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DEGRADACION DE AMINAS BIOGENAS MEDIANTE SISTEMAS MICROBIANOS. IDENTIFICACION Y CARACTERIZACION DE LAS ENZIMAS RESPONSABLES.

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Valencia, Septiembre de 2016

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sois todo para mí, os quiero infinitamente

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Índice

Abreviaturas

AB	Aminas biógenas
ABTS	2,2'-azinobis (ácido 3-etilbenzotiazolina-6-sulfónico)
ADN	Ácido desoxirribonucleico
ADI	Arginina deiminasa
AQC	6-aminoquinolil-N-hidroxisuccinimidil carbamato
AO	Amino oxidasas
ATP	adenosín trifosfato
BA	Bacterias acéticas
BAL	Bacterias lácticas
BLAST	"Basic Local Alignment Search Tool" o Herramienta de búsqueda de alineamientos
BSA	"Bovine Serum Albumin" o Seroalbúmina Bovina
CAO	amino oxidasa dependiente de cobre
CECT	Colección Española de Cultivos Tipo
DAO	diamina oxidasa
DAB	diaminobencidina
DMP	2,6-dimetoxifenol
DO	Densidad óptica
EDC	1-etil-3-(3-dimetilaminopropil)carbodiimida
EDTA	Ácido etilendiaminotetraacético
ELISA	ensayo por inmunoadsorción ligado a enzimas o *"Enzyme-Linked ImmunoSorbent Assay"*
EPR	resonancia paramagnética electrónica
FA	Fermentación alcohólica
FAD	flavín adenín dinucleótido
FAD-AO	amino oxidasa dependiente de flavina
FML	Fermentación maloláctica

HBT	hidroxibenzotriazol
HPLC	"High Performance Liquid Chromatography" o Cromatografía líquida de alta resolución
HRP	"horseradish peroxidase" o peroxidasa de rábano picante
IMAO	inhibidores de la monoamino oxidasa
K.	*Kocuria*
L.	*Lactobacillus*
MALDI	**"***Matrix-Assisted Laser Desorption/Ionization"* desorción/ionización láser asistida por matriz
MALDI-TOF	Técnica MALDI acoplada a un detector de iones o *"Time-Of-Flight"*
MAO	monoamino oxidasa
MCO	multicobre oxidasa
MS	*"Mass spectometry"* o espectrometría de masas
NADH	nicotin adenin dinucleótido
O.	*Oenococcus*
OPA	ortoftaldehído
P.	*Pediococcus*
PAGE	electroforesis en gel de poliacrilamida
PAO	poliamina oxidasas
pb	pares de bases
PCR	"Polymerase Chain Reaction" o Reacción en cadena de la polimerasa
SCSIE	**"**Servei Central de Support a la Investigació Experimental, Universitat de València"
SDS	"Sodium Dodecyl Sulfate" o Dodecil sulfato Sódico
SO_2	Anhídrido sulfuroso
UV	ultravioleta
V	voltios

Resumen

Las aminas son compuestos ampliamente presentes en la naturaleza que contienen uno o varios sustituyentes unidos a un átomo de nitrógeno. Son compuestos endógenos de las plantas que también pueden encontrarse en frutas frescas y verduras. Sin embargo, en los alimentos, las aminas se forman fundamentalmente en los procesos fermentativos, y durante el envejecimiento y la conservación. Las aminas biógenas (AB) se originan por la descarboxilación microbiana de los correspondientes aminoácidos precursores, por ello se denominan 'biógenas'. Entre las AB, las más estudiadas son la histamina y la tiramina por sus implicaciones negativas para la salud. Están presentes en numerosos alimentos tales como queso, vino, cerveza, vegetales, pescado y carnes rojas, entre otros, y son consecuencia de un proceso normal de fermentación o de una alteración microbiana.

Las AB más comunes y con mayor prevalencia en alimentos son la histamina, tiramina y putrescina entre otras. La importancia del estudio de las AB en la industria alimentaria se debe a 2 razones principales, en primer lugar, si se ingieren en grandes cantidades pueden inducir efectos tóxicos. Por ejemplo, el consumo de alimentos con altos niveles de tiramina e histamina se asocia con desórdenes fisiológicos, tales como sensación de ardor en la garganta, rubor, dolor de cabeza, náuseas, hipo o hipertensión arterial, adormecimiento y hormigueo de los labios, pulso rápido y vómitos. La histamina produce, además, reacciones pseudoalérgicas. En segundo lugar, ciertas AB están implicadas en la formación de nitrosaminas carcinogénicas. Finalmente, el nivel de AB puede constituir un buen indicador químico de calidad o del grado de descomposición de los alimentos, como han sugerido varios investigadores. Si bien la histamina es la amina biógena que presenta un mayor potencial tóxico seguida por la tiramina, la presencia de otras AB, como la putrescina o la cadaverina, puede potenciar sus efectos tóxicos. En circunstancias normales el organismo humano es capaz de degradar las AB ingeridas con los alimentos mediante varias enzimas: monoamino oxidasa (MAO, EC 1.4.3.4), diamina oxidasa (DAO, EC 1.4.3.6), y poliamina oxidasa (PAO, EC 1.5.3.11). Pero se debe tener en cuenta que estos mecanismos de desintoxicación pueden no ser capaces de eliminar adecuadamente las AB ya que su efecto puede verse limitado debido a distintos factores: genéticos, alérgicos, ingesta excesiva de aminas, o consumo de medicamentos inhibidores de algunas de estas enzimas, como es el caso de los antidepresivos que inhiben a las MAO. Además, factores como el consumo de bebidas alcohólicas pueden aumentar el potencial tóxico de las AB, al favorecer el transporte de las mismas a través de la pared intestinal. La alta concentración de AB satura las enzimas degradadoras lo que impide que estas desaparezcan totalmente del organismo.

La síntesis de AB es llevada a cabo por enzimas específicas, llamadas descarboxilasas. El proceso de descarboxilación enzimática depende de varios factores tales como la disponibilidad de aminoácidos en forma libre, la presencia de microorganismos productores de enzimas descarboxilasas y la existencia de condiciones adecuadas en el sustrato para que

los microorganismos crezcan y lleven a cabo la descarboxilación. Los aminoácidos precursores o están presentes de manera natural en el alimento crudo o son producidos vía proteólisis por proteasas endógenas de los mismos o por proteasas excretadas por los microorganismos. Los microorganismos productores de descarboxilasas pueden formar parte de la microbiota natural presente en los alimentos, de los cultivos iniciadores utilizados para su fermentación o de la microbiota alterante.

