1	1
40.76	Nonanoic acid†	0	0	0	0	0	0	0	0	0	1
41.35	2-Methyl-5-(1-methylethyl)-phenol†	0	0	0	0	0	0	0	0	1	0
41.45	1,6-Dimethyl-4-(1-methylethyl)-naphthalene†	0	1	0	0	0	0	0	0	0	0
42.01	Dibenzofuran	0	1	1	0	1	0	0	0	0	0
42.45	Decanoic acid	0	1	1	0	1	1	1	0	1	1
42.97	2,4-Bis(1,1-dimethylethyl)-phenol*	1	1	1	1	1	1	1	1	1	1

[a] Rt= Retention time in the DB-WAX column. 1: Presence, 0: Absence. (*) compounds found in all beverages and (†) compounds unique for each type beverage. Bac= bacanora, Ang= mezcal from *A. angustifolia*, Dur= mezcal from *A. durangensis*, Pot= mezcal from *A. potatorum*, Sal= mezcal from *A. salmiana*, Rai= raicilla, Sis= sisal, Sot= sotol, Teq= tequila, and Pul= pulque.

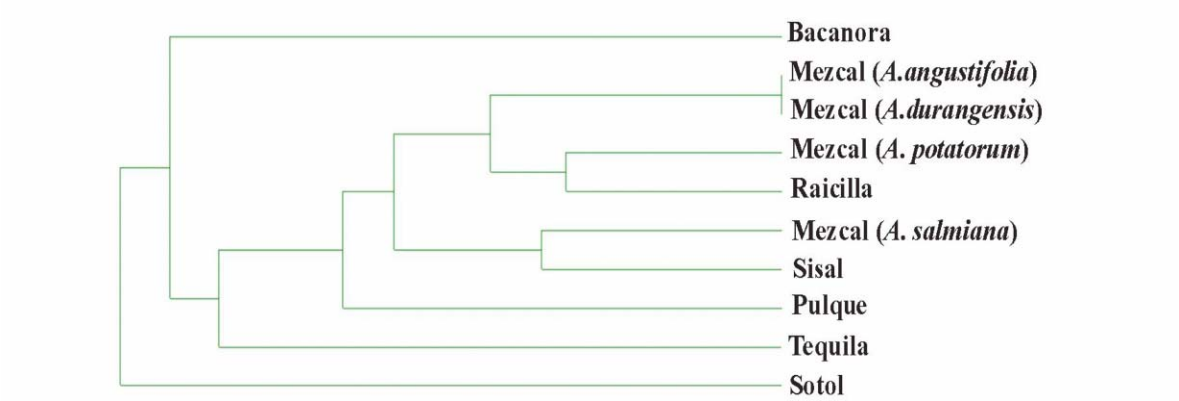

Fig. 5. Dendogram of cluster analysis for the minor compounds in the ethnic *Agave* alcoholic beverages using the UPGMA Method with Jaccard coefficient.

Conclusions

We detected one hundred-five compounds in the *Agave* alcoholic beverages analyzed here, eleven of them were classified as major compounds and the rest were classified as minor compounds. Seventeen minor compounds were unique for each type of beverage and they could be used as authenticity markers. The minor compounds group includes alcohols, ketones, esters, organic acids, furans, terpenes, alkenes and others. According to this study, most of the compounds found in the *Agave* alcoholic beverages are similar to those reported for whisky, cachaça and other alcoholic beverages. However, we found compounds such as azulene, cinnamol, and others that could be used as authenticity markers. Cluster analysis (CA) showed that *Agave* alcoholic beverages could be distinguished by multivariate analysis of major compounds; however, the analysis of minor compounds provides a better fingerprinting.

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References

Aguilar-Cisneros, B.O., López, M.G., Richling, E., Heckel, F., Schreier, P. (2002) Tequila authenticity assessment by headspace SPME-HRGC-IRMS analysis of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios of ethanol. *J Agric Food Chem* **50**, 7520-7523.

Aylott, R.I., Clyne, A.H., Fox, A.P., Walcer, D.A. (1994) Analytical strategies to confirm scotch whisky authenticity. *Analyst* **119***,* 1741-1746.

Berovič, M., Mavri, J., Wondra, M., Kosmerl, T., Dejan, B. (2003) Influence of temperature and carbon dioxide on fermentation of cabernet sauvignon must. *Food Technol Biotechnol* **41**, 353-359.

Cardoso, D.R., Andrade-Sobrinho, L.G., Leite-Neto, A.F., Reche, R.V., Isique, W.D., Ferreira, M.M.C., Lima-Nieto, B.S., Franco, D.W. (2004) Comparision between Cachaça and Rum using pattern recognition methods. *J Agric Food Chem* **52**, 3429-3433.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A.P., Escalante-Minakata, P., López, M.G. (2006) Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana. J Agric Food Chem* **54***,* 1337-1341.

Etiévant, P., Schlich, P., Cantagrel, R., Bertrand, A., Bouvier, J.C. (1989) Varietal and geographic classification of french red wines in terms of major acids. *J Sci Food Agric* **46**, 421-438.

Gentry, H.S. *Agaves* of continental North America, The University of Arizona Press. Tucson, Arizona, USA (1982**)** pp. 670.

González-Arjona, D., González-Gallero, V., Pablos, F., González, A.G. (1999) Authetication and differentiation of Irish whiskeys by higher-alcohol congener analysis. *Anal Chim Acta* **381***,* 257-264.

Janssens, L., De Pooter, H.L., Schamp, N.M., Vandamme, E.J. (1992) Production of flavours by microorganisms. *Process Biochem* **27**, 195-215.

Jelen, H.H., Wlazly, K., Wazowicz, E., Kaminski, E. (1998) Solid-phase microextraction for the analysis of some alcohols and esters in beers: Comparison with static headspace method. *J Agric Food Chem* **46**, 1469-1743.

Lachenmeier, D.W., Sohnius, E.M., Attig, R., López, M.G. (2006) Quantification of selected volatile constituents and anions in Mexican *Agave* spirits (Tequila, Mezcal, Sotol, Bacanora). *J Agric Food Chem* **54***,* 3911-3915.

Ledauphin, J., Barillier, D., Beljean-Leymarie, M. (2006) Gas chromatographic quantification of aliphatic aldehydes in freshly distilled Calvados and Cognac using 3-methylbenzothiazolin-2-one hydrazone as derivate agent. *J Chromatogr A* **1115**, 225-232.

López, M.G. Tequila aroma. In: Flavor Chemistry of Ethnic Foods*,* Shahidi & Ho, New York: Kluwer Academic/Plenum Publishers (1999) pp. 211-217.

López, M.G., Guevara-Yáñez, S.C. Authenticity of three Mexican alcoholic beverages by SPME-GC-MS. *In proceedings of the Annual Meeting of Institute of Food Technologists*, New Orleans, USA (2001) Paper 10-3.

Mexican Ministry of Commerce and Industry, Regulations: NOM-070-SCFI-1994, Alcoholic drinks-Mezcal Specifications, Diario Oficial de la Federación: México, November 28 (1994).

Rohlf,. F. J. NTSYSpc. Numerical Taxonomy and Multivariate Analysis System, Version 2.02i, Applied Biostatistics, Inc. New York (1998).

Szamelan, N., Nowak, J., Jelén, H. (2005) The composition of Jerusalem artichoke (*Helianthus tuberosus* L.) spirits obtained from fermentation with bacteria and yeasts. *Eng Life Sci* **5**, 68-71.

Vallejo-Córdoba, B., González-Córdoba, A.F., Estrada-Montoya, M.C. (2004) Tequila volatile characterization and ethyl ester determination by solid phase microextraction gas chromatography/mass spectrometry analysis. *J Agric Food Chem* **52**, 5567-5571.

Vallejo-Córdoba, B., González-Córdoba, A.F., Estrada-Montoya, M.C. Latest advances in the characterization of Mexican distilled *agave* beverages: tequila, mezcal and bacanora, In *Proceeding of the 229th ACS meeting*, San Diego, CA, USA (2005) paper AGFD-113.

Van Ruth, S.M. (2001) Methods for gas chromatography-olfactometry: a review. *Biomol Eng* **17**, 121-128.

CAPÍTULO II

4. OPTIMIZACIÓN DEL PROCESO DE PRODUCCIÓN DE MEZCAL

Este capítulo trata de la optimización de las condiciones fermentativas del proceso del Mezcal, con el objetivo de maximizar el rendimiento, la productividad y la concentración de etanol. Se utilizó la metodología de superficie de respuesta para el análisis estadístico de los datos obtenidos. Además se presenta una estrategia para el monitoreo en línea de la fermentación a través del potencial redox. El capítulo está formado por los siguientes artículos:

Antonio De León-Rodríguez, Pilar Escalante-Minakata, Ana P. Barba de la Rosa and Hans P. Blaschek. Optimization of fermentation conditions for the production of the mezcal from *Agave salmiana* using response surface methodology. Submitted to: Chemical engineering and processing (Aceptado, en prensa).

P. Escalante-Minakata, V. Ibarra-Junquera, R. González-García, A. De León-Rodríguez and H.C. Rosu. On-line monitoring of Mezcal fermentation base on redox potencial measurements. Submitted to: Biochemical engineering journal (En proceso).

4.1 ***Optimization of fermentation conditions for the production of the mezcal from Agave salmiana using response surface methodology***

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Running title: Production of mezcal from *Agave salmiana*
Submitted to Chemical engineering and processing.

Abstract

Response surface methodology was applied to optimize the fermentative phase for the mezcal production from *Agave salmiana*. A second order and a complete factorial design was used to obtain models describing the relationship between the ethanol production, process productivity, and product yield with respect to the fermentation temperature and the initial sugar concentration. The results shown that the fermentative conditions affected the composition of higher alcohols (referred as a quality indicator) in the mezcal as well as the amount of ethanol. The highest ethanol production was attained by employing the following predicted optimum operational conditions: temperature of 28°C and an initial sugar concentration of 105 g/l. However, the maximum productivity process was attained with 34.6 °C and 90 g/l, whereas the maximum product yield and the best quality mezcal at 28 °C and 77 g/l. Results shows that the simultaneous optimization for high ethanol production and fast production rate are not compatible, since high ethanol production requires a high substrate concentration, which in turn inhibit the growth rate.

KEYWORDS: alcoholic fermentation, spirits, response surface methodology, spirits, substrate inhibition.

Introduction

Mezcal is an alcoholic beverage obtained through the fermentation and distillation of the *Agave* syrup. Species of *Agave* plants such as *Agave salmiana, A. angustifolia* Haw*, A. potatorum, A. durangensis* and others are used as raw materials (NOM-070). However, only wild-type plants of *A. salmiana* are used in the Mexican Altiplano. The mezcal production process includes five phases: cooking, milling, fermenting, distilling and aging. During the cooking phase, the raw material is softened to make easy the milling phase, the inuline and other fructo-oligosacharides are hydrolyzed to single sugars (mainly fructose), and some other organic compounds are generated by the Maillard reactions (Mancilla-Margalli y López, 2002). Events during any of the mezcal production phases have the potential to affect the final quality and yield. However, special attention must be given to the fermentative phase, which produces the ethanol and other compounds that directly define the main characteristics of mezcal.

The goal of this work was the optimization of the fermentative phase for mezcal production. Response surface methodology and the 3k full factorial design were used to determine the influence of the temperature and the initial sugar concentration on the mezcal production from *A. salmiana*.

Materials and Methods

2.1 Culture medium and fermentation conditions

The *Agave* syrup from *A. salmiana* and inocula were kindly provided by Juan Zarur. The *Agave* syrup was centrifuged at 7000 *g* for 10 min and pasteurized at 65°C. Batch cultures were carried out in Erlenmeyer flasks containing 900 ml of

Agave syrup with 1 g/l of ammonium sulfate and inoculated with 100 ml of *Agave* syrup that contain the biomass for an initial optical density (OD_{620nm}) of 0.1. The initial sugar concentration and temperature were fixed according to the experimental design described below. For all experiments, pre-inocula in *Agave* syrup with 1 g/l of ammonium sulfate were grown overnight at 28ºC, after the biomass was washed and resuspended in fresh *Agave* syrup. The potential redox was measured online with an autoclaveable redox electrode (Applikon, Schiedam, The Netherlands) and the data were registered in a PC interfaced with a potentiometer (B&C Electronics, Italy) using a RS232 port. Broth samples were harvested each hour and centrifuged at 5,000 *g* for 5 min; supernatants were collected and stored at 4 ºC.

2.2 Kinetic studies and determination of the fermentation parameters

The fermentations were carried out using the same procedure described in the section 2.1 using initial sugar concentration between 0 and 200 g/l and incubated at 32.5°C. Culture samples were taken each hr and the biomass, sugar and ethanol concentration were determined as described below (section 2.4). The ethanol production (*EP*) is the amount of ethanol produced by liter of culture medium at the end of the exponential phase. The productivity process (*PP*) is the amount of ethanol produced by liter and per hour, and the process yield ($Y_{P/S}$) is the amount of ethanol produced by sugar consumed. The process parameters were obtained as follow:

$$PP = \frac{EP}{t} \tag{1}$$

$$Y_{P/S} = \frac{P_f - P_i}{S_i - S_f} \tag{2}$$

Where *PP* is the productivity process (g/l-h), *EP* the production of ethanol (g/l), *t* the time (h), $Y_{P/S}$ is the process yield (g/g), P_f the final concentration of ethanol (g/l) and P_i is the initial concentration of ethanol (g/l), S_f the final sugar concentration (g/l), S_i the initial sugar concentration (g/l).

The specific growth rate (μ) was determined by linear regression of the plot *Ln* biomass conc. versus time, at the exponential growth phase. The data of μ were fitting to the inhibition by substrate model described by the Eq. (3) and the terms were obtained using Solver algorithm from Excel™ (Microsoft Co.).

$$\mu = \frac{\mu_{\max} S}{k_s + S + \frac{S^2}{k_i}} \tag{3}$$

Where μ is the specific growth rate (h^{-1}), *S* is the initial concentration of sugar (g/l), k_S is the saturation constant (g/l), k_i is the inhibition constant (g/l) and μ_{max} is the maximum specific growth rate (h^{-1}).

2.3 Experimental design

A two-factor factorial experimental design was used to elucidate the influence of the initial sugar concentration (factor X_1) and temperature (factor X_2) as independent variables on the fermentation. The treatments were arranged according to a factorial 3^2 designs; and they were carried out in duplicates as independent experiments in order to take into account the non-adjustable data and allow the calculation of the analysis of variance (ANOVA). The 3k full factorial design was selected instead the 2k, since the expected model has curvature, due to quadratic terminus from the substrate inhibition model. In addition, the wide interval of interest in the initial sugar concentration evaluated (35-105 g/l) requires a third level in the experimental design according to Montgomery (Montgomery, 1997). The treatments were applied randomly in a complete blocks experimental design (Table 1). The Eq. 4 was used to build surfaces graphs for the model for each response variable and for predicting the optimal value.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \tag{4}$$

Where Y is the response variable, X_1 and X_2 are independent variables for the initial substrate concentration and temperature, respectively. β_0 is the intercept term, β_1 and β_2 are the linear coefficients, β_{12} is the interactive coefficient, and β_{11} and β_{22} are quadratic coefficients. The model was evaluated with significance, good fit and the R^2 values. The optimal values were obtained solving the regression Eq. (4) by the Newton-Raphson method and analyzing the response surface contour (Montgomery, 1997; De León-Rodríguez et al., 2004; Paz-Maldonado et al., 2007). The analysis of the response surface, the ANOVA and the optimal conditions were obtained using Statgraphics v 5 (Manugistics Inc. Rockville) software. The adjusted models for *EP*, *PP* and $Y_{p/s}$ were evaluated by the *F*-test from ANOVA. The significant effects on dependent variables were determined by *T*-test with a probability value (*P*-value) smaller than 0.05.

Table 1. Experimental design and summary of results for dependent variables.

	Independent variable		**Dependent variable**		
Treatment No.	**Factor X_1 (g/l)**	**Factor X_2 (°C)**	***EP* (g/l)**	***PP* (g/l-h)**	**$Y_{P/S}$ (g/g)**
1	35	28.0	14.26 ± 1.28	0.95 ± 0.09	0.30 ± 0.01
2	35	32.5	12.36 ± 0.17	1.24 ± 0.02	0.27 ± 0.01
3	35	37.0	13.14 ± 0.64	1.34 ± 0.07	0.30 ± 0.01
4	70	28.0	24.05 ± 0.57	1.35 ± 0.03	0.42 ± 0.00
5	70	32.5	28.76 ± 1.03	2.21 ± 0.08	0.46 ± 0.02
6	70	37.0	23.94 ± 1.02	1.99 ± 0.08	0.40 ± 0.00
7	105	28.0	37.68 ± 0.11	1.60 ± 0.01	0.38 ± 0.01
8	105	32.5	34.48 ± 1.34	2.03 ± 0.08	0.35 ± 0.03
9	105	37.0	33.26 ± 3.11	2.05 ± 0.19	0.36 ± 0.01

Treatments were conducted in a random order. Experimental results are averages of two independent experiments and their respective standard deviation. X_1: Initial sugar concentration, X_2: Temperature, *EP*: Ethanol production, *PP*: Productivity process, $Y_{P/S}$: Product yield.