El vino es el producto de fermentación del mosto de uva en el que las levaduras fermentan los azúcares para formar etanol mediante la fermentación alcohólica. La mayoría de vinos se someten a una segunda fermentación (la fermentación maloláctica, FML), que consiste en la transformación del ácido málico en ácido láctico. Este proceso disminuye la acidez del vino y mejora su sabor. La FML es llevada a cabo por bacterias lácticas (BAL) y la especie que generalmente se asocia a la misma es *Oenococcus oeni*, aunque también se han descrito otras como responsables de la misma (*Lactobacillus plantarum*, y algunas especies de *Pediococcus*). En el vino se han identificado más de 20 AB diferentes, algunas como la putrescina y espermidina pueden estar presente en la uva, mientras que estas dos y otras se pueden formar durante la fermentación. En el vino, las AB más comunes son histamina, tiramina, putrescina, cadaverina, espermina y espermidina. Los microorganismos responsables de la formación de AB durante la fermentación son las BAL. Se ha descrito que *Oenococcus oeni* es la especie con un mayor porcentaje de cepas (80%) que poseen el gen *hdc*, que codifica para la enzima responsable de la síntesis de histamina. Afortunadamente, estas cepas suelen tener actividades de histidina descarboxilasa bajas y no contribuyen demasiado a aumentar la cantidad de histamina en el vino. Por el contrario, algunas cepas *Pediococcus parvulus* y *Lactobacillus hilgardii* pueden llegar a producir entre 40 y 50 mg/L en el vino. Algunos lactobacilos y leuconostocs, también pueden producir cantidades intermedias de histamina. Se han encontrado cepas de *Lactobacillus brevis* y *L. hilgardii* que producen tiramina, mientras que se ha descrito la síntesis de putrescina en cepas de *O. oeni*, *L. hilgardii* y *Leuconostoc mesenteroides*. En el caso de la putrescina y la cadaverina, el problema no es tanto sanitario como organoléptico, ya que estas aminas provocan aromas desagradables. La putrescina provoca el aroma a putrefacción, y la cadaverina a trapo de cocina sucio entre otros. En los vinos la presencia de estas dos AB disminuye la percepción de los aromas propios del vino.

La concentración de AB en el vino es menor que en otros alimentos fermentados. Su contenido varía desde trazas hasta niveles de 130 mg/L. Sin embargo, pese a estar en menor concentración que en otros alimentos, su toxicidad es mayor debido a que el alcohol potencia la toxicidad de las AB. A nivel comercial, la presencia de AB en los vinos compromete la exportación de los mismos a aquellos países que han impuesto límites para la concentración de histamina en los vinos. Las acciones preventivas para evitar la presencia de AB en el vino

serían la limitación del nivel de aminoácidos precursores, la inhibición del crecimiento de las bacterias nativas y el uso de cultivos iniciadores malolácticos que carezcan de enzimas descarboxilásicas. La limitación del nivel de precursores se consigue disminuyendo el tiempo de maceración del mosto con los hollejos, evitando la adición de N_2 orgánico para potenciar el crecimiento de las levaduras y evitando el contacto con las lías. El control del desarrollo de las bacterias nativas se aborda mediante el uso del dióxido de azufre (SO_2), el agente antimicrobiano más utilizado en las bodegas. La utilización de cultivos iniciadores malolácticos no productores de AB evitan que aumenten los niveles de las mismas durante la FML y previene el desarrollo posterior de bacterias autóctonas que puedan sintetizarlas. Sin embargo, algunas de estas estrategias no se pueden aplicar al vino o tienen una eficacia limitada, como ocurre con el uso de SO_2. Estas circunstancias, unidas al hecho de que las enzimas descarboxilasas pueden continuar activas en el vino incluso cuando la bacteria está muerta hacen que las medidas preventivas no sean suficientes para eliminar las aminas del vino. Se requiere por tanto del desarrollo de estrategias paliativas que puedan ser aplicables cuando las preventivas no han conseguido disminuir suficientemente la concentración de AB en vino.

El objetivo principal de esta Tesis es la búsqueda de estrategias para la eliminación de AB mediante el uso de sistemas microbianos o enzimáticos. Este objetivo general se desglosa en los siguientes objetivos específicos:

1. Búsqueda de cepas bacterianas de origen alimentario, capaces de degradar aminas biógenas.

2. Aislamiento, purificación e identificación de las enzimas responsables de la degradación de aminas biógenas. Determinación y caracterización de la actividad de la enzima purificada de *K. varians* LTH 1540.

3. Clonaje y la caracterización de las enzimas degradadoras de aminas biógenas de *Lactobacillus plantarum* J16 CECT 8944 y de *Pediococcus acidilactici* CECT 5930.

4. Aplicación de sistemas biológicos para degradar aminas biógenas en vinos.

Para seleccionar bacterias capaces de degradar aminas, se obtuvieron extractos celulares de las mismas mediante disrupción mecánica y se investigó la presencia de enzimas degradadoras de AB en los mismos mediante un ensayo en gel de poliacrilamida. Las proteínas separadas de esta manera se hacían reaccionar en un tampón fosfato que contenía una mezcla de histamina, tiramina y putrescina, peroxidasa de rábano (HRP) y diaminobenzidina (DAB). De los 77 extractos de BAL probados, 40 de ellos (el 52,6 % de las mismos) y el de *Kocuria varians* dieron reacción positiva frente a la mezcla de las tres aminas. Las cepas que presentaron una reacción positiva frente a las aminas pertenecían a las especies: *Enterococcus faecium, Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus hilgardii, Lactobacillus mali, Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus vini, Pediococcus acidilactici, Pediococcus parvulus y Pediococcus pentosaceus,* además de *Kocuria varians.* Cuando en el tampón de reacción del ensayo en gel se sustituyeron las AB por 2,6-dimetoxifenol (DMP), un sustrato típico de las lacasas, el número de extractos que mostraron una reacción positiva fue de 47; es decir, 7 extractos actuaron sobre el DMP pero no dieron reacción con las aminas. Todos los extractos que mostraron actividad sobre las aminas también lo hicieron sobre el DMP, excepto los de *E. faecium* y el de *K. varians*.

A partir de estos resultados se analizó el comportamiento de las cepas cuyos extractos dieron una reacción más rápida e intensa en el gel tanto en medio de cultivo como en vino. De las 14 cepas seleccionadas, 13 pertenecían al grupo de las bacterias lácticas y procedían de cereales, vinos, salchichas, y pasta fermentada. La otra cepa pertenecía a la especie *K. varians* y procedía de productos cárnicos fermentados. Todas las cepas ensayadas degradaron dos o más aminas tanto en medio sintético como en vino, aunque con diferentes eficiencias, a excepción de *K. varians* LTH 1540 que degradó solo la putrescina. Las cepas que degradaron AB pertenecían a las especies: *Enterococcus faecium*, *Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus hilgardii, Lactobacillus mali, Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus vini, Pediococcus acidilactici, Pediococcus parvulus y Pediococcus pentosaceus*, además de *Kocuria varians.*

Todas las cepas de *Lactobacillus plantarum* y las cepas *Pediococcus acidilactici* CECT 5930 y *Lactobacillus delbrueckii* CECT 286 degradaron histamina, tiramina y putrescina en los dos medios, mientras que, *Lactobacillus farciminis* CRL 678 y *Enterococcus faecium* C1 sólo degradaron dos aminas, esta última cepa únicamente en medio sintético. *Lactobacillus paracasei* ENOLAB Lb 444 únicamente degradó histamina en medio sintético y *Kocuria varians* LTH 1540 la putrescina, tanto en medio como en vino. Las cepas más degradadoras de AB en vino fueron: *Lactobacillus plantarum* J16, Lb 98, Lb 132, Lb 291 y la cepa *Pediococcus acidilactici* CECT 5930, que degradaron histamina, putrescina y tiramina, seguidas de

Lactobacillus farciminis CRL 678 que actuó sobre tiramina y putrescina y, finalmente, *Kocuria varians* LTH 1540.

Por otro lado con este experimento se observó que la capacidad para degradar AB era un carácter dependiente de cepa en las especies *Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus hilgardii*, *Lactobacillus mali, Lactobacillus paracasei, Lactobacillus vini, Pediococcus parvulus y Pediococcus pentosaceus,* mientras que todas las cepas de *Lactobacillus plantarum* presentaron capacidad para degradarlas.

Se seleccionaron 3 de las cepas más degradadoras (*L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540) para llevar a cabo la identificación de las enzimas responsables de la degradación de dichas aminas, para ello se purificaron las enzimas y se enviaron al servicio de proteómica de la Universitat de València para su análisis proteómico por MALDI-TOF MS. La purificación se llevó a cabo en tres pasos, primero se realizó un fraccionamiento con sulfato amónico, seguido de una cromatografía de intercambio aniónico en las tres cepas, en el caso de *L. plantarum* se requirió además una cromatografía de intercambio catiónico adicional. El tercer paso consistió en aplicar las enzimas purificadas en un gel de poliacrilamida en condiciones nativas. Las proteínas responsables de la degradación de aminas se revelaron por la reacción coloreada que se producía en el tampón de reacción en presencia de las tres aminas, peroxidasa de rábano y DAB. Una vez revelada la banda se extrajo del gel y se aplicó en otro gel SDS-page para conseguir un mayor grado de separación. Tras el revelado de esta segunda electroforesis mediante el procedimiento anterior, se escindió la banda coloreada para llevar a cabo su identificación. La concentración de proteína obtenida de las purificaciones de *L. plantarum* J16 fue 0.25 mg/mL, de la de *P. acidilactici* 11.5 mg/mL y de la de *K. varians* 2.8 mg/mL.

Para llevar a cabo los análisis proteómicos, se realizaron digestiones con tripsina y los péptidos resultantes se identificaron mediante MALDI-TOF MS. El análisis de los péptidos significativos, llevado a cabo por el programa MASCOT, mostró que los péptidos obtenidos de la proteína aislada de *L. plantarum* J16 CECT 8944 coincidían con los de la proteína de división celular SufI, cuyo número de acceso es C6VK53. La secuencia de los péptidos cubría el 54 % de la secuencia aminoacídica de dicha proteína. La proteína SufI está clasificada en la base de datos “The laccase engineering database” (LccED) como una multicobre oxidasa (MCO) perteneciente a la subfamilia J (CueO bacteriana). Los péptidos obtenidos por digestión trípsica de la proteína aislada de *P. acidilactici* CECT 5930 cubrían un 36 % de la secuencia completa de una proteína identificada como una presunta MCO cuyo número de acceso es D2EK17. El análisis por MALDI-TOF MS de los péptidos de la proteína de *K. varians* LTH 1540 mostró que se trataba de una proteína ortóloga de la putrescina oxidasa (PuO) de las especies *K. rosea* y *K. rhizophila,* cercanas filogenéticamente a *K. varians*. Por lo tanto, la PuO de *K. varians* se clasificaría en el grupo de enzimas EC 1.4.3.10. En este caso, además, se corroboró

la identidad de la proteína mediante identificación, por espectrometría de masas, del producto de reacción de la putrescina oxidasa: la Δ^1-pirrolina. La aparición de este compuesto indica que la enzima que cataliza la reacción es una putrescina oxidasa dependiente de flavina (PuO).

En los dos primeros casos se trataba de un tipo de enzimas que no se había relacionado previamente con la degradación de AB. En el caso de *K. varians* se trataba de un enzima PuO, cuya actividad ya era conocida, sin embargo es la primera vez que se ha descrito la presencia de este enzima en la especie *K. varians.* La lacasa de *L. plantarum* degradó principalmente la tiramina y en menor grado histamina y putrescina, la de *P. acidilactici* únicamente la tiramina, y la PuO de *K. varians* degradó principalmente diaminas, concretamente, putrescina y cadaverina y, en menor grado poliaminas (espermidina), aunque fue incapaz de degradar monoaminas.

Se identificaron los genes que codifican para estos enzimas en los genomas de las cepas *L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540, para ello se han desarrollado cebadores específicos para amplificar una región interna de los genes de las proteínas resultantes de las identificaciones. Con estos cebadores se comprobó que todas las cepas de *L. plantarum* y *P. acidilactici* presentaban los genes codificantes de las proteínas MCO identificadas, del mismo modo la cepa de *K. varians* también presentaba el gen de la putrescina oxidasa. El tamaño de los fragmentos de amplificación obtenidos para *L. plantarum*, *P. acidilactici y K. varians* fueron 765, 485 y 773 pares de bases, respectivamente. Las secuencias nucleótidicas de estos fragmentos correspondían a las esperadas.

Se caracterizaron bioquímicamente las enzimas identificadas de *L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540. La baja cantidad de proteína obtenida a partir de la purificación de los extractos celulares de las dos primeras cepas requirió del clonaje de los genes codificantes de esas proteínas en *E. coli*. La expresión de ambas proteínas en condiciones estándar (37 ºC y agitación 250 rpm) no dio lugar a proteínas activas, por lo que hubo que optimizar el proceso de expresión. El procedimiento que garantizó mayor actividad de las proteínas consistió en a) la adición de Cu^{+2} durante el crecimiento o durante la fase inducción de las bacterias; b) la reducción de la temperatura y de la agitación (20ºC y 120 rpm durante 2 h durante la inducción; y c) la incubación de las células durante la inducción en condiciones de microaerofilia (cultivo estático durante 16 horas). En el caso de *P. acidilactici* también fue necesaria la coexpresión de los genes que codificaban para las chaperonas GroES y GroEL junto al de la proteína. Estas chaperonas ayudaron al correcto plegamiento de la proteína. De esta manera se obtuvo la cantidad de proteína activa necesaria para la caracterización bioquímica.