2.3 Distillation procedure

The fermented *Agave* syrup was distilled using an eight-plat Vygrux column (SEV, Puebla, Mexico) and a heating mantle (Electrothermal, UK). The distillation temperature was monitored with a thermopar (Hanna Inst. Italy). Two fractions were obtained, the first one (rich in methanol) was collected below 68°C and it was discarded and the second fraction (rich in ethanol) was collected in the range of 68 to 85°.

2.4 Analytical methods

Biomass concentration was determined from OD_{620nm} using a spectrophotometer Cary Bio-50 (Varian Inc., Australia) and converted to dry cell weight with a standard curve. The reducing sugar concentration was determined by the dinitro-salicilic acid (DNS) method using fructose as standard (Miller, 1959) The concentration of ethanol and the other compounds of mezcal were measured in a gas chromatograph 6890N (Agilent technologies, Wilmington, DW) equipped with a FID detector, an auto-sampler 7863 (Agilent technologies, Wilmington, DW) and a capillary column HP-Innowax (30 m x 0.25 mm i.d., 0.25 µm film thickness; Agilent technologies, Wilmington, DW). The analytical conditions are described in (De León-Rodríguez et al., 2006). All samples were analyzed in duplicates, and the average of each compound concentration was used for comparing the different fermentative conditions.

Results and Discussion

Kinetic studies

Kinetic behavior of the batch culture for the treatment 6 at 37°C and an initial sugar concentration of 70 g/l is shown in Fig. 1. Cultures for the other treatments showed similar trends as those in Fig. 1, although rates of the various parameters measured, their maximum concentrations, and times to reach them were different

in each case. For the culture of the treatment 6, the biomass increased exponentially at a specific growth rate of 0.32 h^{-1}. Since the culture is non-axenic, the observed specific growth rate is the average of growth rates of all type of microorganisms. The maximum biomass attained was 1.04 g/l, and thereafter it remained constant (Fig. 1A). In all cultures, ethanol concentration showed a growth-associated behavior and the maximum ethanol concentration attained was 23 g/l for the treatment 6(Fig. 1B). The redox potential decreased from +135 to -163 mV and followed an inverse relationship with respect to ethanol production (Fig. 1B). These results shown that measurements on the redox potential could be used for a rapid and on-line surrogate determination of ethanol during mezcal fermentation and other alcoholic beverages produced from different *Agave* plants. Similar to the results obtained here, Berovič et al. (2003), reported that during the fermentation of cabernet sauvignon must, the redox potential decreased from +190 to -240 mV a the culture temperature of 26°C, while at 18°C it decreased from +190 to -90 mV. They concluded that the must fermented at 26°C was converted into a more stable and reductive environment.

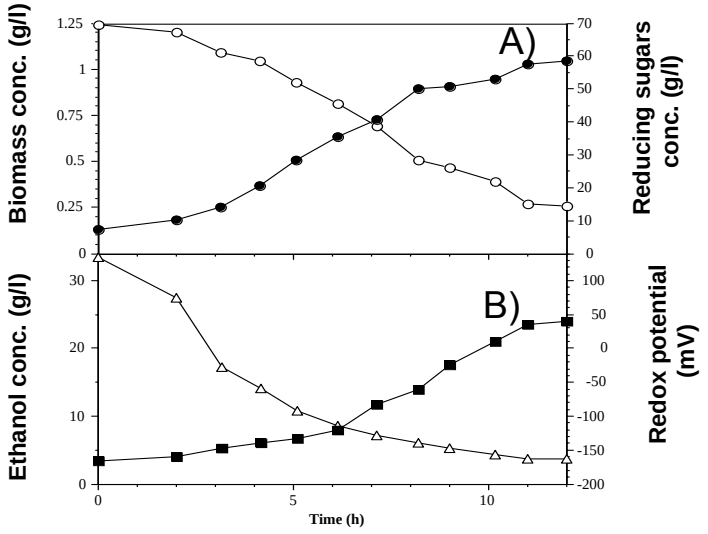

Fig. 1. Typical batch culture for mezcal production at an initial sugar concentration of 70 g/l and temperature of 32.5°C. A) Biomass conc. (●), reducing sugar conc. (○). B) Ethanol conc. (■), redox potential (∆).

The influence of the initial sugar concentration on the specific growth rate is shown in Fig 2. As sugar concentration increased, μ followed a substrate inhibition-type fashion. The maximum specific growth rate, saturation constant and inhibition constant were 0.6 h^{-1}, 16.82 g/l and 47.78 g/l, respectively. Catabolite inhibition of enzymes in the fermentative pathway becomes important at higher substrate concentrations, indicating the onset of substrate inhibition as a result of the high osmotic pressure and low water activity (Stewart et al., 1984). Thatipamala *et al*. (1992), reported a substrate inhibition above 150 g/l for yeast cultures during ethanol batch fermentation at 30°C using a minimum medium with yeast extract. In our case, the substrate inhibition was observed above 40 g/l. Since we used a complex medium, other compounds present in the *Agave* syrup, such as furfural, Maillard products and saponins could cause additional inhibitory effects on the cells (Zaldivar et al., 1999; Yokosuka et al., 2000; Tauer et al., 2004).

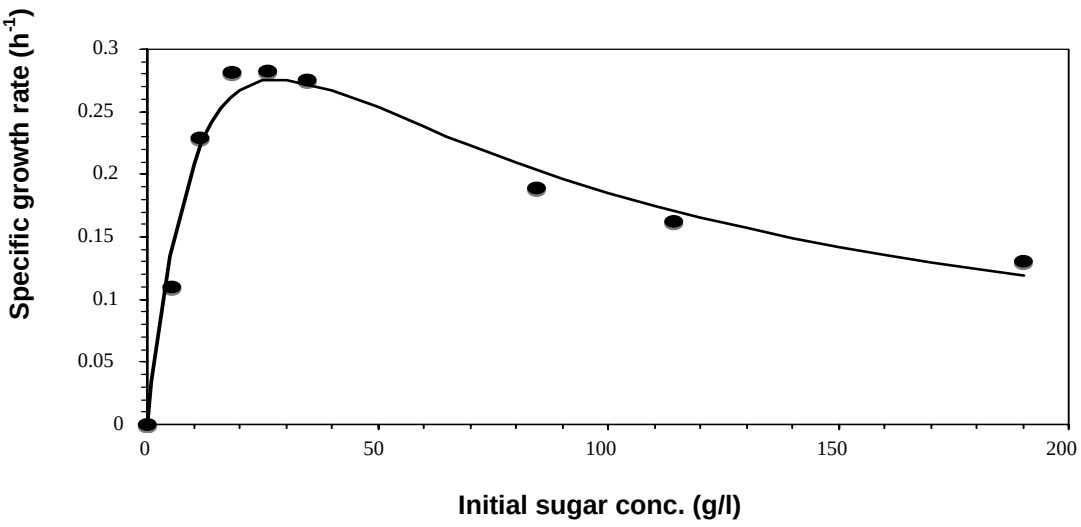

Fig. 2. Effect of the initial sugar concentration on specific growth rate at 32.5°C. Line draws the substrate inhibition-type fashion with a maximum specific rate (μ_{max}), saturation constant (K_s) and inhibition constant (K_i) of 0.6 h^{-1}, 16.82 g/l and 47.78 g/l, respectively.

Optimization of fermentation conditions

Table 1 shows a summary of the results for *EP, PP* and $Y_{p/s}$. The *EP* values varied in a range of 12.36±0.17 to 37.68±0.11 g/l for the treatments 2 and 7 respectively. The analysis of variance for the adjusted model showed that *EP* was only significantly affected by the initial sugar concentration (Table 2). The second-order equation with *EP* as a function of temperature and initial sugar concentration is described by the Eq. (5):

$$EP = -48.779 + 0.641529\,X_1 + 2.76363X_2 - 0.00113331X_1^2 - 0.00523595X_1X_2 - 0.0401X_2 \quad (5)$$

The highest predicted *EP* of 36.63 g/l was attained when the temperature and initial sugar concentration were 28°C and 105 g/l, respectively. The predicted dependence of *EP* on the temperature and initial sugar concentration, based on Eq. (5) is shown in the Fig. 3. Neither a maximum nor a minimum point was observed in the response surface within the range of study. Thus, it is possible to conclude that increasing the sugar concentration in the culture medium, the ethanol production increase as well.

Table 2. Analysis of variance for the adjusted model for ethanol production

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	*F*-Ratio	*P*-Value
Constant	-48.779					
X_1	0.641529	81.607	1	81.607	17.59	0.0015
X_2	2.76363	0.493174	1	0.493174	0.11	0.7505
X_1^2	-0.00113331	7.70951	1	7.70951	1.66	0.2238
X_1X_2	-0.00523595	5.44055	1	5.44055	1.17	0.3020
X_2^2	-0.0401	2.63754	1	2.63754	0.57	0.4666
Total error		51.0201	12	4.63819		
Total (corr.)		1515.79	17			

X_1: Initial sugar concentration, X_2: Temperature, DF: Degrees of freedom, *F*: Fisher test, *P*-value: probability distribution value. The correlation coefficient (R^2) was 0.966 and the standard error was 2.1536.

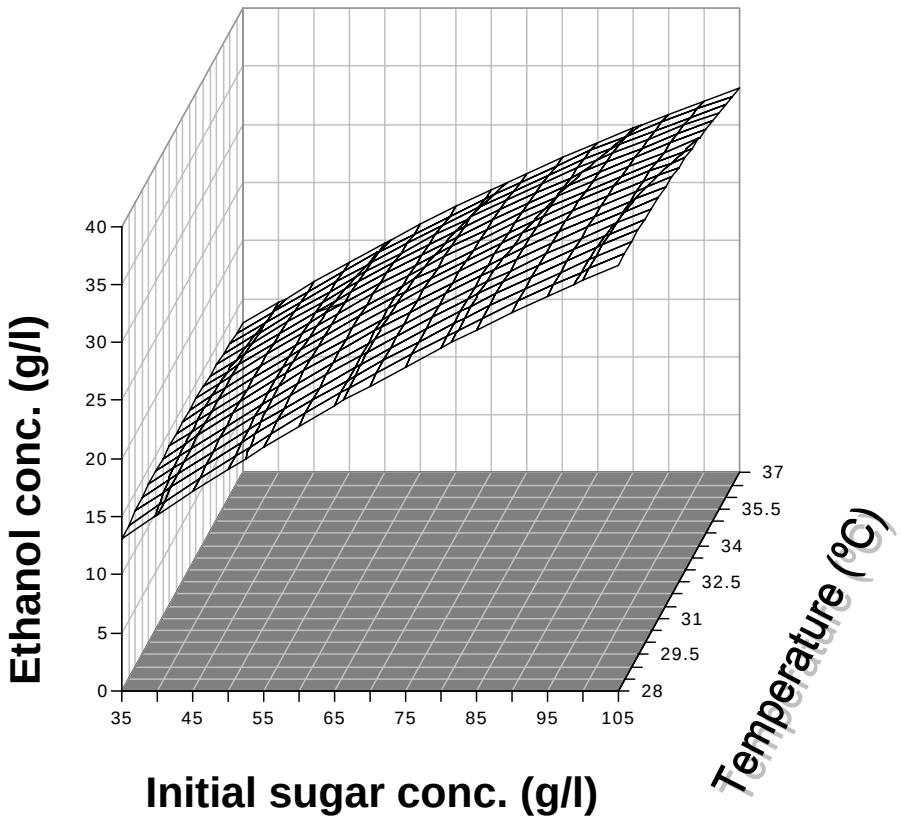

Fig. 3. Dependence of ethanol production on the temperature and the initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

The *PP* values varied in the range of 0.95±0.09 to 2.21±0.08 g/l-h for the treatments 1 and 5, respectively. The analysis of variance for the adjusted model showed that *PP* was significantly affected by X_1, X_2, X_1^2 and X_2^2 (Table 3). The mathematical model representing *PP* as a function of X_1 and X_2 in the experimental region studied is expressed by Eq. (6).

$$PP = -15.9837 + 0.0437542\, X_1 + 0.938191\, X_2 - 0.000261204\, X_1^2 + 0.0000951587\, X_1X_2 - 0.0136975 X_2^2 \quad (6)$$

The maximum predicted *PP* of 2.2 g/l-h was attained when the temperature and the initial sugar concentration were 34.6°C and 90 g/l, respectively. The predicted dependence of *PP* on the temperature and the initial sugar concentration, based

on Eq. (6) is shown in the Fig. 4. The plot shows that, both linear and quadratic coefficients of the temperature and the initial sugar concentration affected the *PP*. Chen (1981), observed that during the alcoholic fermentation from glucose syrup by *Saccharomyces cerevisiae*, the highest alcohol productivity was 21 g/l-h at a substrate concentration of 12°Brix (approx. 120 g/l). In our case we observed a maximum *PP* of 2.2 g/l-h. The low productivity attained could be the result of the inhibitory effect of other compounds in the *Agave* syrup.

Table 3. Analysis of variance for the adjusted model for process productivity

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	*F*-Ratio	*P*-Value
Constant	-15.9837					
X_1	0.0437542	1.55297	1	1.55297	70.65	0.0000
X_2	0.938191	0.722114	1	0.722114	32.85	0.0001
X_1^2	0.000261204	0.409536	1	0.409536	18.63	0.0012
X_1X_2	0.0000951587	0.001797	1	0.001797	0.08	0.7802
X_2^2	0.0136975	0.307748	1	0.307748	14.00	0.0033
Total error		0.241788	12	0.0219807		
Total (corr.)		3.2404	17			

For abbreviations, see Table 2. The correlation coefficient (R^2) was 0.925 and the standard error was 0.14823.

The $Y_{P/S}$ values varied in a range of 0.27±0.01 to 0.46±0.02 for the treatments 2 and 5 respectively. The analysis of variance for the adjusted model showed that $Y_{P/S}$ was significantly affected by X_1 and X_1^2 terms (Table 4). The mathematical model representing $Y_{P/S}$ in the range of study is expressed by Eq. (7):

$$Y_{P/S} = 0.0729124 + 0.0134648\,X_1 - 0.0078323\,X_2 - 0.000083217\,X_1^2 - 0.000023730\,X_1X_2 + 0.0001226X_2^2 \quad (7)$$

In this case, the maximum predicted $Y_{P/S}$ of 0.44 was attained when the temperature and the initial sugar concentration were 28°C and 77 g/l, respectively.

The predicted dependence of $Y_{P/S}$ on the temperature and initial sugar concentration, based on Eq. (7) is shown in the Fig. 5. It can be observed that, both linear and quadratic coefficients of the sugar concentration affected the $Y_{P/S}$ and a maximum response was observed within the range of study. Since, the ethanol is a biofuel, several reports on the optimization of ethanol production have been published (Chen, 1981; Balusu et al., 2005; Bandaru et al., 2006). Criteria such as yields, productivity and ethanol production are used to evaluate the fermentations. In our case, simultaneous optimization is not an easy task. For instance, a high ethanol production and high productivity process are not compatible, because the first one requires high substrate concentration, which cause substrate inhibition leading to a low ethanol production rate.

Table 4. Analysis of variance for the adjusted model for product yield

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	*F*-Ratio	*P*-Value
Constant	0.0729124					
X_1	0.0134648	0.0493078	1	0.0493078	79.42	0.0000
X_2	-0.0078323	4.3517E-7	1	4.3517E-7	0.00	0.9794
X_1^2	-0.000083217	0.0415684	1	0.0415684	66.96	0.0000
X_1X_2	-0.000023730	0.000111751	1	0.000111751	0.18	0.6796
X_2^2	0.0001226	0.0000246678	1	0.0000246678	0.04	0.8456
Total error		0.00682916	12	0.000620833		
Total (corr)		0.0652157	17			

For abbreviations, see Table 2. The correlation coefficient (R^2) was 0.895 and the standard error was 0.02492.