Los resultados de la caracterización se detallan a continuación. La lacasa recombinante de *L. plantarum* J16 presentó una masa molecular de 62.5 kDa, un pH óptimo de 3.5 para el ABTS y de 7 para el DMP, y una temperatura óptima de 60ºC. Además, la proteína retuvo más del 60% de actividad a 70ºC durante 10 min. Las constantes cinéticas K_m para el ABTS y DMP fueron 0.21 y 1.67 mM respectivamente, la $V_{máx}$ obtenida para el ABTS fue 0.54 U/mg y para el DMP fue 0.095 U/mg. La actividad de la enzima disminuyó en presencia de los compuestos semicarbazida, bipiridilo, fenantrolina y EDC. La lacasa recombinante de *P. acidilactici* CECT 5930 presentó una masa molecular de 60 kDa, un pH óptimo de 4 y una temperatura óptima de 28ºC para el ABTS. Esta proteína retuvo más del 80% de actividad a 60ºC durante 10 min. Las constantes cinéticas K_m y $V_{máx}$ para el ABTS fueron 1.7 mM y 24.05 U/mg, respectivamente. La actividad de la enzima disminuyó en presencia de los compuestos semicarbazida, EDTA, SDS y EDC. La caracterización bioquímica demostró que a pesar de tratarse de enzimas pertenecientes al mismo grupo, las lacasas de *L. plantarum* y de *P. acidilactici* diferían en sus masas moleculares, en su rango de sustratos, en sus constantes cinéticas y en sus pH y temperaturas óptimas de actuación. En cuanto a la degradación de aminas, las lacasas recombinantes de *L. plantarum J16* y *P. acidilactici* CECT 5930 diferían en el rango de sustratos sobre los que actuaban, ya que la primera degradó principalmente la tiramina y en menor grado histamina y putrescina, y la segunda sólo la tiramina. Se asemejaban en que la adición de 100 mM de $CuCl_2$ al tampón de reacción aumentaba su actividad sobre la tiramina, además ambas presentaron una temperatura óptima de 28ºC y los dos mismos pH óptimos de actuación de 4 y 9.5 para la tiramina. Además, el ABTS actúa como mediador para las dos enzimas recombinantes aumentando considerablemente la degradación de tiramina.

Por otro lado también se caracterizó la enzima nativa obtenida de *K. varians* LTH 1540, la enzima purificada presentó una masa molecular de 43 kDa, un pH óptimo de 8.5 y una temperatura óptima de 45ºC para la putrescina. Esta enzima retuvo más del 50% de actividad a 55ºC durante 10 min. Las constantes cinéticas K_m y $V_{máx}$ para la putrescina y la cadaverina fueron: 94 µM y 2.3 µmol/min·mg y 75 µM y 0.15 µmol/min·mg, respectivamente. La actividad de la enzima disminuyó en presencia de los compuestos EDC, rasagilina, pargilina y con el deprenil y la semicarbazida a la máxima concentración ensayada.

Las principales diferencias entre las lacasas de bacterias lácticas y la putrescina oxidasa de *K. varians* se hallaron en los sustratos susceptibles de ser degradados y en que la PuO tiene un pH óptimo de actuación neutro o ligeramente alcalino típico de las enzimas amino oxidasas, y una estabilidad térmica mucho más baja.

Los logros más importantes derivados de este trabajo son:

Es la primera vez que se ha descrito la degradación de aminas en vino por parte de las bacterias lácticas de las especies: *Lactobacillus farciminis, Lactobacillus plantarum* y *Pediococcus acidilactici,* así como de la cepa *Kocuria varians* LTH 1540 de origen alimentario.

Es el primer trabajo donde se ha demostrado que las enzimas multicobre oxidasa (EC 1.10.3.2) son capaces de degradar las AB.

Es la primera vez que se han identificado las enzimas responsables de la degradación de histamina, tiramina y putrescina en las especies *L. plantarum* y *P. acidilactici*.

Se ha identificado una putrescina oxidasa en la especie *K. varians* que no se había descrito previamente.

Es la primera vez que se ha descrito que la enzima lacasa del hongo filamentoso *T. versicolor* es capaz de degradar tiramina, histamina y putrescina en tampón, y las dos primeras también en vino.

Introducción

1. Aminas biógenas: definición y efectos sobre la salud.

Las aminas biógenas (AB) son compuestos nitrogenados de bajo peso molecular que pueden estar presentes en alimentos frescos o que pueden formarse a partir de los aminoácidos presentes en ellos mediante la acción de distintos microorganismos. Las reacciones enzimáticas que conducen a la síntesis de estos compuestos son descarboxilaciones, transaminaciones, aminaciones reductoras y degradaciones (Sebastian et al. 2011). Las enzimas responsables de estas actividades están presentes en muchos microorganismos asociados a las matrices alimentarias bien como microbiota alterante o como microbiota funcional (Alvarez y Moreno-Arribas 2014). Atendiendo a la estructura química las AB se pueden clasificar en alifáticas (putrescina, espermidina, espermina, cadaverina), aromáticas (tiramina, feniletilamina) o heterocíclicas (histamina, triptamina). En función del número de grupo aminos de la molécula, podemos hablar de monoaminas (histamina, feniletilamina, tiramina), diaminas (putrescina, cadaverina) o poliaminas (espermidina, espermina) y, de acuerdo con el número de sustituyentes alquilos o arilos unidos al nitrógeno, se clasifican en aminas primarias, secundarias y terciarias (Tabla 1). Las principales aminas utilizadas en esta tesis se muestran en la Figura 1.

Tabla 1. Clasificación de las aminas biógenas.

Amina	Precursor	Clasificación
Histamina	Histidina	Heterocíclica, primaria, monoamina
Tiramina	Tirosina	Aromática, primaria, monoamina
Feniletilamina	Fenilalanina	Aromática, primaria, monoamina
Triptamina	Triptófano	Heterocíclica, primaria, monoamina
Putrescina	Ornitina, arginina, agmatina	Alifática, primaria, diamina
Cadaverina	Lisina	Alifática, primaria, diamina
Espermina	Putrescina	Alifática, primaria, poliamina
Espermidina	Putrescina	Alifática, primaria, poliamina

Desde un punto de vista biológico, las AB son moléculas con funciones fisiológicas esenciales para los seres vivos. En plantas, la putrescina y algunas poliaminas como la espermidina y la espermina, están implicadas en diversos procesos celulares de respuesta al estrés y al envejecimiento. En animales están implicadas en procesos tan relevantes como la división celular o la transmisión nerviosa. Así, por ejemplo, la histamina actúa como neurotransmisor y la tiramina es un intermediario de las rutas de biosíntesis de otros neurotransmisores (ten Brink et al. 1990).

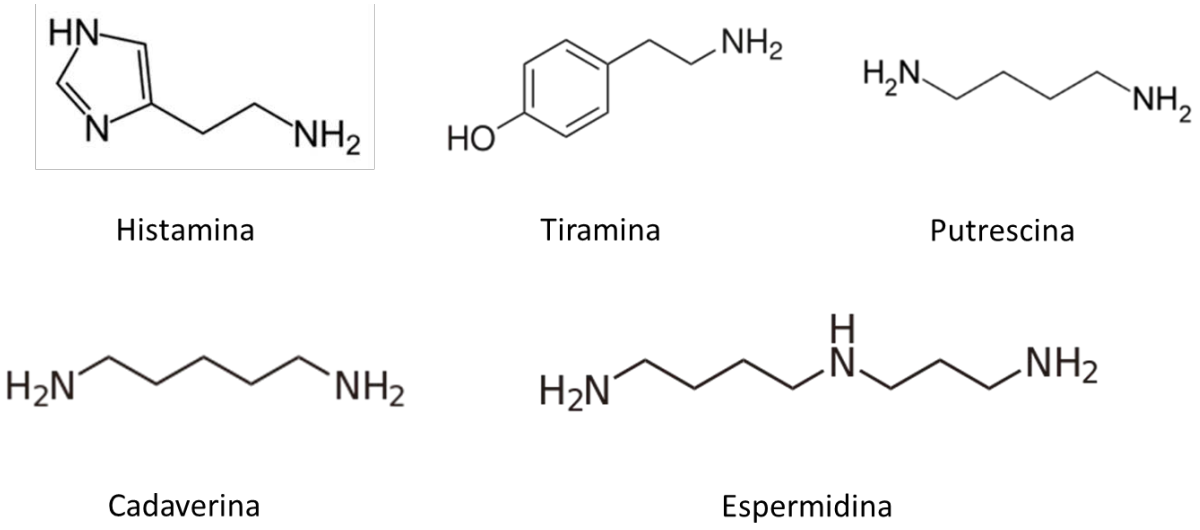

Figura 1. Principales aminas utilizadas en esta tesis. La histamina, tiramina y putrescina han sido las más estudiadas por ser las más abundantes en alimentos.

El interés del estudio de las AB en relación con los alimentos surgió en los años 60 a raíz de las crisis hipertensivas graves causadas por la interacción entre alimentos ricos en aminas (tiramina en queso) y los medicamentos inhibidores de la monoamino oxidasa (IMAO), utilizados básicamente como antidepresivos (Bover-Cid et al. 2005). Desde entonces, las AB como tiramina e histamina se han considerado microcomponentes indeseables de los alimentos por su potencial efecto negativo sobre la salud del consumidor, y por tanto se han enmarcado en el ámbito de la seguridad alimentaria. La acumulación de AB en el organismo debida a la ingesta de alimentos con grandes concentraciones de éstas produce intoxicaciones alimentarias. Las aminas pasan a la circulación sanguínea ejerciendo una toxicidad que se manifiesta con distinta sintomatología. Entre los síntomas que aparecen en los consumidores destacan: malestar, náuseas, alteraciones respiratorias, sofocos, sudoración, palpitaciones, migrañas, fuertes dolores de cabeza, picor de ojos, hiper e hipotensión, problemas estomacales e intestinales y reacciones pseudoalérgicas (Ladero et al. 2010). La tiramina y la histamina están principalmente implicadas en estas alteraciones fisiológicas. Mientras que la tiramina tiene una acción principalmente vasoactiva (provoca constricción de los vasos

sanguíneos y aumento de la presión sanguínea), la histamina es vasoactiva (dilata los vasos sanguíneos y disminuye la presión sanguínea) y psicoactiva (afecta a los transmisores neuronales del sistema nervioso central, y provoca somnolencia) (Ruiz-Capillas y Jiménez-Colmenero 2010). La histamina además, es un mediador característico de las enfermedades alérgicas, por lo que el consumo de alimentos con histamina puede dar lugar a los mismos síntomas que los procesos alérgicos (Ladero et al. 2010). Otras aminas abundantes en alimentos, como la cadaverina y la putrescina, no son tóxicas por sí mismas pero potencian el efecto de histamina y tiramina, bien favoreciendo su absorción en el intestino o bien compitiendo por los sistemas de desintoxicación (Moreno-arribas 2007).

Además las AB pueden también desempeñar un papel activo en otros tipos de procesos nocivos para la salud humana. Algunas de ellas (espermidina, espermina, tiramina, cadaverina y putrescina) pueden reaccionar con nitritos para formar nitrosaminas que son compuestos con propiedades carcinogénicas, mutagénicas y teratogénicas (Collins et al. 2011) (Figura 2). Su presencia constituye un riesgo toxicológico adicional en los productos con altos niveles de aminas y que además contienen sales de nitrito y nitrato, que se utilizan como agentes de curado en el procesamiento de productos cárnicos (Herrmann et al. 2015).

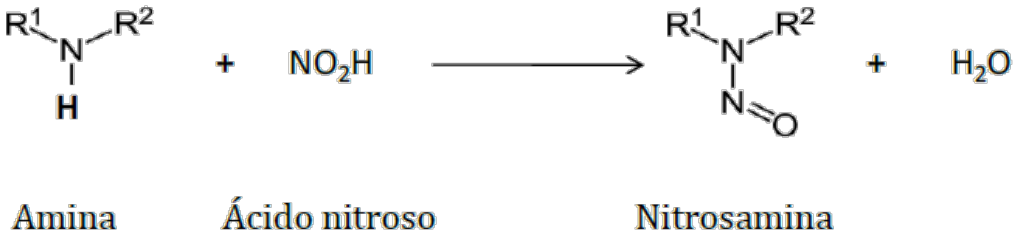

Figura 2. Reacción de formación de nitrosaminas a partir de las aminas**.** Obtenida de http://www.hablandodeciencia.com/articulos/2013/01/28/cuidado-con-las-aminas/

La histamina es la amina que se encuentra presente en cantidades más elevadas en el pescado y sus derivados, principalmente en los productos fermentados derivados de los mismos. Esta amina biógena se ha relacionado principalmente con la conocida como "intoxicación de los escómbridos" o "intoxicación histamínica" (Taylor y Eitenmiller 1986). En la carne y los productos cárnicos derivados, la histamina se encuentra en pequeñas cantidades, siendo la tiramina, la amina biógena más significativa. La tiramina también está presente en elevadas concentraciones en el queso. Los síntomas típicos de la intoxicación por tiramina es la migraña, dolor de cabeza y aumento de la presión arterial (ten Brink et al. 1990). Además de estos efectos tóxicos, estudios recientes han demostrado que la tiramina facilita la adhesión a la mucosa gástrica de distintos microorganismos patógenos (p.e. *Escherichia coli* O157:H7) (Lyte 2004).

Las AB procedentes de la dieta se metabolizan en el hígado y en el intestino por la acción de distintas enzimas presentes en el tracto digestivo, la monoamino oxidasa (MAO), la diamino oxidasa (DAO) y poliamina oxidasa (PAO, EC 1.5.3.13). Diversos factores pueden potenciar la toxicidad de las aminas biógenas porque reducen su capacidad de metabolización por el organismo. Estos factores pueden ser de tipo genético (actividad MAO y/o DAO reducida), patológico (enfermedades gastrointestinales) o de tipo transitorio (ingesta de medicamentos inhibidores de amino oxidasas) (Maijala y Eerola 2003; Önal 2007). Otro tipo de factores que potencian la actividad tóxica de las aminas biógenas son el consumo de alcohol, la sensibilidad individual del organismo y la presencia de otras aminas en alimentos (Gennaro et al. 2003; Maijala y Eerola 2003; Önal 2007). La presencia de otras AB como las relacionadas con el deterioro de los alimentos, cadaverina y putrescina fundamentalmente, provocan la saturación del sistema de desintoxicación debido a que interactúan con las amino oxidasas lo que causa la acumulación de las aminas tóxicas y potencian su efecto perjudicial (Maijala y Eerola 2003; Pinho et al. 2004). Algunos agentes farmacéuticos usados en el tratamiento de la depresión y del Parkinson, son inhibidores de las enzimas monoamino oxidasa y diamino oxidasa implicadas en el mecanismo de desintoxicación de aminas vasoactivas (Figura 3) (Komprda et al. 2008; Maijala y Eerola 2003; Önal 2007). En el caso del vino, la presencia de etanol y acetaldehído, aumenta el efecto tóxico de las AB al inhibir los sistemas de desintoxicación (Pozo-Bayon et al. 2012).