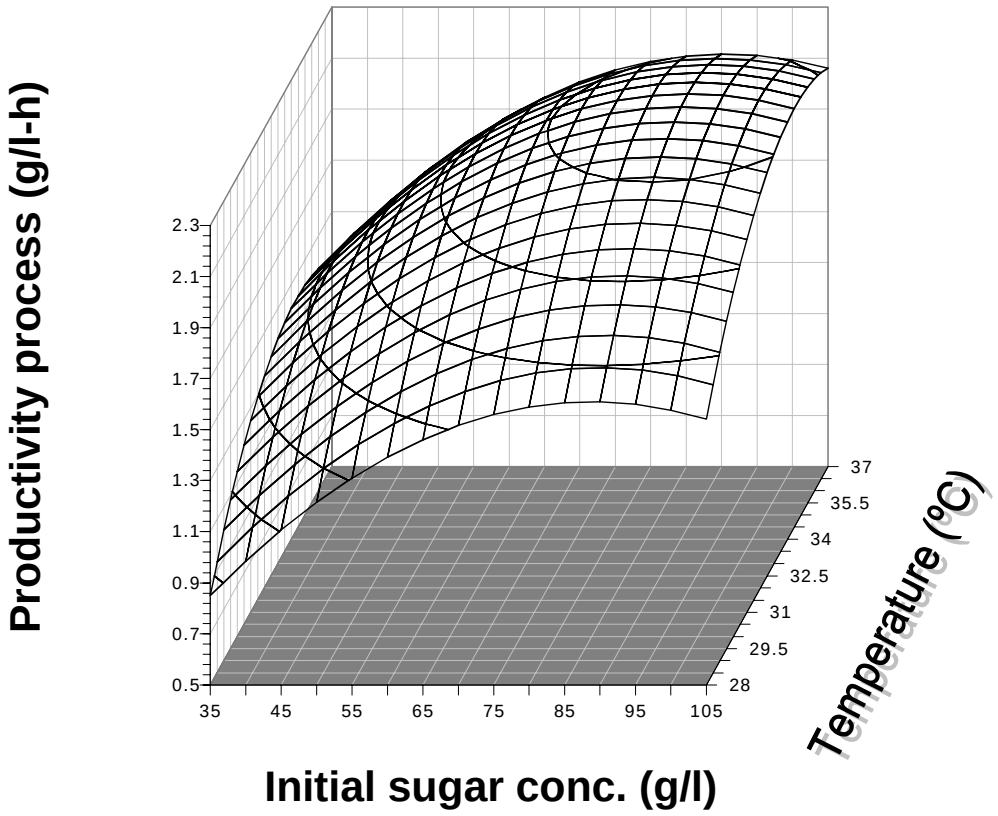

Fig. 4. Dependence of productivity process on the temperature and the initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

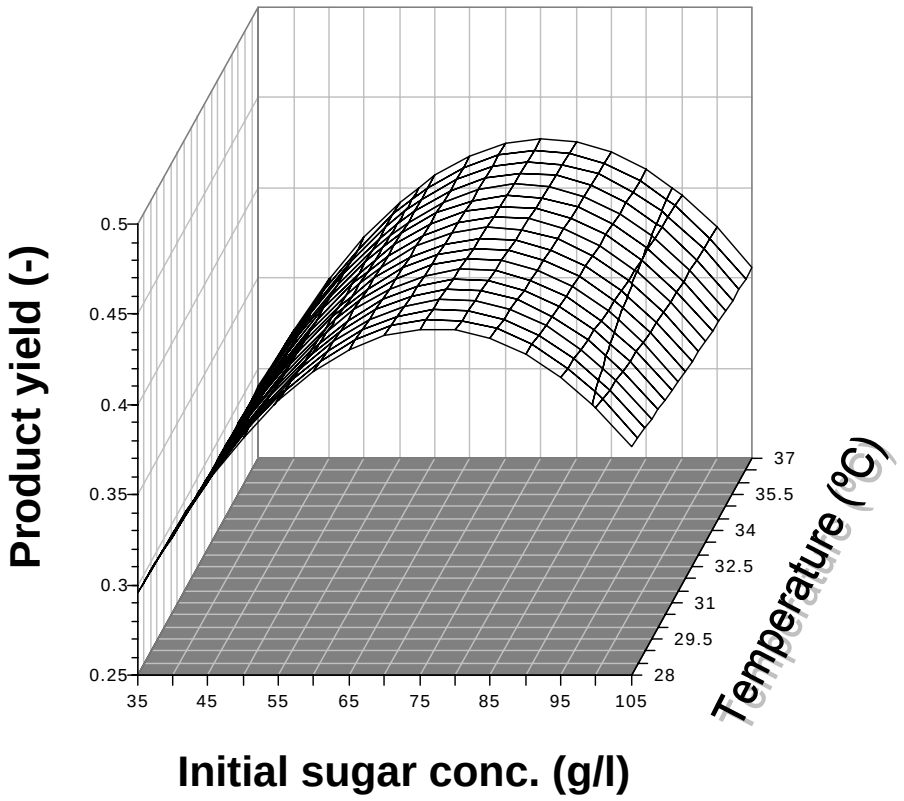

Fig. 5. Dependence of product yield on the temperature and the initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

Effect of the temperature and the initial sugar concentration on the mezcal composition

Table 5 shows a summary of the concentration of volatile compounds present in the mezcals obtained in this work. In all cases the ethanol concentration was set at 36 % (v/v) according to the Official Mexican Norm (1994). It can be observed that the composition of volatile compounds in the mezcals depends on the fermentative conditions. Since, the culture conditions affect the microbial dynamic and metabolic pathways resulting in mixtures of alcohols with different composition. Methanol is produced from pectin and lignin present in the vegetal-cell wall (Cedeño, 1995),

whereas higher alcohols such as propanol, n-butanol, 2-methyl-propanol, 2/3 methyl-1-butanol are produced by the catabolism of amino acids (Pronk et al., 1996). Higher alcohol concentration is used as quality indicator during tequila production, grape wine and spirits obtained from Jerusalem artichoke (Berovič, 2003; Pinal et al., 1997; Szamelan et al., 2005), because they contribute to the organoleptic properties and the bouquet of alcoholic beverages. Then, we used the same criteria as indicator of the mezcal quality. The amount of higher alcohols obtained in mezcals produced under different fermentative conditions varied in the range of 201 to 313 mg/l, for the treatments 3 and 4 respectively. Thus, mezcal obtained at 28°C and 70 g/l could be considerate as the best mezcal. Pinal et al. (1997), reported that the type of yeast strain, temperature and C/N ratio had a significant influence in the amount of higher alcohols produced during the tequila production. It has been reported that *Lactobacillus* genus produces propanol (Readler y Zorg, 1986), whereas *S. cerevisiae, Pichia fermentans* and others yeasts produce higher alcohols such as n-butanol, 2-methyl-propanol and 2/3 methyl-1-butanol (Clemente-Jiménez et al., 2005). The origin of ethyl 2-hydroxypropanoate and ethyl acetate is not clear; they could be produced by *Lactobacillus* or by extra-cellular esterification reaction (Davis et al., 1985). Since, the major compounds are the main responsible of conferring aroma and organoleptic properties to the mezcal. Therefore, differences on the composition of alcoholic beverages result from differences in the microbial community and their metabolism during the fermentation phase. Further identification of the microorganisms involved on the fermentative phase, and the subsequent selection of main strains could improve the understanding of the process and the quality of the mezcal as well.

Table 5. Concentration (mg/l) of volatile compounds present in mezcal produced under different fermentative conditions.

Rt[a] (min)	Compound	Treatment No.								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
4.4o6	Ethyl acetate	269±1	146±0	50±1	103±2	400±7	104±0	115±8	780±1	158±2
4.63	Methanol	1795±2	1782±3	1537±37	1583±13	1648±22	1682±3	1554±105	1671±2	1640±2
6.91	n-Propanl	218±0	195±1	191±4	272±3	234±3	216±0	270±17	276±0	244±1
7.87	2-Methyl-propanol	ND[c]	ND	ND	ND	ND	ND	5±0	ND	ND
8.64	n-Butanol	ND	10±1	ND	29±5	14±6	12±2	8±3	7±0	10±2
9.62	2/3-Metyl-1-Butanol	ND	8±0	ND	12±0	9±0	9±0	13±1	14±0	12±0
11.72	Ethyl 2-hydroxypropanoate	104±1	121±1	124±3	235±3	182±1	140±1	124±8	158±1	70±0
13.26	Acetic acid	67±0	58±1	90±4	29±3	72±4	63±1	23±1	68±2	77±4
13.524	Furfuraldehyde	15±0	33±0	ND	11±0	ND	ND	ND	ND	ND
	Higher Alcohols[b]	212±0	213±2	201±17	313±3	257±3	237±3	296±15	297±0	266±1

[a]Rt: Retention time in the HP-Innowax column. [b]Sum of alcohol with three or more carbons. [c]ND: Not detectable. Ethanol concentration was fixed to 36 % v/v. Data are the average ± standard deviation of two independent experiments as described in materials and methods.

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References

Balusu, R., Paduru, R.R., Kuravi, S.K., Seenayya G., Reddy, G. (2005) Optimization of critical medium components using response surface methodology for ethanol production from cellulosic biomass by *Clostridium thermocellum* SS19. *Process Biochem* **40**, 3025-3030.

Bandaru, V.V.R., Somalanka, S.R., Mendu, D.R., Madicherla N.R., Chityala, A. (2006) Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology. *Enz Microbial Technol* **38**, 209-214.

Berovič, M., Mavri, J., Wondra, M., Kosmerl T., Dejan, B. (2003) Influence of temperature and carbon dioxide on fermentation of cabernet sauvignon must. *Food Technol Biotechnol* **41**, 353-359.

Cedeño, M. (1995) Tequila production. *Crit Rev Biotechnol* **15**, 1-11.

Chen, S.L. (1981) Optimization of batch alcoholic fermentation of glucose syrup substrate. *Biotechnol Bioeng* **23**, 1827-1836.

Clemente-Jiménez, J.M., Mingorance-Carzola, L., Martínez-Rodríguez, S., Las Heras-Vázquez F.J., Rodríguez-Vico, F. (2005) Influence of sequential yeast mixtures on wine fermentation. *Int J Food Microbiol* **98**, 301-308.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee T.H., Flee, G.H. (1985) Practical implications of malolactic fermentation: a review. *Am J Enol Vitic* **36**, 290-301.

De León-Rodríguez A., Jiménez-Islas, H., González-Cuevas, M., Barba de la Rosa, A.P. (2004) Analysis of the expression of the *Trichoderma harzianum ech*42 gene in two isogenic clones of *Escherichia coli* by response surface methodology. *Proc Biochem* **39**, 2173-2178.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A.P., Escalante-Minakata P., López, M.G. (2006) Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana. J Agric Food Chem* **54**, 1337-1341.

Jones A.M., Ingledew, W.M. (1994) Fuel Alcohol Production: Optimization of Temperature for Efficient Very-High-Gravity Fermentation. *Appl Environ Microbiol* **60**,1048-1051.

Mancilla-Margalli N.A., López, M.G. (2002) Generation of Maillard compounds from inulin during the thermal processing of *Agave tequilana* Weber Var. azul. *J Agric Food Chem* **50**, 806-812.

Mexican Ministry of Commerce and Industry, Regulations: NOM-070-SCFI-1994, Alcoholic drinks-Mezcal Specifications México: *Diario Oficial de la Federación* (1994 November 28).

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducting sugar. *Anal Chemistry* **31**, 426-428.

Montgomery, D.C. Response surface methods and other approaches to process optimization. In: *Design and analysis of experiments*. 4th ed. USA: Wiley, 1997, p. 372–422.

Paz-Maldonado, L.M.T., Balderas-Hernández, V.E., Medina Rivero, E., Barba De la Rosa, A.P., Flores-Flores, J.L., Ordónez Acevedo L.G., De León Rodríguez, A. (2007) Optimization of culture conditions for a synthetic gene expression in *Escherichia coli* using response surface methodology: the case of human interferon beta. *Biomol Eng* **24**, 217-222.

Pinal, L., Cedeño, M., Gutiérrez H., Alvarez-Jacobs, J. (1997) Fermentation parameters influencing higher alcohol production in the tequila process. *Biotechnol Lett* **19**, 45-47.

Pronk, J.T., Steensma H.Y., Van Dijken, J.P. (1996) Pyruvate metabolism in *Saccharomyces cerevisiae. Yeast* **12**, 1607-1633.

Readler, F., Zorg, J. (1986) Characterization of the enzyme involved in formation of 2-butanol form meso-2, 3-butandiol by lactic acid bacteria. *Am J Enol Vitic* **37**, 206-209.

Stewart, G.C., Pancha, C.J., Rusell I., Sillis, A.M. (1984) Biology of ethanol producing microorganisms. *Crit Rev Biotechnol* **1**, 161-188.

Szamelan, N., Nowak J., Jelén, H. (2005) The composition of Jerusalem artichoke (*Helianthus tuberosus* L.) spirits obtained from fermentation with bacteria and yeasts. *Eng Life Sci* **5**, 68-71.

Tauer, A., Elss, S., Frischmann, M., Tellez P., Pischetsrieder, M. (2004) Influence of themally processed carbohydrate/amino acid mixture on the fermentation by *Saccharomyces cerevisiae*. *J Agric Food Chem* **52**, 2042-2046.

Thatipamala, R., Rohani S., Hill, G.A. (1992) Effects of high product and substrate inhibitions on the kinetics and biomass and product yields during ethanol batch fermentation. *Biotechnol Bioeng* **40**, 289-297.

Yokosuka, A., Mimaki, Y., Kuroda M., Sashida, Y. (2000) A new steroidal saponin from the leaves of *Agave Americana. Planta Med* **66**, 393-396.

Zaldivar, J., Martínez A., Ingram, L.O. (1999) Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli. Biotechnol Bioeng* **65**, 24-33.

4.2 *On-line monitoring of mezcal fermentation based on redox potential measurements*

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Abstract

The present study describes an algorithm for the continuous monitoring of the biomass and ethanol concentrations as well as the growth rate in the Mezcal fermentation process. The algorithm performs its task having only available the online measurements of redox potential. The procedure includes an Artificial Neural Network (ANN) that relates the redox potential to the ethanol and biomass concentrations. Then a nonlinear-observer-based algorithm uses the biomass estimations to infer the growth rate of this fermentation process. The results show that the redox potential is a valuable indicator of microorganism metabolic activity during the Mezcal fermentation. In addition, the estimated growth rate can be considered as a direct evidence of the presence of mixed culture growth in the process. Usually, mixtures of microorganisms could be intuitively clear in this kind of processes, however the total biomass data do not provide definite evidence by themselves. In this paper, the detailed design of the software-sensor is presented, as well as its experimental application at laboratory level.

Key words: Mezcal, mixed-cultures, software-sensor, *redox potential* , growth rates

Introduction

Mezcal, is a Mexican distilled spirit made from the core of the *Agave* plant (the "piñas"). Several species are used for the Mezcal production, i.e. *Agave salmiana*, *potatorum*, *angustifolia*, *tequilana*, etc, and each produces a slightly different Mezcal. Therefore, the tequila can be considered a regional type of Mezcal, restricted to the usage of *Agave tequilana* as raw material (Cedeño, 1995). It is important to mention that during this Mezcal fermentative process the syrup obtained from the juice of cooked "piñas" is left to naturally (spontaneously) ferment. By natural alcoholic fermentations we refer to the ones that start by themselves when a wild mixture of different microorganism starts fermenting. Therefore, the fermentation of *Agave* syrup into Mezcal is a complex biochemical process involving a whole realm of interactions between microorganisms.

During fermentation, the microorganisms employ sugars and other constituents of *Agave* syrup as substrate for their growth, converting them into ethanol, carbon dioxide, higher alcohols and their esters, and other metabolic compounds that contribute to the chemical composition and sensory qualities of the Mezcal (De León et al., 2006). We also notice that the qualitative and quantitative composition of the microbiota in fermenting musts could depend on the following factors: region of the *Agave* origin, production procedure, initial cell concentration, temperature, and ethanol concentration. As consequence, the organoleptic properties are also the result of the diversity and composition of microorganisms and their dynamics and frequency of occurrence. Consequently, this fermentative process is a vital stage in Mezcal making. Thus, it is very important to know more about the dynamics of the entire microflora during the alcoholic fermentation process. In other words, an algorithm that enables a monitoring process could be fundamental for a quality control that ensures, at least, homogeneity in the final product.
Nevertheless, a bottleneck in all biochemical monitoring process is often the lack of sensors for biological variables. Moreover, it is a well-known issue that in order to monitor many biotechnological processes, the problem of growth rates estimations

represent a strategic feature. That is why several techniques have been developed to estimate on-line the biological variables from the available measurements, which are usually dealing with physicochemical variables. Depending on the obtainable information about the process, there exist many possible types of estimators that can be used (Bastin y Dochain, 1986; Locher et al., 1992; Farsa et al. 1998).

Previously, various attempts of relating the *redox potential* to fermentation processes have been made taking into account that *redox potential* assesses the growth ability of microorganisms, as well as the physiological activity in a given environments (Kwong et al., 1992; Berovič, 1999; van Dijk et al., 2000; Cheraiti et al., 2005). Particularly, the practical significance of redox potential and oxygen content at various stages of winemaking was examined by (Kukec et al., 2002). Many chemical, enzymatic and biological processes in wine are correlated with the oxidative state of the wine.

Monod kinetics, which were originally derived from laboratory experiments with pure cultures and single substrates, are frequently applied to describe the behavior of undefined mixed cultures growing with single substrates or complex substrate mixtures (Novák et al., 1994; Wanner, 1994; Gujer et al., 1995; Reeves et al., 2004). In this case, the growth parameters that have been used represent overall values reflecting the growth constants of many different strains with respect to the multicomponent substrate and the frequencies and concentrations of both the different substrates and microbial strains. Nevertheless, it is well known from control processes that an accurate model leads to better control design and therefore to better closed-loop performance. There is recent progress shedding light on the dynamical processes underlying the growth of mixed culture in a mixture of substrates (Reeves et al., 2004; Ibarra-Junquera et al., 2006). However, the exact determination of the growth rates, under such complex situations, is still an open subject.

The work is organized as follows. Section Materials and Methods is devoted to a concise presentation of the fermentation experiment performed to illustrate our

approach. The software sensor, which is a combination of an ANN and an adaptive observer scheme, is described in Section called The Software Sensor. The results obtained by applying this software sensor to the Mezcal fermentation process together with brief comments are included in Section 3. Finally, the paper ends up with some concluding remarks.

Materials and Methods

Microorganism and culture conditions

In order to evaluate experimentally the performance of the estimation algorithm, we performed six individual batch experiments using inocula of native microorganisms (without the addition of any commercial strain). The must (*Agave* syrup) was obtained from *Agave salmiana*, a species from the Mexican plateau (or altiplano) of the geographical region of San Luis Potosí. This must was centrifuged at 8000 *rpm x* 10 *min* and stored in a frozen state at -20°*C* prior to experiments.