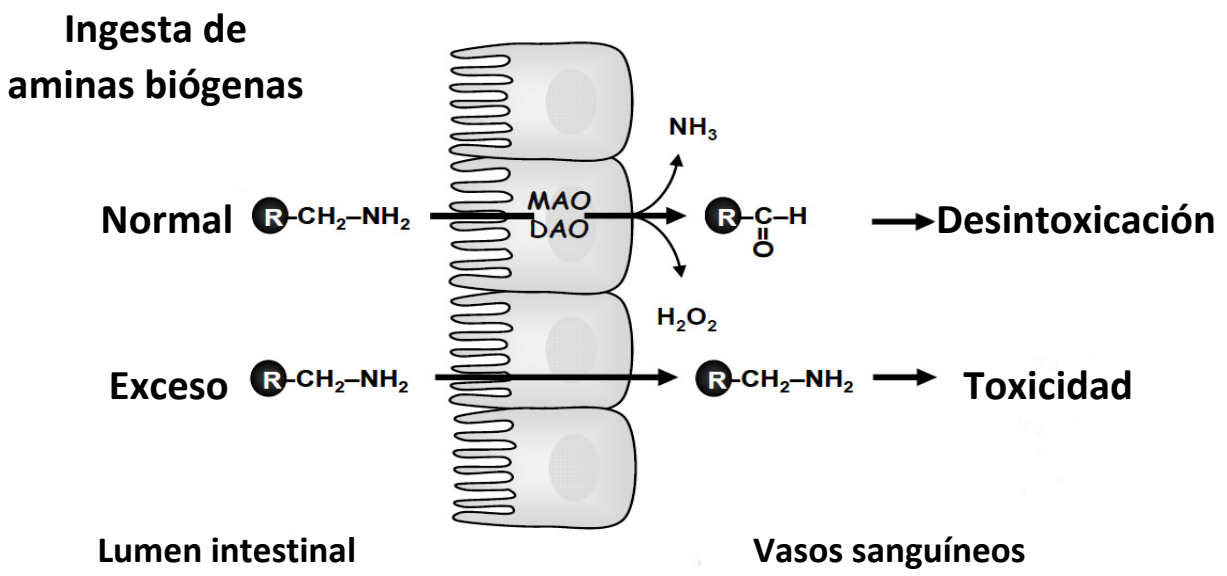

Figura 3. Posible escenario que puede darse tras la ingesta de aminas biógenas en el intestino.

Adaptado de Ladero et al. (2010)

2. Aminas biógenas en alimentos

2.1 Presencia de aminas biógenas en alimentos

En casi todos los alimentos que contengan proteínas o aminoácidos libres y microorganismos con las actividades bioquímicas necesarias, cabe esperar la formación de AB (Halász et al. 1994). Las AB más comunes encontradas en los alimentos son la histamina, tiramina, cadaverina, 2-feniletilamina, espermina, espermidina, putrescina, triptamina y agmatina (Naila et al. 2010). Las poliaminas, como la putrescina, cadaverina, agmatina, espermina y espermidina, están naturalmente presentes en los alimentos y están involucrados en el crecimiento y proliferación celular (Bardocz et al. 1995). Hay otras aminas que se encuentran en concentraciones mucho menores y que actúan como neurotransmisores, como por ejemplo la dopamina, serotonina y noradrenalina. También podemos encontrar aminas volátiles que son las moléculas responsables del olor a pescado presente en peces varios días después de la captura y se utilizan comúnmente como criterio para la evaluación de la calidad del pescado (Veciana-Nogués et al. 1997). De todas las aminas citadas las que mayor prevalencia tienen y se encuentran en mayor cantidad en alimentos son la histamina, tiramina y putrescina.

Las AB están presentes en muchos alimentos (Tablas 2 y 3) pero adquieren mayor protagonismo en los productos fermentados, como por ejemplo queso, vino, cerveza, chucrut y en alimentos mal conservados o alterados como el pescado, hígado de ternera y carnes preparadas (Krizek y Pelikanova 1998). Por una parte, la carne cruda, los productos frescos y los tratados térmicamente sólo deberían contener poliaminas fisiológicas: espermidina y espermina.

Tabla 2. Aminas biógenas encontradas en alta concentración en distintos alimentos.

Amina biógena	Alimento
Histamina	Atún, caballa, boquerón, sardina, huevos, quesos añejos, frutos secos, patatas y col fermentadas, anchoa en conserva, embutidos, espinacas, berenjena, aguacate, tomate, cerveza, vino
Tiramina	Quesos curados, arenques en vinagre, hígado, embutidos, aguacate, plátano, alubias, col, caviar, chocolate, pepino, berenjena, guisantes, pasas, espinacas, higos, uvas, carne de bovino, vino
Putrescina	Quesos maduros y vino
Cadaverina	Quesos maduros
Feniletilamina	Cereales, frutos secos, carne, pescado, lácteos, judías, chocolate, quesos, embutidos
Triptamina	Cereales, frutos secos, carne, pescado, lácteos, judías, quesos, embutidos
Dopamina	Cereales, frutos secos, carne, pescado, lácteos, judías, plátano, aguacate
Serotonina	Plátano, avellana, tomate, chocolate
Noradrenalina	Plátano

Las otras aminas no deberían estar presentes, o en todo caso, sólo en cantidades insignificantes. La presencia de diaminas (cadaverina y putrescina) e histamina se relaciona con la alteración de la carne y otros alimentos, ya que, son producidas generalmente por enterobacterias y pseudomonas, que son también los principales agentes microbianos causantes del deterioro de la carne. Precisamente, se ha sugerido que se tenga en cuenta la concentración de aminas biógenas como índice químico objetivo para la evaluación del estado higiénico de la carne (Bover-Cid et al. 2005). Estas AB son compuestos termoestables, no volátiles, que no desaparecen durante el tratamiento térmico que se aplica a los alimentos cocinados. Por ello, la concentración de AB es un índice útil para valorar el estado higiénico de la materia prima aunque ésta haya sido cocinada (Hernández-Jover et al. 1996). Algunos productos crudos o curados se someten a un proceso de fermentación y/o maduración durante el cual se desarrollan una gran variedad de microorganismos (potencialmente aminogénicos). Además, los fenómenos proteolíticos (que aumentan la disponibilidad de aminoácidos precursores) y una ligera acidificación, constituyen factores particularmente favorables para la formación de AB. Ello explica que los niveles de aminas en los productos fermentados sean más elevados que en los productos frescos y cocidos.

Tabla 3. Concentraciones de AB por familias de alimentos. Resultados expresados como mg/Kg de alimento. Datos tomados de Collins et al. (2011)

Categoría alimento	Sub categoría	Histamina	Tiramina	Putrescina
Bebidas alcohólicas	Cerveza	1.4	6.1	3.3 - 3.5
	Vino fortificado	1.1	6.0	1.4
	Vino tinto	3.6-3.7	2.7-2.9	4.2-4.8
	Vino blanco	0.8-0.9	1.1-1.2	1.4-1.5
	Vino blanco espumoso	1.0	4.9	5.2
Salsas	Salsas de pescado	196-197	105-107	98.1-99.3
	Otras salsas	0.5-10.1	1.5-10.5	6-13.6
Pescado y derivados	Anchoas	348	-	-
	Fermentados	7.7-11.4	47.2-49.1	13.4-17
	Otros	26.8-31.2	6.8-14.6	4.1-11.7
Productos cárnicos	Salchichas fermentadas	23.0-23.6	136	84.2-84.6
	Productos madurados	5.9-6.4	44-44.2	32.8
	Otros	3.9-4.4	16.1-16.2	17.4-17.6
Lácteos	Queso	20.9-62	68.5-104	25.4-65
	Queso fresco	3.2-38.5	12.8-48	5.5-41.3
	Queso duro	25.2-65.1	82.9-113	26.6-65.5
	Queso corteza	8.5-54.4	31.6-76.1	32.3-72.3
	Queso azul	21.8-63.8	63.2-104	20.9-62.2
	Requesón	51.3-55.3	335	449
	Yogurt	0.5	1.9	0.7
	Otros	0.3	0.3	0.7
Vegetales	Fermentados	39.4-42.6	45.0-47.4	264
	Otros	2.9-3.1	1.8	37.2

Cabe considerar aparte los productos madurados a partir de piezas enteras (tipo jamón o lomo) en los que el crecimiento microbiano y la actividad aminogénica se encuentran fuertemente limitados por las elevadas concentraciones de sal que se usan en su procesamiento. En estos productos los contenidos de las diferentes AB podría equipararse a los de los productos cocidos. Por otro lado, los tejidos de los peces de las familias *Scombridae* contienen altos niveles de histidina libre, que los microorganismos asociados a esos peces pueden convertir en histamina. El atún y otras especies de peces de las familias *Scombridae* y *Clupeidae* se han caracterizado por contener altos niveles de histamina, como resultado de la manipulación y conservación inadecuada.

2.2 Formación de aminas biógenas en alimentos y microorganismos responsables

La formación de AB supone una ventaja energética para los microorganismos que las producen, ya que el catabolismo de aminoácidos por descarboxilación o la desaminación producida por microorganismos anaerobios-fermentativos supone la obtención de energía metabólica en medios pobres de nutrientes por el antiporte precursor/producto. La descarboxilación de un aminoácido para dar su correspondiente amina y dióxido de carbono (Figura 4) genera energía metabólica y regula el pH intracelular, gracias a un mecanismo común de una gran variedad de descarboxilasas, como por ejemplo las que descarboxilan el aspartato (Abe et al. 1996; WN Konings 1995) y la histidina (Molenaar et al. 1993). En el citoplasma, la descarboxilación de un aminoácido consume un protón. El transporte de la correspondiente amina fuera de la célula provocaría una extrusión protónica indirecta (Maloney et al. 1992), la cual puede ser usada para regular el pH intracelular. Además, el transporte de la amina genera un gradiente electroquímico que puede ser utilizado por la célula para realizar reacciones que consumen energía tales como el transporte de nutrientes o la generación de ATP vía F1F0ATPasa (Konings et al. 1997). Otras aminas como la tiramina, feniletilamina, cadaverina y triptamina se generan de manera similar y provocan los mismos efectos fisiológicos que las dos anteriores (Rice et al. 1976).

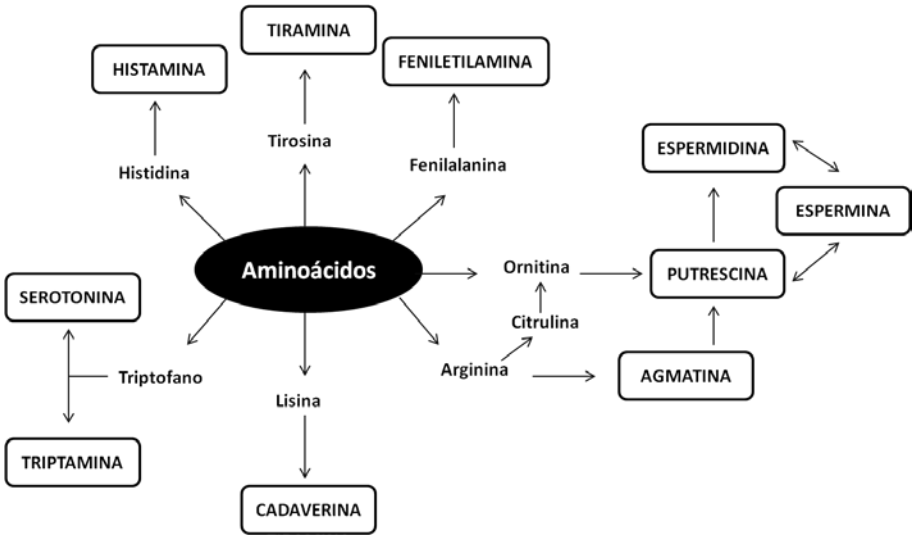

Figura 4. Origen metabólico de las aminas biógenas.

Los microorganismos responsables de la formación de AB en los alimentos fermentados suelen ser los mismos implicados en el proceso fermentativo, sin embargo, en los no fermentados, las AB se producen por la acción de bacterias alterantes, por lo que en estos casos, la concentración de AB es un parámetro útil como indicador de la descomposición del alimento (Lázaro et al. 2015; Veciana-Nogués et al. 1997; Vinci y Antonelli 2002) como ya se ha mencionado anteriormente.

La presencia de actividades descarboxilásicas implicadas en la síntesis de AB, es una característica dependiente de cepa y no de especie. Las bacterias implicadas en la sínteis de AB pueden ser tanto Gram positivas como Gram negativas y pertenecen a especies de diversos géneros como *Aeromonas, Bacillus, Citrobacter, Clostridium, Klebsiella, Escherichia, Proteus, Pseudomonas, Salmonella, Shigella, Staphylococcus, Micrococcus, Kocuria, Photobacterium, Morganella, Serratia*, *Lactobacillus, Enterococcus, Carnobacterium, Pediococcus y Lactococcus*, éstos cinco últimos pertenecientes al grupo de las bacterias del ácido láctico (BAL). También se ha descrito la producción de AB por algunas levaduras y hongos, como se puede ver en la Tabla 4 donde se muestra las especies de los microorganismos descritos con capacidad de producir AB en alimentos.