The batch fermentations were carried out in a Bioreactor (Applikon, Schiedam, the Netherlands) of 1 liter. The bioreactor is equipped with pH and redox sterilizable electrodes (Pt-Ring, Applisens, Sensor Innovation, Applikon). The electrodes are connected to a console for data acquisition (Bioexpert, Data Acquisition Control Program, Version 1.1x, Applicon), a device which is connected to a computer where the data are stored and computed. The schematic representation of the process appears in Fig. 1.

The bioreactor was filled with 900 *mL* of must as a culture medium, 100 *mL* of the inoculum in its exponential growing phase (biomass 0.1 *g/L*) and 0.1 % of ammonium sulfate at final concentration. The initial conditions of the fermentation were settled at a temperature of 32.5 °*C* and initial sugar concentration of 70 *g/L*. The pH does not show a dynamic evolution, maintaining itself at a value of 4 during the whole process. For a schematic representation of the data acquisition process see Fig 1.

Analytical procedures and measurements

The batch processes have been monitored for 14 hours, through sampling under sterile conditions. In order to quantify biomass and ethanol concentrations, 5 *mL* samples of culture was removed every 30 *min*. The samples were cleared by centrifugation at 6000 *rpm* for 5 minutes at room temperature. The next step was to collect the supernatant phase and store it frozen at -20 °*C* prior to be analyzed. The obtained pellet was resuspended in distilled water in order to proceed with biomass analysis.

Biomass and ethanol quantification

The biomass measurements have been performed using (varian) UV spectroscopy at 600 *nm*. The obtained values were interpolated with a standard curve of cell dry weight concentration.

For the determination of the ethanol, 1 *mL* of each sample with a final dilution 1:10 and 1 *mL* of 1-butanol in vortex motion for 5 *min* and followed by centrifugation at 6000 *rpm* for 5 *min*. The organic phase was analyzed by gas chromatograpy (6890N Agilent technologies, Wilmington, DE) provided with a capilar column HP-Innowax (30 *m* x 0.25 *mm* i.d., 0.25 *m* film thickness; Agilent technologies, Wilmington, DE) and an auto-sampler 7863 (Agilent technologies, Wilmington, DE) with a split relation of 25 :1. The chromatographic conditions were 35 °*C* for 2 *min*, increased at the rate of 10 °*C/min* up to 80 °*C*, and maintained at the latter temperature for 15 *min*. The carrier gas was helium at a flow rate of 1.5 *mL/min*. The temperatures of the injector and flame ionization detector (FID) were set at 220 °*C* and 250 °*C*, respectively. The ethanol concentration of the samples was determined by means of a calibration curve of known standard solutions of ethanol.

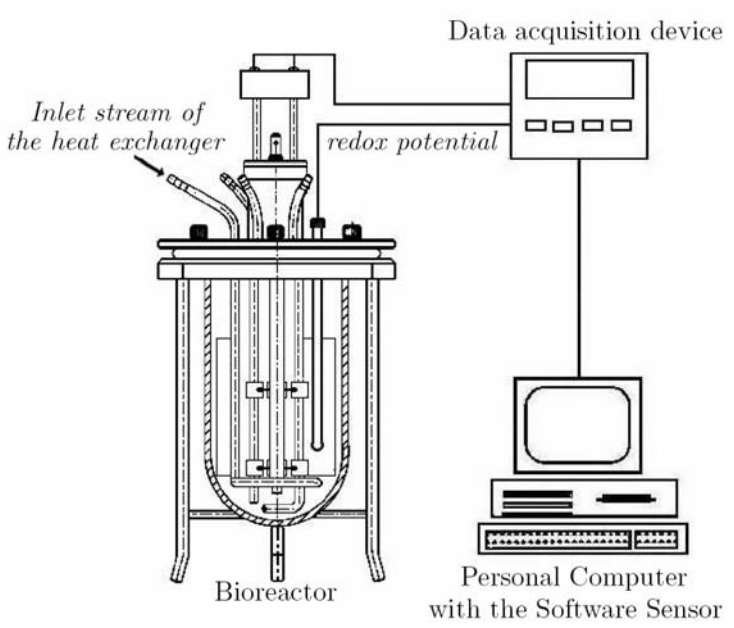

Fig. 1. The figure shows the schematic representation of the experiments carried out in our laboratory.

Redox potential

The measurement of *redox potential* is relatively fast, accurate and reliable and its values give an insight into the oxidation process as well as the inherent ability of reduction in the process, which is well established in the case of wine (Kukec et al., 2002). We mentioned that improvements in manufacturing processes and equipment have allowed beer, wine, as well as Mezcal producers to increase both the quantity and quality of these products. It is crucial for the commercial competition to maintain good quality control practices. Higher good-quality productions of the manufacturing plants require faster and more sophisticated analytical techniques to achieve them.

The measured values of the *redox potential* can give information on redox reactions in wine, which have an important influence on its quality and stability (Kukec et al., 2002). During storing and aging of wine, oxidation and reduction processes affect the character and taste of wine to a considerable extent (Cheraiti

et al., 2005; Kukec et al., 2002). We surmise that the same features could occur in the case of the Mezcal making. Checking this assumption is one of the main goals of this research. In the case of tequila, there exist previous efforts to understand the relation of the organoleptic properties with the process stages (Benn y Peppard, 1996; López, 1999; López y Dufour, 2001; Vallejo-Córdoba et al., 2004). However, neither in Tequila nor in Mezcal there exist a report of an online-measured variable that allows its fermentation monitoring in real time.

The *redox potential* measurements were acquired periodically each 0.01 *hr* during 14 *hrs*, and the data were stored and computed on-line in a PC (see Fig. 1).

The Software Sensor

By the software sensor we mean the algorithm generated by the coupling of the ANN and the adaptive observer. In this section we develop step by step this type of algorithm. First, we present the mathematical model that stays as the background of our approach. Then we explain the relationship with the measured output, the *redox potential*, and the ANN scheme. We end the section with a discussion of the adaptive scheme used to infer the growth rate function.

Mathematical model of the fermentation process

Batch microbial growth in a completely stirred bioreactor is commonly described by the following differential equations (Bastin y Dochain, 1986; Nielsen et al., 2003).

$$\frac{dX_1}{dt} = X_1\mu(t)$$

$$\frac{dX_2}{dt} = -k_1 X_1\mu(t)$$

$$\frac{dX_3}{dt} = k_2 X_1\mu(t)$$

where X_1 represent the total biomass concentration ([*g/l*]), X_2 refers to the total substrate concentration ([*g/l*]) and X_3 is the product concentration ([*g/l*]) (ethanol in this case), k_1 represents the biomass yield, k_2 the product yield and μ (t) is the growth rate. All the parameters that have been used represent overall values reflecting the many different strains with respect to the multicomponent substrate. It is important to note that the scheme here proposed does not need the values of k_1 or k_2 and μ (t) it is infer by the software sensor.

Artificial neuronal network

In order to relate the redox measurements to the ethanol and biomass concentration an ANN procedure is applied. The methodology that we carried out includes a forward-propagation training algorithm for the ANN using some of our experimental data. In order to perform our task we construct a model of the following form:

$$X_1 = f(X_4) \quad (1)$$

where X_4 represents the redo*x* potentia*l* measurement data (*mV*), X_1 is the set of biomass concentration data (*mg/L*) and the function f (X_4) is approximated by means of the ANN procedure. The ANN architecture is of the standard type (Lanchenmeier, 2006) with a single ANN hidden-layer containing 10 units. The same scheme was used for the case of the ethanol but using $X_3 = f(X_4)$ as the ANN model.

Each unit of this network uses a sigmoid function as the activation function. On the other hand, the output contains a linear activation function, in our case the identity. The feed-forward training algorithm considered here is the conjugate gradient method (Rumelhart y McClelland, 1986). Three of the six individual batch experiments were used to provide data for the training process. The ANN after the training gives an error of only 0.0029.

The adaptive observer

The analysis of the adaptive observer scheme used here is based on the following realistic assumptions:

(A1) The specific growth rate $\mu(t)$ is positive and bounded, that is μ_{max} exists but is unknown, although it is bounded: $0 < \mu(t) < \mu_{max}$

(A2) There is no growth without substrate: $X_2 = 0 \Rightarrow \mu(t) = 0$

(A3) The time derivative of $\mu(t)$ is bounded: $\left|\frac{d\mu(t)}{dt}\right| \leq M_1$ where $M_1 \in \mathrm{R}_+$.

On-line estimation of μ(t) from measurements of X_1 Since X_1 is available through the neuronal algorithm mentioned in the previous section, we can now rewrite the output of the system as:

$$y_S = X_1, \qquad (2)$$

where y_S is the set of on-line measurements of the system which is available indirectly through ANN means. Then, following (Bastin y Dochain, 1986), the following algorithm can be used to estimate $\mu(t)$:

$$\frac{d\hat{X}_1}{dt} = y_s\,\hat{\mu}(t) + K_1 y_s(y_s - \hat{X}_1)$$

$$\frac{d\hat{\mu}}{dt} = K_2 y_s(y_s - \hat{X}_1),$$

where $\hat{X}_1$ and $\hat{\mu}$ represent the estimated value of X_1 and μ, respectively. In others words, $\hat{X}_1$ stands for the estimated biomass concentration. The constants K_1 and K_2 must be chosen such that:

$$0 > K_2 > \frac{K_1^2}{4}.$$

The above condition ensures the asymptotic convergence of the observer error to a neighborhood of zero (Bastin y Dochain, 1986).

Results and Discussion

In the previous section, the general idea as well as the detailed steps for the construction of the software sensor were been given. To complete the analysis of the approach, this section presents the experimental results obtained at the laboratory level at which the software sensor was tested.

In total, six series of experiments of Mezcal fermentation at temperature of 32°*C* were performed. The temperature has been maintain constant using a heat exchanger device, see Fig. 1. First, we perform three experiments to generate the necessary biomass and ethanol concentration data for the training of the ANN. Once the error given by the ANN goes below the value of 0.003 biomass units, the adaptive scheme was added to complete the software sensor procedure. Then, three more experiments were carried out in order to further testing the scheme. The results concerned with the performance of the ANN to infer the ethanol and biomass concentrations from the *redox potential* measurements are presented in Figs. 2-3, where one can appreciate the degree of accuracy of the algorithm. Notice that the Fig. 3 presents several slope changes that do not allows to infer the presence of a mixture of microorganisms during the fermentation process.

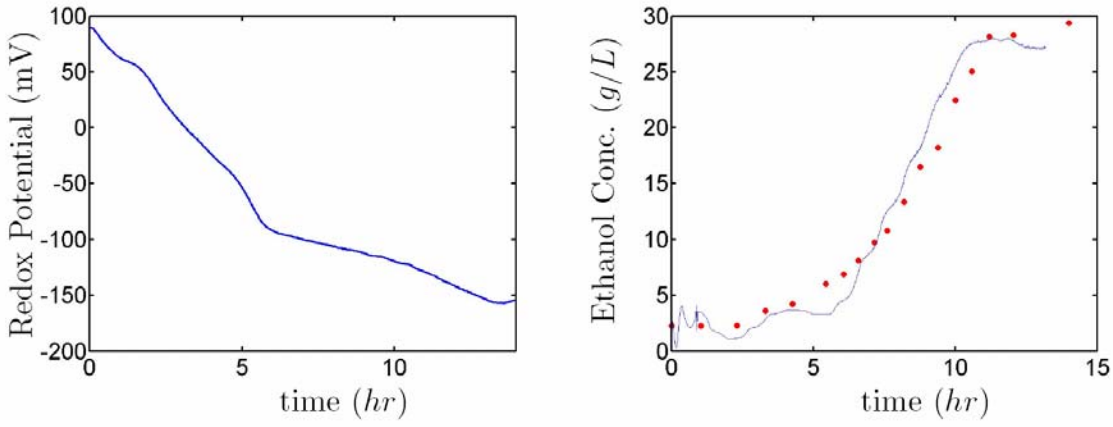

Fig. 2. Right plot illustrates the time evolution of the redox potential in the fermentation process. In the left plot the blue solid lines represent ANN-estimated ethanol concentration and red dots stand for its experimental values.

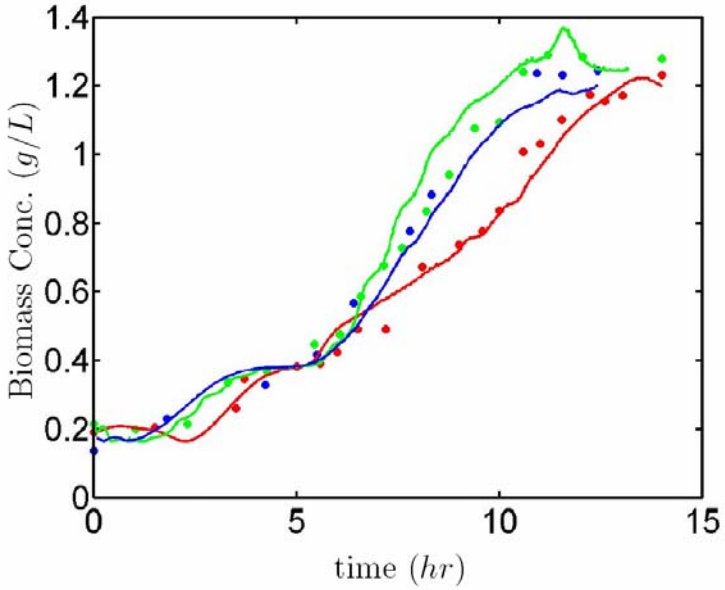

Fig. 3. Continuos lines correspond to the estimated biomass data and dots to the experimental data. Blue, red and green correspond to each of the experiments carried out in our laboratory.

Although the main goal is to estimate $\mu(t)$, the biomass estimation is also performed, and the difference between this estimation and the value predicted by the ANN procedure is used as a correction term in the algorithm. Fig 4 shows the ability of the software sensor to rebuild the biomass concentration data. It should be highlighted that the simplicity associated with the implementation of this algorithm and the necessity of a unique measured signal (*redox potential*) are very promising features from the technological and industrial stand points.

From Fig. 5, it is possible to discern three regions, labeled as A, B and C. In general terms, in region A one is not able to get any conclusion on the dynamical behavior of the process during this span of time since it corresponds to the transient behavior of the observer. the span of time corresponding to region A is given by the time that the observer takes up to minimize the error between its estimated biomass concentration and that given b the ANN procedure.

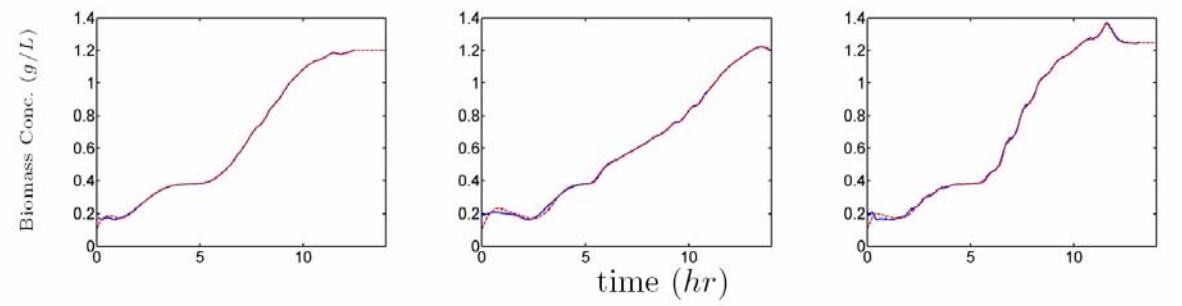

Fig. 4. (Blue) solid lines represent the estimated biomass concentration given by the software sensor and (red) dash-dotted lines stand for the ANN-predicted biomass. From the left to the right appears the plots corresponding to the three experiment performed to teste our approach are displayed.

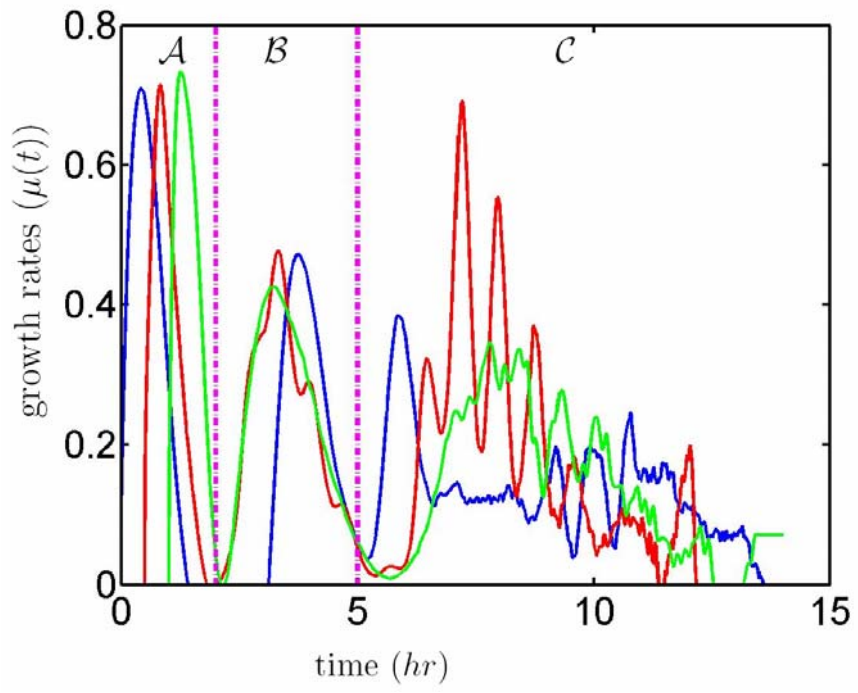

Fig. 5. The figure shows growth rates as estimated by the adaptive algorithm. The blue, red an green lines correspond to the estimation results for each of the experiments carried out in the bioreactor.

On the other hand, we associate regions B and C with the presence of two growth rates ($\mu(t)$) different groups of microorganisms, namely bacteria and yeasts. This

assumption is based on the fact that the growth rate of bacteria is faster than that of yeasts. Thus, from Fig. 5, the presence of a mixed culture growth comes out naturally, a fact which is not so obvious when one examines only the biomass data given in Fig. 3. Note in addition that the end of the fermentation process is quite clear in Fig. 5.

Conclusions

In this work, we have shown that in the Mezcal fermentation process the *redox potential* could give relevant information on the microorganism metabolism, including both ethanol and biomass concentration. Besides, the problem of estimating the specific growth rates in the Mezcal fermentation process is treated. Moreover, the strategy here presented clearly detects the end of this fermentative process. The latter fact is quite relevant from the production point of view since it is the piece of information by which one can save time and avoid the degrading process due to the conversion of the ethanol to acetic acid, improving in this way the quality of the product. In addition, the computational scheme gives a very appropriate tool for quality control, helping to ensure the homogeneity of the final product. The methodology presented in this paper is general and can be also used for automatic control applications.

References

Bastin, G., Dochain, D. (1986) On-line Estimation of microbial specific growth rates. *Automatica* **22**, 6705-709.

Benn, S.M., Peppard T.L. (1996) Characterization of tequila flavor by instrumental and sensory analysis. *J Agric Food Chem* **44**, 557-556.

Berovič, M., Mavri, J., Wondra, M., Kosmerl T., Dejan, B. (2003) Influence of temperature and carbon dioxide on fermentation of cabernet sauvignon must. *Food Technol Biotechnol* **41**, 353-359.

Cedeño, M. (1995) Tequila production. *Crit Rev Biotechnol* **15**, 1-11.

Cheraiti, N., Guezenec, S., Salmon, J.M. (2005) Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. *Appl Environmental Microbiol* 255-260.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A.P., Escalante-Minakata P., López, M.G. (2006) Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana. J Agric Food Chem* **54**, 1337-1341.

Farza, M., Busawon, K., Hammouri, H. (1998) Simple Nonlinear Observers for on-line estimation of kinetic rates in bioreactors. *Automatica* **34**, 3301-318.

Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.R. (1995) The activated sludge model no. 2: biological phosphorus removal. *Water Sci Technol* **31**, 1-11.

Ibarra-Junquera, V., Escalante-Minakata, P., Murguía-Ibarra, S., Rosu, H.C. (2006) Inferring mixed-culture growth from total biomass data in a wavelet approach. *Physica A* **370**, 777-792.

Kukec, A., Berovič, M., Celan, S., Wondra, M. (2002) The Role of On-line Redox Potential Measurement in *Sauvignon blanc* Fermentation. *Food Technol Biotechnol* **40**, 149-55.

Kwong, S.C.W., Randers, L., R. Govin. (1992) On-line assessment of metabolic activities based on culture redox potential and dissolved oxygen profiles during aerobic fermentation. *Biotechnol Prog* **8**, 576-579.

Lachenmeier, D.W., Sohnius, E.M., Attig, R., López, M.G. (2006) Quantification of selected volatile constituents and anions in Mexican Agave spirits (Tequila, Mezcal, Sotol, Bacanora). *J Agric Food Chem* **54**, 3911-3915.

Lapedes, A.S., Farber, R.M. Nonlinear signal processing using neural networks: Prediction and system modeling, Los Alamos Report LA-UR-87(1987)2662.

Locher, G., Sonnleitner, B., Fiechter, A. (1992) On-line measurement in biotechnology: Techniques. *J Biotech* **25**, 23-53.

López, M.G. Tequila aroma. In Flavor Chemistry of Ethnic Foods, Shahidi and Ho Eds. Kluwer Academic/Plenum Publishers, New York, 1999, pp. 211-217.

López, M.G., Dufour, J.P. Tequilas: Charm analysis of blanco, reposado, and aejo tequilas. In Chromatography-Olfactometry. The state of the art. Leland, J.V., Schieberle, P., Buettner, A., Acree, T.E., Eds.;ACS Sympusium Series 782; American Chemical Society: Washington, DC 2001; pp 60-72.

Nielsen, J. Villadsen, J. Liden, G. Bioreaction Engineering Principles, Kluwer Academic/Plenum Publishers 2003.

Novák, L., Larrea, L., Wanner, J. (1994) Estimation of maximum specific growth rate of heterotrophic and autotrophic biomass: a combined technique of mathematical modelling and batch cultivations. *Water Sci Technol* **30**, 171-180.

Reeves, G.T., Narang, A., Pilyugin, S.S. (2004) Growth of mixed cultures on mixtures of substitutable substrates: the operating diagram for a structured model. *J Theor Biology* **226**, 143-157.

Rumelhart, D., McClelland, J. Parallel Distributed Processing: Explorations in microstructure of cognition, Vol. 1, Foundations, MIT Press, Cambridge, MA., 1986, pp. 318-362.

Vallejo-Córdoba, B., Gonzordoba, A.F., Estrada-Montoya, M.C. (2004) Tequila volatile characterization and ethyl ester determination by solid phase microextraction gas chromatography/mass spectrometry analysis. *J Agric Food Chem* **52**, 5567-5571.

van Dijk, C., Ebbenhorst-Selles, T., Ruisch, H., Stolle-Smits, T., Schijvens, E., van Deelen, W., Boeriu, C. (2000) Product and redox potential analysis of Sauerkraut fermentation. *J Agric Food Chem* **48**, 132-139.

Wanner, J. Activated sludge bulking and foaming control, Technomic Publishing Co., Inc., Lancaster, Pa. 1994.

CAPÍTULO III

5. IDENTIFICACIÓN MOLECULAR DE LOS MICROORGANISMOS PRESENTES EN LA FERMENTACIÓN DEL MEZCAL

En el presente capítulo se muestran los resultados obtenidos de la identificación de los microorganismos, mediante técnicas moleculares, presentes al inicio de la fermentación. El trabajo aquí presentado se encuentra en proceso para ser sometido a la revista: Lettlers in Applied Microbiology.

P. Escalante-Minakata, H.P Blaschek, A.P. Barba de la Rosa and A. De León-Rodríguez. Identification of yeasts and bacteria involved in the mezcal fermentation process (En proceso).

5.1 Identification of yeasts and bacteria involved in the mezcal fermentation process

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Abstract

Aims: The objective of this work was to identify the yeasts and bacteria present in the early phases of mezcal fermentation of *Agave salmiana*.

Methods and Results: The restriction and sequence analysis of the amplified region, between 18S and 28S rDNA and 16S rDNA genes, were used for the identification of yeast and bacteria, respectively. Eleven different microorganisms were identified in the early step of the Mezcal fermentation. Three of them were the following yeasts: *Clavispora lusitaniae, Pichia fermentans* and *Kluyveromyces marxianus.* The bacteria found were *Zymomonas mobilis* subsp mobilis and *Zymomonas mobilis* subsp pomaceae, *Weissella cibaria, Weissella paramesenteroides, Lactobacillus pontis, Lactobacillus kefiri, Lactobacillus plantarum* and a new heterofermentative strain which was reported as *Lactobacillus farraginis*.

Conclusions: The phylogenetic analysis of 16S and ITS sequences showed that microbial diversity present in mezcal is dominated by bacteria, mainly LAB species and *Zymomonas mobilis*. It is important to note that the *L. pontis* and *Z. mobilis* species were the most abundant microorganisms present in the inoculum and that

the presence of the *P. fermentans* and *K. marxianus* could be responsible for the production of some volatile compounds.

Significance and Impact of the Study: We have shown the identification of bacteria and yeast responsible for *Agave salmiana* mezcal fermentation at the early phase. With this we have broaden the knowledge of the microbial diversity in the alcoholic fermentation.

Keywords: Mezcal, fermentation, phylogenetic analysis, ITS rDNA, 16S rDNA

Introduction

Mezcal is a traditional Mexican distilled beverage produced from the fermented juices of the cooked heart (or core) named o dubbed the “piñas”, of the agave plants. The *Agave salmiana* is used in the Altiplano region for the mezcal production (De León-Rodríguez et al., 2006). The “piñas” are cooked in stone ovens to hydrolyze the inulin into fructose (Peña-Alvarez et al., 2004). During the process, the syrup obtained is naturally fermented with its own microorganisms. Along the fermentation the sugars are converted mainly into ethanol, high alcohols, and other compounds such as esters and organic acids (Cedeño, 1995). Therefore, the taste and aroma of mezcal is determined by the composition of a mixture of compounds produced during the fermentation and others extracted from the *Agave* plant. It is worth to mention that a spontaneous fermentation contrains the mezcal process. Then, in order to provide a homogeneous quality between batches is convenient to initiate the fermentation process using well identified inocula. For this, the analysis of the microbial diversity and its role in the sensorial features is crucial.

Many techniques have been previously developed for the identification of microorganisms. For instance, Lachance (1995), used microbiological methods and 13 different yeasts species of the microorganisms involved in the fermentation

of tequila (spirit obtained from *Agave tequilana weber*). The use of the molecular biology methods has allowed a rapid and accurate identification of yeasts and bacteria. Methods based on the analysis of restriction fragment length polymorphism (RFLP) of the DNA that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) and the non-coding internal transcribed spacers (ITS) have been applied successfully by several groups (Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; Las Heras-Vazquez et al., 2003). Flores Berrios (2006), used the Amplified Fragment Length Polymorphism (AFLP) technique to study the genetic yeast diversity and showed the underlying relationship between the molecular profiles, strain origin and fermentation process for yeasts isolated from Mexican *Agave* spirit (tequila, raicilla, sotol and mezcal), Italian and African wines.

Recently, the bacteria identification in natural environment has been extensively studied in several works (Dojka et al., 1998; Escalante et al., 2004; Coton et al., 2005). The phylogenetic analysis of the 16S rDNA region was proved to be a powerful tool to explore the biological diversity in such environments.

The aim of this work was the identification of the yeast and bacteria present in the early steps of mezcal fermentation. For this purpose molecular techniques such as PCR-RFLP were used to compare a specific ribosomal DNA region for both organisms classes. Those microorganisms with differences in the restriction pattern were identified by cloning, sequencing and lastly, compared against the GenBank database.

Materials and Methods

Mezcal origin and sampling

A sample of inoculum was kindly provided by Juan Zarur from the Mezcal factory “La Perla” located in San Luis Potosí State, Mexico.

Sampling, isolation and selection of bacteria and yeast

The inoculum for mezcal production were collected aseptically and stored at -80°C with glycerol. The sample was diluted in sterile saline solution (NaCl, 0.85% wt v^{-1}) and plated on seven different media which were prepared for bacteria and/or yeast growth respectively (Table 1).

The plates were incubated at 30°C and 28°C for bacteria and yeast for a period of 48h. From each different colony type, three colonies with the same morphological features were selected for further characterization. The purification from each colony was performed on the same medium by successive subculturing at the same temperature (Beuchat, 1993; Deák et al., 1998).

DNA extraction

Bacterial DNA was isolated using a physical breakdown method with glass beans and for yeast the DNA was isolated according to Querol et al. (1992), with some modifications described below.

Physical breakdown

Bacteria cells were grown overnight in 5ml of the same broth in which they were isolated. Cells were washed with distilled water, centrifuged and resuspended in 0.2ml of extraction mixture (2% triton, 1% SDS, 10mM NaCl, 10mM TRIS-HCl pH 8, USB, Cleveland, USA), EDTA 1mM, adding 0.2ml of 25:24:1 phenol:chloroform:isoamylic alcohol, 0.06g of 0.5-mm glass beads and 0.1ml sterile distilled water. Finally, the cells were homogenized by vortex during 1 min at high speed for 8 times. The tubes were chilled on ice for 1 min between runs. The upper layer was recovered and transferred to a clean tube and 1 volume of pure chloroform was added, the tube was mixed carefully and centrifuged at 13 000 *g* for 5 min. The aqueous phase was saved in a clean tube and 20μg ml^{-1} RNAase was added and incubated for 1 hour at 37°C. After 1 volume of 24:1

chloroform:isoamylic alcohol was added and centrifuged again. The aqueous phase was transferred to a clean tube with 10μl of isopropanol and 4μl of ammonium acetate (4M) and was carefully mixed and incubated for 15 min at room temperature. Supernatant precipitated was centrifuged for 10 min and the DNA was washed with 70% v v^{-1} ethanol, vacuum-dried and dissolved in 30μl of TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

Enzymatic breakdown

Yeast cells were grown overnight in 5ml of TGYC broth, then the cells were centrifuged, at 6000 *g* for 5 min, washed with distilled water and resuspended in 500μl of 0.9M sorbitol (Sigma), 0.1M EDTA pH 7.5, and 40μl of 2.5mg ml^{-1} Zymolyase 20T (Seikagaku, Tokyo, Japan). Tubes were incubated at 37°C for 20 min. After centrifugation for 5 min at 7000 *g*, spheroplasts were resuspended in 500μl of 50mM TRIS-HCl, 20mM EDTA pH 7.4, adding 2.5μl of 10% w v^{-1} SDS, and the mixture was incubated at 65°C for 30 min. Inmediately, 200μl of 5M potassium acetate was added, and the tubes were placed on ice for 1-2 hours. Then, they were centrifuged at 13000 *g* at 4°C during 20 min. The supernatant was precipitated by adding 1 volume of isopropanol, and after incubation at room temperature for 15 min, was centrifuged for 10 min. The DNA was washed with 70% v v^{-1} ethanol, vacuum-dried and dissolved in 30μl of TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

PCR amplification and cloning of 16S rDNA and ITS rDNA

The 16S rDNA regions of the bacteria were amplified using the forward primer 533F 5'- GTG CCA GCA GCC GCG GTA A -3' and the reverse primer 1492R 5'- GGT TAC CTT GTT ACG ACT T -3'. (Dojka et al., 1998). To amplify the ITS rDNA region from yeast the primers used were ITS1 5'- TCC GTA GGT GAA CCT GCG- 3' and ITS4 5'- TCC TCC GCT TAT TGA TAT GC -3' which were described elsewhere by White *et al.* 1990. PCR amplification of ITS region was performed in 50μl volume using plastic tubes on a thermocycler (Peltier PTC-200, M.J. Research, Wattham. MA, USA). Each reactions contained 2μl template, 8μl of

dNTP's mixture (2.5mM, Roche Diagnostics, Germany), 5μl Extaq buffer 10X, 5μl $MgCl_2$ 25 mM, 0.25μl of Extaq polymerase 5U (Takara, Japan), and 1μl of each primer (100pM, Invitrogen, Carlsbad, CA). Cycling conditions were 94°C for 4 min, followed by 30 cycles of (94°C for 1 min), annealing temperature of 55°C (for 30 seconds), extension (72°C for 2min) and a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis with a 1.5% agarose gel in TAE buffer (40mM Tris-acetate, 1mM EDTA), 100 bp DNA ladder was used for the characterization of band size, following ethidium bromide staining and UV illumination.

The 16S rDNA from each bacterial colony was amplified by PCR using the pair of primers 533F and 1492R. The PCR was performed in 50μl volume containing 0.25μl of Taq Polymerase (Invitrogen, Carlsbad, CA), 5μl of Taq Polymerase buffer 10X , 8μl of dNTP mixture, 1.0μl of each primer (100pM, Invitrogen, Carlsbad, CA) and 2μl of bacterial DNA extracted from each different colony as template in a final reaction volume on 50μl. For the amplification of 16S rDNA, the samples were incubated for 14 min at 94°C to denature the target DNA and then cycled 30 times at 94°C for 1 min, 50°C for 45s and 72°C for 2 min. The samples were then incubated for 12 min at 72°C for a final extension and were maintained at 4°C until tested.

Each PCR product amplified for the different bacteria and yeasts (16SrDNA and ITS region respectively) were ligated into the pCR 4-TOPO vector kit sequencing (Invitrogen, Carlsbad, CA). Two microliters of ligation reaction were used to chemically competent *E. coli,* One shot TOP10 cells included in the TOPO TA cloning kit. Transformed cells were plated on LB-ampicillin plates. For bacteria, positive clones carrying approx. 1000 pb, 16S rDNA were identified by colony PCR. In contrast, the yeasts presented different size of amplified product between 300-800 pb and they were identified by colony PCR using one ITS primer and one primer of the cloning kit.

A 15μl of each PCR amplified product (ITS and 16S rDNA) was digested with 1μl of restriction enzyme *HaeIII* (New England Biolabs Ltd., Hertfordshire, England) and 2μl of buffer and sterile water. The mixture was incubated for 2 hours at 37°C and inactivated for 20 min at 80°C. The restriction fragments were separated on a 3% (w v-1) agarose gel. The resulting gels were stained with ethidium bromide and photographed. The lengths of both amplified products and restriction fragments were estimated by comparison against a 100 bp DNA ladder (Gibco-BRL).

Identification and phylogenetic analysis

In order to identify unique sequences of bacteria and yeasts, we performed amplified ribosomal DNA restriction analysis (ARDRA) profiling for each positive clone. The unique ARDRA profiles belonging to partial sequences corresponding to *E. coli* 16S rDNA and ITS, were submitted to the non-redundant nucleotide database at GenBank using the BLAST program (www.ncbi.nlm.nih.gov) in order to determine the identity of the clones inserts.

A multiple alignment of 16S rDNA clones and reference 16S rRNA or rDNA sequences retrieved from GenBank database was performed using Clustal W program. A distance matrix calculation of nucleotide substitution rates and a phylogenetic tree was constructed with the Kimura 2-parameter and the neighbor-joining (NJ) method, respectively, using MEGA 3.1. In order to provide confidence estimated for the tree topology in the NJ method bootstrap methods were used (1000 replicates).

Results

Phylogenetic analysis of ITS and 16S rDNA

Figure 1 shows a gel of the different restriction patterns obtained of the ITS region amplified. The size of the PCR amplification products and their restriction patterns corresponding to three different yeasts isolated in selective media and the positive

control, are shown in Table 2. The PCR-RFLP analysis of the rDNA ITS region corresponded with the restriction patterns reported elsewhere by Esteve-Zarzoso et al. (1999). Therefore, the use of PCR-RFLP to identify yeasts such as *Clavispora lusitaniae, Pichia fermentans* and *Kluyveromyces marxianus* in the mezcal fermentation, was only a first approximation. In order to perform a clear-cut positive identification the amplified ITS1, ITS2 regions and 5.8S rDNA were cloned and sequenced by duplicate. The results of the alignment of the sequenced region with the GenBank database confirmed the PCR-RFLP analysis. The organisms identified and their identities with the sequenced clones are shown in Table 2. The ITS sequences cloned as well as the sequences of the organisms identified in database were used to construct a phylogenetic tree using the neighbor joining method (Figure 2).

A total of 120 colonies of bacteria were isolated using the different culture media. The PCR product of each colony corresponding to 16SrDNA, of 1000 pb approx., was digested with a endonuclease enzyme *HaeIII*. The results showed clearly at least fifteen different restriction patterns. Some of them have shown slight differences, i.e., only one band had a different size. The sequences of these different clones were aligned with the database of GenBank. The bacteria identified were *Zymomonas mobilis subsp mobilis* and *pomaceae*, *Weissella cibaria, Weissella paramesenteroides, Lactobacillus pontis, Lactobacillus kefiri, Lactobacillus plantarum,* and *Lactobacillus farraginis* a new heterofermentative strain which is very close to *Lactobacillus hilgardii* (Endo and Okada, 2007). Finally, the phylogenetic tree was constructed with the 16S rDNA sequences obtained and the sequences of the organisms identified as the closest neighbor in databases. The Figure 3 shows the phylogenetic tree and the bootstrap analysis revealed that most of the branches in the tree have bootstrap values up to 80%. Moreover, high bootstrap values validated the robustness of the branching pattern obtained.

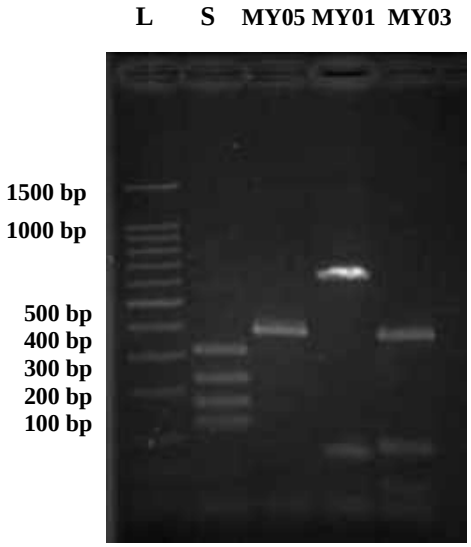

Figure 1. ARDRA patterns obtained from digestion with *HaeIII*. Lanes: L, size ladder (100 pb DNA ladder; Gibco-BRL); S, positive control (*Sacharomyces cerevisiae*); MY05, *Clavispora lusitaniae*; MY01, *Kluyveromyces marxianus*; MY03, *Pichia fermentans*.

Table 1. Formula to elaborate the different media (1L)

Media	Components
TYGC	Tripton 30g, yeast extract 10g, glucose 20g, agar 15g, chloramphenicol 100mg l^{-1}
MRS	Protease peptone No. 3 10g, beef extract 10g, yeast extract 5g, dextrose 20g, polysorbate 80 1g, ammonium citrate 2g, sodium acetate 5g, magnesium sulfate 0.1g, manganese sulfate 0.05g, dipotassium phosphate 2g, agar 15g
MYGP	Malt extract 3g, yeast extract 3g, glucose 10g, peptone 5g, agar 15g, actidione 20mg l^{-1}, ethanol 3%
PD	Potato starch 4g, dextrose 20g, agar 15g
AA	Pancreatic digest of casein 20g, sodium chloride 5g, dextrose 10g, agar 20g, sodium thioglycollate 2g, formaldehyde sulfoxylate 1g, methylene blue 0.002g
PCA	Tryptone 5g, yeast extract 2.5g dextrose 1g, agar 15g
MA	Potato starch 4g, dextrose 20g, agar 15g and filtered agave juice for obtained a final concentration of 5° Brix.

Table 2. Yeast identified, lengths (in bp) of the 5.8S-ITS region amplified by PCR and the fragments obtained after digestion with restriction endonuclease *HaeIII.*

Yeast identified	Amplified product (pb)	Restriction fragments ***HaeIII***
Saccharomyces cerevisiae	880	320+230+180+150
Clavispora lusitaniae (MY05)	390	360
Kluyveromyces marxianus (MY01)	740	655+80
Pichia fermentans (MY03)	450	340+80+30

Table 3. Organism which showed the highest percent of identity in the output result from analysis in the non-redundant nucleotide database from NCBI with BLAST program.

Organism identified	**Gene Bank Accession number**	**Clones identify and identity with Gene bank database**
Clavispora lusitaniae	AY939811	MYO5(99%), MYO6(100%)
Kluyveromyces marxianus	AY939806	MYO1(99%), MYO2(99%)
Pichia fermentans	AF411062	MYO3(98%), MYO4(98%),
Lactobacillus farraginis	AB262732	MB02(93%)
Lactobacillus kefir	AY363303	MB01(97%)
Lactobacillus plantarum	DQ239698	MB03(98%)
Lactobacillus pontis	AJ422032	MB04(95%), MB05(98%), MB06(97%), MB07(98%), MB08(98%)
Weissella cibaria	WC1422031	MB10(98%)
Weissella paramesenteroides	AB023238	MB09(98%)
Zymomonas mobilis	AF281033	MB14(97%), MB15(98%)
Zymomonas mobilis subsp mobilis	AY670648	MB11(96%), MB12(98%), MB13(97%)
Zymomonas mobilis subsp pomaceae	AY670649	MB15(98%), MB16(99%)

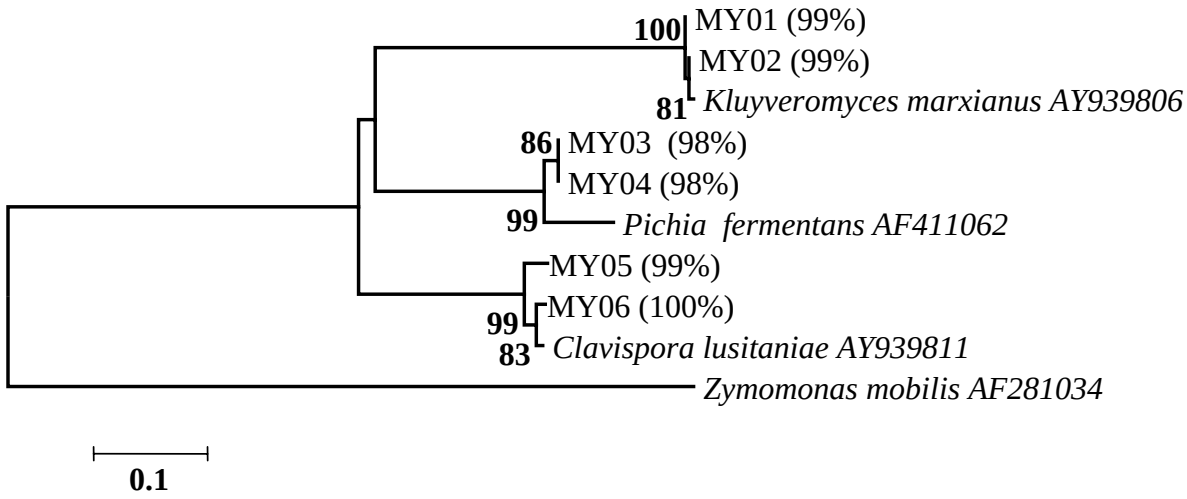

Figure 2. Phylogenetic relationship of 5.8S-ITS region from mezcal clones and sequences of closest neighbor ITS rDNA from identified yeast in NCBI database. The sequences were aligned by using Clustal W. The distance matrix and phylogenetic tree were calculated by the Kimura 2-paramenter and Neighbor joining algorithms, respectively. Percent of identity with closest references ITS rDNA clones in the database is indicated in parenthesis. The sequence of *Zymomonas mobilis* served as outgroup sequence. Bootstrap 1000.

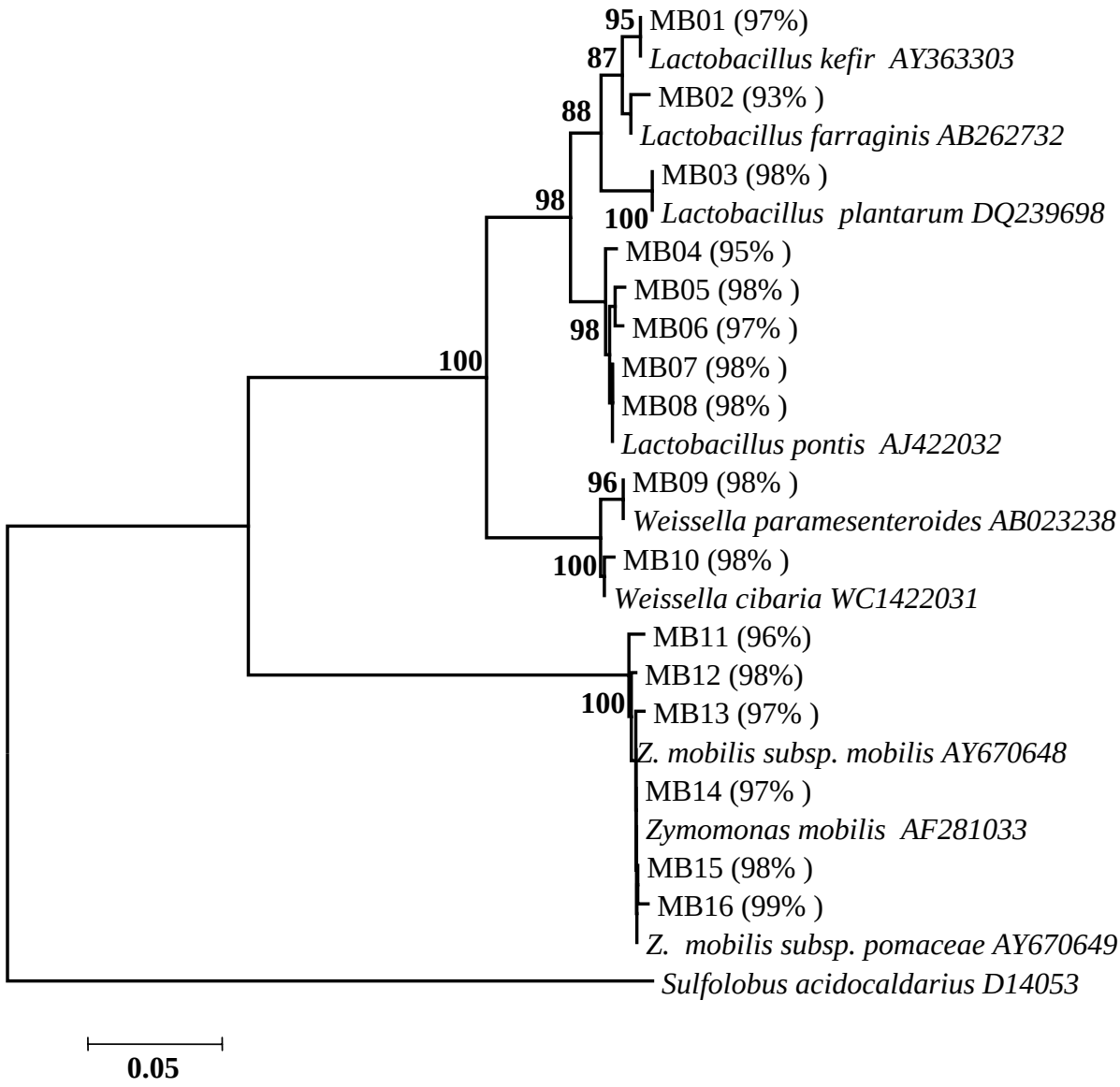

Figure 3. Phylogenetic relationship of 16S rDNA from mezcal clones and sequences of closest neighbor 16S rDNA from identified bacteria in NCBI database. The sequences were aligned by using Clustal W. The distance matrix and phylogenetic tree were calculated by the Kimura 2-paramenter and Neighbor joining algorithms, respectively. Percent of identity with closest references 16S rDNA rDNA clones in the database is indicated in parenthesis. The sequence of *Sulfolobus acidocaldarius* served as outgroup sequence. Bootstrap 1000.

Discussion

Although, the mezcal is an ancient alcoholic beverage, its fermentation process is scarcely known. Recent reports confirmed that a complex mixture of volatile compounds such as acids, alcohols, esters, and terpenes are the responsible components for the spirit aroma (De León-Rodríguez et al., 2006; Lachenmeier et al., 2006). When referring to the aroma of the mezcal, it is important to distinguish between the compounds extracted from i) the raw material (*Agave* plant), ii) those compounds produced by fermentation, and iii) the ones corresponding to the bouquet produced by the transformation of some compounds during the ageing. Throughout the fermentation, the yeast present influences the ester production and the formation of aroma. Furthermore, the higher alcohols are important precursors of the esters during the ageing process (Clemente-Jimenez et al., 2005).

Our results point out the fact that the presence of *Kluyveromyces marxianus* and *Pichia fermentans,* may possibly be the key organisms for the mezcal fermentation process. Furthermore, the higher alcohols produced by yeast are important precursors of the esters during the ageing process (Clemente-Jimenez et al., 2005). In some wines, the spontaneous fermentation drives the process. Then, the first period of aroma production is carried out by the so-called *non-Saccharomyces* strains, such as *Kloeckera, Hanseniaspora, Candida, Pichia, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Hansenula* and *Metschnikowia* (Rojas et al., 2001; Clemente-Jimenez et al., 2005). The aforementioned strains produce high concentrations of some compounds with a remarkable influence on the sensory quality of wine (Rojas et al., 2001; Clemente-Jimenez et al., 2005). It is known that *Hanseniaspora* and *Pichia sp*. promote the esterification of various alcohols such as ethanol, geraniol, isoamyl alcohol and 2-phenylethanol, increasing the concentrations of esters with a fruity aroma (Rojas et al., 2001). Conversely, *P. fermentans* has proved to be a good starter strains for must fermentation in the winemaking industry. This yeast has a high capacity to produce volatile compounds, which increase the aromatic properties of wine, although it has shown

a low capacity to produce ethanol (Clemente-Jimenez et al., 2005). In spirits like Mezcal, the presence and concentration of volatile compounds is crucial for the quality of the beverage (De León-Rodríguez et al., 2006). Another identified yeast was the *K. marxianus* that belongs to the GRAS (Generally Regarded As Safe) group of microorganisms (Medeiros et al., 2000). It has been reported previously that *K. marxianus* was used to produce aroma compounds such as monoterpene alcohols and isoamyl acetate (fruity aroma) in liquid fermentations. Thus, this could be also used for goals in industry. *Clavispora lusitaniae* has been recovered from some cacti in Southern Mexico and also occurs in fruits, *Agave*, effluents, and warm-blooded animals (Starmer et al., 2003). There is no information about the production of volatile compounds during the fermentation with *C. lusitaniae* alone*;* this could be because they do not appear naturally in grape must.

The restriction patterns of the 5.8S-ITS region in Figure 2 shows it is an easy and rapid method of identification of yeast. The confirmation of the identity of the yeast was performed by means of the alignment with redundant sequences in the database. On the other hand, the phylogenetic tree showed clearly the formation of three different branches and the bootstrap values of 96-100% validating the robustness of the branching pattern obtained.

Analyzing the PCR-amplified 16S rDNA sequences, it was found that several Lactic acid bacteria (LAB) species are the most abundant in the inoculum used for mezcal production. Lactic acid bacteria such as *L. plantarum* (facultative heterofermentative), *L. hilgardii,* and *L. kefir* (obligately heterofermentative LAB) have been reported as members of the normal microbiota present in fermentations carried out by a mixed yeast/bacteria microbial population (Van Beek et al., 2002; Simpson et al., 2001). The heterofermentative LAB such as *L. hilgarddi* and *L. kefir*, produce lactic acid, ethanol, acetic acid and carbon dioxide as main products, but also diacetyl, acetoin, 2-3 butanediol and formate in minor proportions depending on the oxidizing potential of the environment (Liu et al., 2003). Therefore, it is possible that these organisms have a negative impact on mezcal

flavor. The presence of *Lactobacillus pontis* was identified; it is a typical *Lactobacillus* isolated from endemic cereal fermentations (Müller et al., 2000). It is interesting to note that one of the identified microorganisms the *Weissella cibaria* belongs to the very recent detected species; it is a species with both genomic and phenotypic similarities to *Weissella confuse* (Björkroth et al., 2002). A common characteristic of both species that allows them to be distinguished from each other from *Weissella* species is the ability to grow at 45°C. *W. cibaria* had been isolated for the first time from Greek traditional wheat sourdoughs (Vuyst et al., 2002). *W. cibaria* and *W. paramesenteroides* have not been previously isolated from *Agave* fermented beverage.

Zymomonas mobilis is one of the few bacteria able to produce ethanol as final product by an almost quantitative conversion of glucose into ethanol and CO_2 via the Entner-Doudoroff pathway (Coton et al., 2005). Due to its ability to decouple growth and ethanol production and yields of up to 12% (w v^{-1}) ethanol, it has been proposed as a potential candidate for fuel ethanol production. (Buchholz et al., 1987; O'Mullan et al., 1995). *Z. mobilis* is responsible for the natural fermentations of sugar cane, *Agave* sap and palm sap (Swings and DeLey, 1977). Another biotechnological interest of this bacterium includes its production of acetaldehyde, a molecule often used as a flavor compound (Coton et al., 2005). The *Zymomonas* could be the responsible for the highest production of ethanol. This is supported by the fact that the identified yeast does not produce elevated concentrations of ethanol. Moreover, it has been previously proposed that *Z. mobilis* is an essential microorganism in the fermentation of *pulque* (Mexican beverage), along with yeast for ethanol production (Sanchez-Marroquín, 1967). In addition, several strains of *Z. mobilis* have been previously isolated from pulque samples and have been reported as high ethanol producers (Escalante et al., 2004).

The phylogenetic analysis of 16S rDNA and ITS rDNA sequenced clones showed that microbial diversity present in mezcal is dominated by bacteria, mainly LAB species and *Zymomonas mobilis.* This result could be interpreted as an indicator of

selective influences in the relative bacterial diversity induced by the must of *Agave* and the environment. It is important to note that the *L. pontis* and *Z. mobilis* species were the most abundant microorganisms present in the inoculum and that the presence of the yeast, *P. fermentans* and *K. marxianus* could be responsible for the contribution of the volatile compounds. Thus, a good selection of the starter cultures will improve the quality of this beverage. To the best of our knowledge, this work provides the first evidence of mixed cultures in the alcoholic fermentation of mezcal.

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References

Beuchat, L. (1993) Selective media for detecting and enumerating foodborne yeast. *Int J Food Microbiol* **19**, 1-4.

Björkroth, K.J., Schillinger, U., Gersen, R., Weiss, N., Hoste, B., Holzapfel, W.H., Korkeala, H.J. and Vandamme P. (2002) Taxonomic study of *Weissella confus*e and description of *Weissella cibaria* sp. nov detected in food and clinical samples. *Int J Syst Evol Microbiol* **52**, 141-148.

Buchholz, S.E., Dooley, M.M. and Eveleigh, D.E. (1987) *Zymomonas*: an alcoholic enigma. *Trends in Biotechnology* **5**, 199-204.

Cedeño, M. (1995) Tequila Production. *Crit Rev Biotechnol* **15**, 1-11.

Clemente-Jimenez, J.M., Mingorance-Carzola, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J. and Rodríguez-Vico, F. (2005) Influence of sequential yeast mixtures on wine fermentation. *Int J Food Microbiol* **98**, 301-308.

Coton, E. and Coton, M. (2003) Microbiological origin of "framboisé" in French ciders. *Journal of the Institute of Brewing* **109**, 299-304.

Coton, M., Laplace, J.M. and Coton, E. (2005) Zymomonas mobilis subspecies identification by amplified ribosomal DNA restriction analysis. *Lett Appl Microbiol* **40**, 152-157.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A. P., Escalante-Minakata, P. and López, M.G. (2006) Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana. J Agric Food Chem* **54**, 1337-1341.

De Vuys, L., Schrijvers, V., Paramithiotis, S., Hoste, B., Vancanneyt, M., Swings, J., Kalantzopoulos, G., Tsakalidou, E. and Messens, W. (2002) The biodiversity of lactic acid bacteria in greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. *Appl Environ Microbiol* **68**, 6059-6069.

Deák, T., Beuchat, L.R., Guerzoni, M.E., Lillie, A., Meter, G., Rohm, H., Schnürer, P.V., Tabajdi, P.V. and Westphal, S. (1998) A collaborative study on media for the enumeration of yeast in foods. *Int J Food Microbiol* **43**, 91-95.

Dojka, M.A., Hugenholtz, P., Haack, S. and Pace, N. (1998) Microbial diversity in a hydrocarbon and chlorinated solvent contaminanted aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**, 3869-3877.

Endo, A. and Okada, S. (2007) Lactobacillus farraginis sp. nov. and Lactobacillus parafarraginis sp. nov., heterofermentative lactobacilli isolated from a compost of distilled shochu residue. *Int J of Syst Evol Microbiol* **57**, 708-712.

Escalante, A., Rodríguez, M.E., Martínez, A., López- Murguía, A., Bolívar, F. and Gosset, G. (2004) Characterization of bacterial diversity in *Pulque*, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. *FEMS Microbiol Lett* **235**, 273-279.

Esteve-Zarzoso, B., Belloch, C., Uruburu F. and Querol, A. (1999) Identification of yeast by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* **49**, 329-337.

Fernandez-Espinar, M.T., López, V., Ramón, D., Bartra, E. and Querol, A. (2001) Study of the authenticity of comercial wine yeast strain by molecular techniques. *Int J Sys Bacteriol* **49**, 329-337.

Flores Berrios, E.P., Alba González, J.F., Arrizon Gaviño, J.P., Romano, P., Capece, A. and Gschaedler, A.M. (2006) The uses of AFLP for detecting DNA polymorphism, genotype identification and genetic diversity between yeast isolated from Mexican agave-distilled beverages and from grape musts. *Lett Apl Microbiol* **41**, 147-152.

Lachance, M.A. (1995) Yeast communities in natural tequila fermentation. *Antonie van Leeuwenhoeck* **68**, 151-160.

Lachenmeier, D.W.; Sohnius, E.M.; Attig, R.; López, M.G. (2006) Quantification of selected volatile constituents and anions in Mexican *Agave* spirits (Tequila, Mezcal, Sotol, Bacanora). *J Agric Food Chem* **54**, 3911-3915.

Las Heras-Vazquez, F.J., Mingorance-Carzola, L., Clemente-Jimenez, J.M., Rodríguez-Vico, F. (2003) Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. *FEMS Yeast Reserch* **3**, 3-9.

Liu, S.Q. (2003) Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *Int J Food Microbiol* **83**, 115-131.

López, V., Querol, A., Ramón, D. and Fernández-Espinar, M. (2001) Simplified procedure to analyze mitochondrial DNA from industrial yeast. *Int J Food Microbiol* **68**, 75-81.

Müller, M.R.A., Ehrmann, M.A. and Vogel R.F. (2000) Multiplex PCR for the detection of *Lactobacillus pontis* and two related species in a sourdough fermentation. *Appl Environ Microbiol* **66**, 2113-2116.

Peña-Alvarez, A., Diáz, L., Medina, A., Labastida, C., Capella, S. and Vera, L.E. (2004) Characterization of three *Agave* species by gas chromatography and solid-phase-gas chromatography-mass spectrometry. *J Chromatogr A* **1027**, 131-136.

Sánchez-Marroquín, A. (1967) Estudios sobre la microbiología del pulque XX. Proceso industrial para la elaboración técnica de la bebida. *Rev Lat Microbiol Parasit* **9**, 87-90.

Simpson, K.L., Pettersson, B. and Priest, F.G. (2001) Characterization of lactobacilli from Scotch malt whisky distilleries and description of *Lactobacillus ferintoshensis* sp. nov., a new species isolated from malt whisky fermentations. Microbiology **147**, 1007-1016.

Starmer, W.T., Schmedicke, R.A. and Lachance, M.A. (2003) The origin of the cactus-yeast community. *FEMS Yeast Research* **3**, 441-448.

Van Beek, S. and Priest, F.G. (2002) Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study. *Appl Environ Microbiol* **68**, 297-305.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In:PCR protocols. A guide to methods and applications (Eds: Innis, M.A., Gelfand, D.H., Sninsky, J. J., White, T.J.) Academic Press, San Diego, 315-322.

CONCLUSIONES

Los resultados del análisis cromatográfico de los destilados de *Agave,* revelaron la presencia de 105 compuestos volátiles. De los cuales once fueron clasificados como compuestos mayoritarios y el resto como minoritarios. El análisis multivariable permitió distinguir las diferentes bebidas alcohólicas de *Agave*. Las diferencias en las concentraciones fueron atribuidas a la comunidad de microorganismos, materia prima y a las condiciones de fermentación y destilación. Por otro lado se identificaron 17 compuestos volátiles específicos que podrían ser utilizados como marcadores de autenticidad de las bebidas.

El análisis de superficie de respuesta permitió la optimización de las variables de operación, temperatura y concentración inicial de azúcares. Los valores máximos del rendimiento, la producción de etanol y la productividad se obtuvieron a diferentes condiciones iniciales. Esto indica que no es posible optimizar las tres variables de respuesta simultáneamente.

El estudio cinético reveló que el crecimiento de los microorganismos, durante la fermentación, se ve afectado con el incremento en la concentración de azúcares. Los datos experimentales siguieron el comportamiento de una cinética de inhibición por sustrato.

El análisis de los compuestos volátiles obtenidos durante la fermentación del mezcal reveló que a 28 °C se obtiene la mayor cantidad de alcoholes superiores. Esto se observó independientemente de la concentración inicial de azúcares utilizada en el experimento. Por lo tanto, la temperatura afecta la producción de compuestos volátiles deseable en el proceso fermentativo del mezcal.

Los datos experimentales obtenidos de la medición del potencial redox presentaron un comportamiento gráfico inversamente proporcional al crecimiento

de la microbiota. Lo cual permitió establecer un criterio para finalizar la fermentación.

El análisis de la señal producida por el electrodo de redox y la implementación de una red neuronal artificial (RNA) permitió estimar la concentración de biomasa y etanol. A partir de estos datos se logró calcular los parámetros cinéticos de la fermentación. Por lo tanto, mediante la implementación de una RNA es posible conocer la concentración de biomasa y etanol en línea durante la fermentación.

La identificación molecular de la microbiota en el inóculo mostró que las bacterias acidolácticas se encuentran en mayor concentración respecto a los demás microorganismos.

La *Zymomona mobilis* fue la bacteria productora de etanol más abundante en el inóculo. Se identificó a *Pichia fermentans* y a *Kluyveromyces marxianus*, levaduras capaces de producir compuestos volátiles deseables en bebidas fermentadas.

El presente trabajo muestra la primera evidencia científica de un cultivo mixto en la fermentación del mezcal.

BIBLIOGRAFÍA

Abbott J.A. (1999) Quality measurement of fruits and vegetables. *Postharvest Biology and Technology* **15**, 207-225.

Amann R. I. (1995) Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology* **4**, 543-554.

Arthur C.L. y Pawliszyn J. (19909 Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry* **62**, 2145-2148.

Benn, S.M., Peppard T.L. (1996) Characterization of tequila flavor by instrumental and sensory analysis. *J Agric Food Chem* **44**, 557-556.

Berovič M., Mavri J., Wondra M., Kosmerl, T. y Dejan, B. (2003**)** Influence of temperature and carbon dioxide on fermentation of cabernet sauvignon must*. Food Technol Biotech* **41***,* 353-359.

Borneman J., Skroch P., OSullivan K., Palus J., Rumjanek N., Jansen J., Nienhus J. y Triplett E. (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl Environ Microbiol* June, 1935-1943.

Carro D. y Piña B. 2000.
Identificación de cepas de levadura de interés enológico ACE Revista de Enología CIENCIA http://www.acenología.com/ciencias52_1.htm

De León-Rodríguez A., González-Hernández L., Barba de la Rosa Ana P., Escalante-Minakata P. y López, M.G. (2006) Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana. J Agric Food Chem* **54***,* 1337-1341

Deák T., Beuchat L.R., Guerzoni M.E., Lillie A., Meter G., Rohm H., Schnürer P.V., Tabajdi P.V. y Westphal S. (1998) A collaborative study on media for the enumeration of yeast in foods. *Int J Food Microbiol* **43**, 91-95.

Díaz G. y Watcher C. (2003) Métodos para el estudio de comunidades microbianas en alimentos fermentados. *Revista Latinoamericana de Microbiología* **45**, 30-40.

Dojka M.A., Hugenholtz P., Haack S. y Pace N. (1998) Microbial diversity in a hydrocarbon and chlorinated solvent contaminanted aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**, 3869-3877.

Escalante A., Rodríguez M., Martínez A., López-Munguía F. Gosset G. (2004) Characterization of bacterial diversity in Pulque, a tradicional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. *FEMS Microbiol Letters* **235**, 273-279.

Escalante A., Wacher C., Farrés A. (2001) Lactic acid baterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. *Intl J Food Microbiol* **64**, 21-31.

Esteve-Zarzoso B., Belloch C., Uruburu F. y Querol A. (1999) Identification of yeast by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* **49**, 329-337.

Fujita S., Senda Y., Nakaguchi S. y Hashimoto T. (2001) Multiplex PCR using internal transcribed spacer 1 and 2 region for rapad detection of yeast strain. *J Clinical Microbiol* **39**, 3617-3622.

Garrett D., Dobson A., (2004) Potential of using real-time PCR-based detection of spoilage yeast in fruit juice- a preliminary study. *Int J Microbiol* **91**, 327-335.

Goff S.A. y Klee H.J. (2006) Plant Volatile Compounds: Sensory Cues for Health and Nutritional Value?. *Science* **311**, 815-819.

Granchi L., Bosco M., Messini y Vicenzini M., (1999) Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J Appl Microbiol* **87**, 949-956.

Harris D. C. *Análisis Químico Cuantitativo*, 3ª. Ed., Grupo Editorial Iberoamérica S.A. de C.V., EAU, 1992.

Las Heras-Vazquez F., Mingorance-Cazorla L., Clemente-Jimenez J., Rodríguez-Vico F. (2003) Identification of yeast species from orange fruti and juice by RFLP and sequence análisis of the 5.8S rRNA gene and the two internal transcribed spacers. *FEMS Yeast Research* **3**, 3-9.

López, M.G. (1999) Tequila aroma. In: Flavor Chemistry of Ethnic Foods, Shahidi & Ho, New York: Kluwer Academic/Plenum Publishers pp. 211-217.

Kataoka H., Lord Heather L. y Pawliszyn J. (2000) Applications of solid-phase microextraction in food analysis. *J Chromatogr A* **880**, 35-62.

Mexican Ministry of Commerce and Industry. Regulations: NOM-070-SCFI-1994. Alcoholic drinks-Mezcal Specifications; Diario Oficial de la Federación: México, November 28, 1994.

Nielsen J., Villadsen J. y Liden G. (2003) Bioreaction Engineering Principles. Kluwer Academic Plenum Publishers, USA.

Nyström T. (2003) Nonculturable bacteria: programmed survival forms or cells at death´s door?. *BioEssays* **25**, 204-211.

Orberá Ratón Teresa (2004) Métodos moleculares de identificación de levaduras de interés biotecnológico. *Revista Iberoamericana de Micología* **21**, 15-19.

Penderson, M.B. (1986) DNA sequence polymorphism in the genus Saccharomyces III. Restriction endonuclease fragment patterns of chromosomal regions in brewing and other yeast strains. *Carlsberg Research Community* **51**, 163-183.

Peña-Alvarez, A., Díaz, L., Medina, A., Labastida, C., Capella, S. and Vera, L.E. (2004) Characterization of three *Agave* species by gas chromatography and solid-phase-gas chromatography-mass spectrometry. *J Chromatogr A* **1027**, 131-136.

Querol A., Barrio E. y Ramón D. (1992) Molecular monitoring of wine fermentations conduced by active dry yeast strains. *Appl Environ Microbiol* **58**, 2948-2953.

Riu A.M. (2005) Caracterización de compuestos volátiles en bebidas derivadas de fruta. Tesis de grado de Doctor en Ciencias, Universidad de Barcelona, España.

Rivas R., Velázquez E., Zurdo-Piñeiro J.L., Mateos P. F. y Martínez M. E. (2004) Identification of microorganisms by PCR amplification and sequencing of a universal amplified ribosomal region present in both prokaryotes and eukaryotes. *J Microbiol Methods* **56**, 413-426.

Ruiz Hernández M. y Martínez Garoña M. (1997) *Curso popular de cata de vinos.* Edita: Gobierno de la Rioja, Consejería de Agricultura, Ganadería y Desarrollo Rural. España.

Sabate J., Cano J., Esteve-Zarzoso B. y Guillamón J. (2002) Isolation and identification of yeast associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiology Research* **157**, 1-8.

Torija M.J., Rozes N., Poblet M., Guillamon J.M. y Mas A. (2001) Yeast population dynamics in spontaneous fermentations: Comparison between two different wine-producing areas over a period of three years. *Antonie van Leeuwenhoek* **79**, 345-352.

Torija Martínez M.J. (2002) Ecología de levaduras selección y adaptación a fermentaciones vínicas. Universitat Rovira I Virgili. Departament de Bioquímica I Biotecnología. Facultad d´Enologia.

Vallejo-Córdoba, B., González-Córdoba, A.F., Estrada-Montoya, M.C. (2004) Tequila volatile characterization and ethyl ester determination by solid phase microextraction gas chromatography/mass spectrometry analysis. *J Agric Food Chem* **52**, 5567-5571.

Vasdinyei R. y Deák T. 2003. Characterization of yeast isolates originating from Hungarian dairy products using tradicional and molecular identification techniques. Internacional *Journal Food Microbiol* **86**, 123-130.

Wade L.G., *Química Orgánica*., 2ª. Ed., Prentice-Hall Hispanoamericana S.A., 1993.

White, T.J., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In:PCR protocols. A guide to methods and applications (Eds: Innis, M.A., Gelfand, D.H., Sninsky, J. J., White, T.J.) Academic Press, San Diego, 315-322.

I. ANÁLISIS CROMATOGRAFICO DE LOS DESTILADOS DE AGAVE

1. Compuestos volátiles mayoritarios

El análisis se realizó en un Cromatógrafo de gases, modelo 6890N de Agilent Technologies provisto de un puerto de inyección capilar split/splitless, con detector de ionización de llama (FID), torreta de inyección automática marca Agilent modelo 7683 con capacidad para 8 viales (viales de rosca con septum de 1.5 mL), columna capilar Agilent HP-INNOWAX (Polietilenglicol), 30 m de longitud nominal, 0.25 mm de diámetro nominal y 0.50 µm de espesor de película y jeringa Agilent de 10 µl.

- *Condiciones de operación del equipo*

Inyector (split/splitless) a una temperatura de 220 ºC, razón de split (10:1), gas acarreador helio 1.5 mL/min (flujo constante), programa de temperatura: 35 ºC (2 min), incremento a una tasa de 10ºC/min hasta 210 ºC. El tiempo total de análisis fue de 20.5 min y la temperatura del detector (FID) 250 ºC.

- *Preparación de la muestra*

Se toman 8 mL (destilado de Agave) y se coloca en un matraz volumétrico de 10 mL, se agrega 1 mL de la solución del estándar interno (2-Pentanol, 350 mg/100 mL de etanol al 40% v/v) y se afora a 10mL con etanol al 40% v/v. Se introduce en el automuestreador del cromatógrafo de gases, el cual toma 1µL de muestra. Los resultados se expresan en mg/100 mL de alcohol anhidro.

2. Compuestos volátiles minoritarios

Cromatógrafo de gases, modelo HP 6890N (Agilent Technologies), acoplado al detector de espectrometría de masas HP 5973N (Agilent Technologies), biblioteca NIST (Nacional Institute of Standard Tests), columna capilar DB-WAX, 30 m de longitud, 0.32 mm de diámetro y 0.53 µm de espesor de película. Viales de rosca con septum, capacidad de 4 mL. Dispositivo para Microextracción en fase

sólida (MEFS) con una fibra de 65 µm de Carbowax/Divinylbenzene (CW/DVB)- (naranja) y Carboxen/Polydimethylsiloxane (CAR/PDMS)- (negra)

- *Condiciones de operación del equipo*

Las condiciones de operación fueron: Helio como gas acarreador 1 mL/min; programa de temperatura 40 ºC (3min) incremento a una tasa de 3ºC/min hasta 120 ºC y de 6ºC/min hasta 200 ºC; splitless; las temperaturas para el inyector y detector fueron 180 y 230 ºC, respectivamente.

- *Preparación de la muestra*
    1. Se toman 2 mL del destilado, sin tratamiento previo ni alteración alguna, se colocan en un vial de 4 mL e inmediatamente se tapa.
    2. Las muestras se incuban por 2 horas a temperatura ambiente.
    3. Se expone la fibra por una hora.
    4. La fibra se retrae saca del vial y se introduce inmediatamente en puerto de inyección durante 30 segundos.

II. METODOLOGÍA PARA LA FERMENTACIÓN DE MEZCAL

- **Fermentación en matraz**

1. El mosto e inóculo se mantienen en matraces erlenmeyer de 1000mL dentro de un baño de agua a temperatura constante según se desee.
2. Se miden con ayuda del refractómetro los º Brix del mosto y del inóculo.
3. Se estandarizan los º Brix del mosto y del inóculo a los que se desea trabajar en la fermentación por medio de la ecuación: $C_1V_1=C_2V_2$ donde C_1= º Brix del mosto, V_1=Volumen de mosto total requerido en la fermentación, C_2= º Brix requeridos en la fermentación y V_2=Volumen del mosto.
4. Se toma una muestra de 5 mL de inóculo se centrifuga a 3500 rpm por 10 min, se retira el sobrenadante, la pastilla se enjuaga con agua destilada y se resuspende. Se mide la absorbancia en el espectro UV-vis a 600nm.
5. Se realiza la dilución para obtener una concentración inicial de 0.1 con el mosto estandarizado. Volumen total de 1000mL.
6. Se adiciona 1g/L de sulfato de amonio.
7. Los matraces se colocan inmediatamente en el baño de agua y se mantienen a temperatura constante.
8. Durante toda la fermentación se toman muestras de 5 mL y se procesan como se explicó en el paso 4. Solo que en este caso el sobrenadante se congela para análisis futuros de azucares reductores y etanol.
9. La fermentación termina cuando la espuma “cae” es decir, cesa la producción de CO_2
10. El líquido fermentado se etiqueta y congela para posteriormente ser destilado.

- **Determinación de azúcares reductores DNS**

1. Preparación de soluciones

   - Solución DNS: Para preparar 100mL pesar 1 g de hidróxido de sodio, 1 g de ácido dinitrosalicílico, 20 g de tatrado de sodio y potasio, 0.05g de metabisulfito de sodio, 0.2 g de fenol. Se disuelven todos los reactivos excepto el ácido en 60 mL de agua destilada. Se agrega el ácido en pequeñas cantidades hasta lograr la disolución se afora y se guarda en un frasco forrado ya que la solución es sensible a la luz

   - Solución patrón de glucosa 1g/L: Se pesan 0.1 g de glucosa y se disuelve en un poco de agua destilada, finalmente se afora a 100mL.

2. Curva patrón de glucosa: Se preparan concentraciones de glucosa de 0.1 a 1.0 g/L.

3. Preparación de la muestra: Se centrifuga la muestra durante 20 min a 6000 rpm, y se prepara una dilución 1:100.

4. Se toman 0.5 mL de cada estándar o muestra y se agrega 1.5 mL de solución de DNS. La mezcla se agita y calienta a baño maría (hirviendo) durante 15 min. Se agregan 8 mL de agua destilada, se agita y se mide la absorbancia.

- **Determinación del porcentaje de alcohol**

El análisis se realiza en un Cromatógrafo de gases con FID (descrito anteriormente). Se prepara una curva estándar de etanol en acetona al 10% como estándar interno. Los estándares y las muestras se tratan de la misma forma.

- *Condiciones de operación del equipo*

Inyector (split/splitless) a una temperatura de 220 ºC, razón de split (25:1), gas acarreador helio 1.5mL/min (flujo constante), programa de temperatura: 35 ºC (2 min), incremento a una tasa de 10ºC/min hasta 80 ºC. El tiempo total de análisis fue de 6.5 min, post-análisis 100 ºC (2 min); temperatura del detector (FID) 250 ºC.

- *Preparación de la muestra*

1. En un tubo falcon se coloca 1mL de mosto fermentado y 1 mL de n-butanol
2. Se agita por 5 minutos en un vortex.
3. Posteriormente se centrifuga durante 10 min a 6000 rpm. La fase orgánica se filtra y se reserva.
4. Se toman 100 µL del filtrado y se agregan 250 µL de la solución patrón de acetona al 10%. Se afora con agua destilada a 10 mL. Un volumen de aprox. 1mL se coloca en un vial y se introduce en el automuestreador del cromatógrafo de gases. Los resultados se expresan en g/L de etanol.

III. IDENTIFICACIÓN DE MICROORGANISMOS EN MEZCAL

- **Aislamiento y selección de bacterias y levaduras**

El inóculo obtenido de la mezcalera se centrifuga y se almacena la pastilla con glicerol a -80°C hasta su uso. Posteriormente la muestra se diluye en solución salina (NaCl, 0.85% p/v) y se siembra en los diferentes medios de cultivo.

Medios de cultivo fórmula por litro

- **TYGC**: 30g triptona, 10g levadura de cerveza, 20g glucosa, 15g agar y 100mg/l del antibiótico cloranfenicol.

- **MRS**: 10g peptona No. 3, 10g extracto de res, 5g extracto levadura, 20g dextrosa, 1g polisorbato 80, 2g citrato de amonio, 5g acetato de sodio, 0.1g sulfato de magnesio, 0.05g sulfato de manganeso, 2g fosfato dipotasico y 15g de agar.

- **MYGP**: 3g extracto de malta, 3g extracto de levadura, 10g, peptona 5g, agar 15g, actidione 20mg/l y etanol al 3%.

- **PD**: 4g almidón de papa, 20g dextrosa y 15g agar.

- **AA**: 20g caseína, 5g cloruro de sodio,10g dextrosa, 20g agar, 2g tioglicolato de sodio, 1g formaldehído sulfoxilado y 0.002g de azul de metileno.

- **PCA**: 5g triptona, 2.5g extracto de levadura, 1g dextrosa y 15g de agar.

- **MA**: Adicionar asépticamente jugo de agave filtrado al agar de papa dextrosa. (Concentración final de 5° Brix).

Las placas se incuban durante 48 horas, a 30°C para las bacterias y 28°C para las levaduras. Se seleccionan tres colonias que tengan las mismas características morfológicas. Se purifica la colonia resembrando a las mismas condiciones, medio de cultivo y temperatura (Beuchat 1993; Deák *et al*. 1998).

- **Extracción de DNA (perlas de vidrio)**

1. Sembrar una colonia de la bacteria seleccionada en un tubo con 5 mL del mismo medio en que se aisló.
2. Incubar durante 48 horas a 30°C.
3. Centrifugar durante 5 minutos a 6000 rpm.
4. Lavar la pastilla con agua destilada.
5. Resuspender con 200μl de mezcla de extracción (2% triton, 1% SDS, 10mM NaCl, 10mM TRIS-HCl pH 8, USB, Cleveland, USA), EDTA 1mM, adicionar 200μl de fenol:cloroformo:alcohol isoamílico (25:24:1), 0.06g de 0.5-mm perlas de vidrio y 100μL de agua destilada estéril.
6. Homogeneizar durante un minuto 8 veces, enfriando en hielo durante 1 minuto antes de volver a agitar.
7. Centrifugar 5 minutos a 13 000 *rpm* a 4°C.
8. Transferir el sobrenadante a un tubo limpio y adicionar un volumen de cloroformo puro, mezclar cuidadosamente.
9. Centrifugar durante 5 minutos a 13 000 *rpm* a 4°C.
10. Trasferir la fase acuosa a un tubo limpio con 20μg/mL de RNAasa e incubar durante 1 hora a 37°C.
11. Adicionar 1 volumen de cloroformo:alcohol isoamílico 24:1, Centrifugar nuevamente.

12. Transferir la fase acuosa a un tubo limpio con 10μL de isopropanol y 4μL de acetato de amonio (4M), mezclar cuidadosamente e incubar durante 15 minutos a temperatura ambiente.
13. Centrifugar 10 minutos a 13 000 *rpm* a 4°C.
14. Retirar cuidadosamente la fase acuosa y lavar el DNA con etanol al 70% v/v, dejar secar y resuspender en 30μL de TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

- **Extracción de DNA (Zymolyasa)**

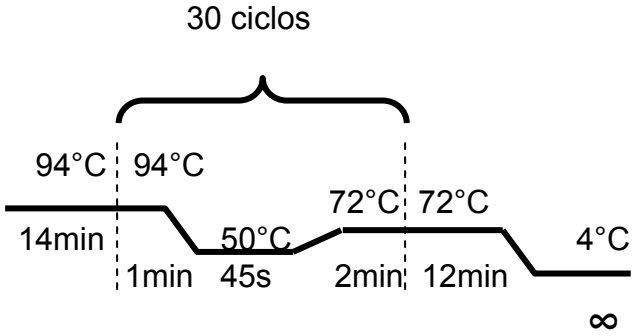
1. Inócular 5ml de caldo TGYC con una colonia de levaduras e incubar durante 48 horas.
2. Centrifugar a 6000 *rpm* durante 5 minutos, lavar la pastilla con agua destilada estéril.
3. Resuspender en 500μL de 0.9M sorbitol (Sigma), 0.1M EDTA pH 7.5 y 40μL de 2.5mg/mL de la enzima Zymolyasa 20T (Seikagaku, Tokyo, Japan).
4. Incubar los tubos a 37°C durante 20 minutos, después centrifugar durante 5 min a 7000 *rpm*.
5. Resuspeder los esferoplastos en 500μL de 50mM TRIS-HCl, 20mM EDTA pH 7.4 y adicionar 2.5μL de 10% w/v SDS, incubar a 65°C durante 30 minutos.
6. Inmediatamente después adicionar 200μL de 5M acetato de potasio y colocar los tubos sobre hielo de 1 a 2 horas.
7. Centrifugar a 13000 *rpm* a 4°C durante 20 minutos.
8. Adicionar 1 volumen de isopropanol e incubar durante 15 minutos a temperatura ambiente para precipitar el DNA.
9. Centrifugar durante 10 minutos a 13000 *rpm* y 4°C.

10. Lavar el DNA con etanol al 70% v/v, secar (haciendo pasar una corriente de nitrógeno) y disolver en 30μL de TE (10mM Tris-HCl, 1mM EDTA pH 7.5).

- **Condiciones para amplificar la región del 16S rDNA en bacterias**

Oligos: 533F 5'- GTG CCA GCA GCC GCG GTA A -3'
1492R 5'- GGT TAC CTT GTT ACG ACT T -3'
Dojka *et al.* 1998

DNA	2μL
dNTP's mezcla 2.5mM	8μL
Taq Polymerasa 5U Invitrogen, Carlsbad, CA	0.25μL
Buffer 10X	5μL
$MgCl_2$ 25mM	5μL
Oligos 100pM Invitrogen, Carlsbad, CA	1.0μL c/u
Agua	27.75μL
Volumen total	50μL

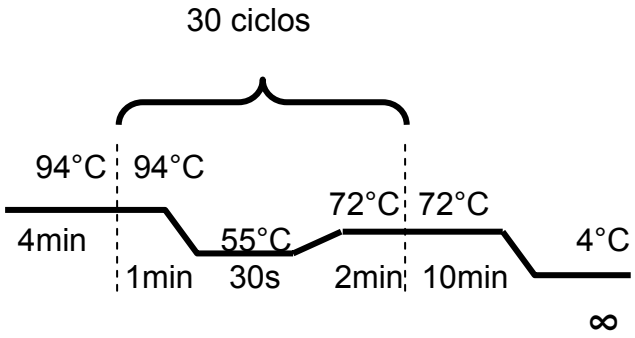


Los productos de PCR se corren en una cámara de electroforesis 80V, en un gel de agarosa al 1.5% con TAE (40mM Tris-acetate, 1mM EDTA) usando un marcador de peso molecular para 100pb. El gel se tiñe en una solución de bromuro de etidio y se visualiza la banda en un transiluminador.

- **Condiciones para amplificar la región del ITS rDNA en levaduras**

Oligos: ITS1 5'- TCC GTA GGT GAA CCT GCG-3'
ITS4 5'- TCC TCC GCT TAT TGA TAT GC -3'
White *et al.* 1990.

DNA	2μL
dNTP's mezcla 2.5mM	8μL
Taq Polymerasa 5U Invitrogen, Carlsbad, CA	0.25μL
Buffer 10X	5μL
$MgCl_2$ 25mM	5μL
Oligos 100pM Invitrogen, Carlsbad, CA	1.0μL c/u
Agua desionizada estéril	27.75μL
Volumen total	50μL



Los productos de PCR se corren en una cámara de electroforesis 80V, en un gel de agarosa al 1.5% con TAE (40mM Tris-acetate, 1mM EDTA) usando un marcador de peso molecular para 100pb. El gel se tiñe en una solución de bromuro de etidio y se visualiza la banda en un transiluminador.

- **Clonación**

Se realiza siguiendo las condiciones propuestas por Invitrogen para el vector pCR 4-TOPO (vector kit sequencing). Se utilizan las células competentes *E. coli,* One shot TOP10 cells incluidas en el kit de clonación.

1. Para la transformación de las células se usan 2 μL de la reacción de ligación.
2. Las transformantes se siembran en platos con medio LB-ampicilina.
3. Para identificar las clonas positivas se realiza el PCR de una colonia utilizando un oligo del vector y otro de la región amplificada.

- **ARDRA**

Un volumen de 15μL de cada producto de PCR (ITS y 16S rDNA) se digiere con 1μL de la enzima de restricción *HaeIII* (New England Biolabs Ltd., Hertfordshire, England), 2μL de buffer y 2μL de agua estéril. La mezcla se incuba durante 2 horas a 37°C, después se inactiva durante 20 min a 80°C.

Los fragmentos son separados en una cámara de electroforesis 80V con TAE 1X, en un gel de agarosa al 3% (w/v). Posteriormente se tiñen con bromuro de etidio y se visualizan las bandas en el transiluminador.