Tabla 4. Microorganismos responsables de la producción de AB en alimentos.

Amina	Microorganismo	Familia / Género / Especie	Referencia
Histamina	Hongos y levaduras	*Debaryomyces hansenii*	Roig-Sagués et al. (2002)
		Geotrichum candidum	Gardini et al. (2006)
		Kloeckera apiculata	Caruso et al. (2002)
		Brettanomyces bruxellensis	
	Gram-	*Enterobacteriaceae*	Marino et al. (2000)
		Morganella morganii	Coton et al. (2012)
		Morganella psychrotolerans	
		Photobacterium phosphoreum	
		Photobacterium psychrotolerans	
		Hafnia alvei	
	Gram+	*Lactobacillus buchneri*	Martín et al. (2005)
		Lactobacillus curvatus	Burdychova y Komprda
		Lactobacillus helveticus	(2007)
		Lactobacillus parabuchnen	Calles-Enríquez et al. (2010)
		Streptococcus thermophilus	
Tiramina	Hongos y levaduras	*Yarrowia lipolytica*	Gardini et al. (2006)
	Gram+	*Enterococcus casseliflavus*	
		Enterococcus durans	Fernández et al. (2007)
		Enterococcus faecalis	Bonetta et al. (2008)
		Enterococcus faecium	Ladero et al. (2012)
		Enterococcus hirae	Lucas et al. (2007)
		Lactobacillus brevis	Buňková et al. (2009)
		Lactobacillus curvatus	La Gioia et al. (2011)
		Streptococcus thermophilus	
Putrescina	Hongos y levaduras	*Debaryomyces hansenii*	Wyder et al. (1999)
		Yarrowia lipolytica	
		Saccharomyces cerevisiae	Caruso et al. (2002)
		Kloeckera apiculata	
		Brettanomyces bruxellensis	
		Metschnikovia pulcherrima	
	Gram-	*Enterobacteriaceae*	ten Brink et al. (1990)
		Proteus	Coton et al. (2012)
		Pseudomonadaceae	López-Caballero et al. (2001)
		Shewanellaceae	
	Gram+	*Enterococcus faecalis*	Llácer et al. (2007)
		Enterococcus hirae	Lucas et al. (2007)
		Lactobacillus brevis	Victor Ladero et al. (2011)
		Lactobacillus curvatus	
		Lactococcus lactis	
Cadaverina	Hongos y levaduras	*Yarrowia lipolytica*	Wyder et al. (1999)
		Kloeckera apiculata	Caruso et al. (2002)
		Brettanomyces bruxellensis	
		Metschnikovia pulcherrima	
	Gram-	*Enterobacteriaceae*	Meng y Bennett (1992)
		Halamonas sp.	
		Morganella morganii	Coton et al. (2012)
		Pseudomonas putida	

Amina	Microorganismo	Familia / Género / Especie	Referencia
Triptamina	Hongos y levaduras	*Saccharomyces cerevisiae* *Kloeckera apiculata* *Candida estellata*	Caruso et al. (2002)
	Gram-	*Morganella morganii* *Proteus* *Serratia*	Marino et al. (2000) Coton et al. (2012)
Feniletilamina	Hongos y levaduras	*Yarrowia lipolytica* *Saccharomyces cerevisiae* *Kloeckera apiculata* *Metschnikovia pulcherrima* *Brettanomyces bruxellensis*	Wyder et al. (1999) Caruso et al. (2002)
	Gram-	*Halomonas* *Serratia*	Marino et al. (2000)
	Gram+	*Enterococci*	Marcobal et al. (2006a)

3. Métodos de detección de BAL productoras de AB y cuantificación de estas aminas:

3.1 Métodos de detección de microorganismos productores:

Debido a que se han descrito muchas especies de BAL productoras de AB y que estas bacterias abundan en los alimentos, se han desarrollado métodos microbiológicos de detección que se basan en el crecimiento de los microorganismos en medios diferenciales, que contienen el aminoácido sustrato de la reacción de descarboxilación que da lugar a la AB correspondiente y, en ocasiones, un indicador de pH (Choudhury et al. 1990; Maijala 1993b). Para la detección de bacterias lácticas productoras de histamina se suelen utilizar medios que, además de histidina contienen un indicador de pH que vira como consecuencia del aumento de pH que provoca la síntesis de la amina (Landete et al. 2005; Maijala 1993b). Para detectar las bacterias productoras de tiramina Landete et al. (2007c) desarrollaron un medio indicador que contenía tirosina. La tirosina (insoluble) confería al medio un aspecto opaco, sin embargo, cuando ésta se transformaba en tiramina (soluble) se producía un aclaramiento del medio. Los principales inconvenientes de estos métodos de detección son la dificultad para el crecimiento de algunas cepas de BAL, los largos períodos de incubación necesarios (72 horas) y los bajos niveles de sensibilidad.

Otra alternativa para detectar bacterias productoras de aminas son los métodos moleculares basados en PCR, que permiten detectar los genes que codifican las enzimas descarboxilásicas. En el trabajo realizado por Landete et al (2007a) se hace una extensa revisión de diversos cebadores que tienen como diana los genes de la histidina descarboxilasa (*hdc*), tiramina descarboxilasa (*tdc*), ornitina descarboxilasa (*odc*) y lisina descarboxilasa (*ldc*). La detección de estos genes se puede llevar a cabo de uno en uno o de forma conjunta realizando para ello una PCR múltiple (Coton et al. 2010; de las Rivas et al. 2005; Marcobal

et al. 2005b). Se considera que las cepas que dan una banda de amplificación del tamaño y secuencia esperada son potenciales productoras de AB. En la Tabla 5 se muestran diversos cebadores que se han descrito para detectar los genes codificantes de varios enzimas descarboxilásicas presentes tanto en bacterias Gram+ como Gram-.

Tabla 5. Cebadores publicados para la detección de genes implicados en la producción AB.

Amina	Cebador	Secuencia 5′ → 3′	Referencia
Histamina	*Gram-positivas*		
	CL1	CCWGGWAAWATWGGWAATGGWTA	Le Jeune et al. (1995)
	CL2	GAWGCWGTWGTCATATTWATTTGWCC	
	JV16HC	AGATGGTATTGTTTCTTATG	
	JV17HC	AGACCATACACCATAACCTT	
	JV17	AGACCATACACCATAACCTTG	Coton et al. (1998a) Rollan et al. (1995)
	CL1mod	CCAGGWAACATTGGTAATGGATA	Landete et al. (2005)
	HDC3	GATGGTATTGTTTCKTATGA	Coton y Coton (2005)
	HDC4	CAAACACCAGCATCTTC	
	PHDC1	CCGTGCGGAAACAAAGAAT	Costantini et al. (2006)
	PHDC2	CCAAACACCAGCATCTTCA	
	HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	de las Rivas et al. (2006) de las Rivas et al. (2007)
	HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA	
	Hdc1	TTGACCGTATCTCAGTGAGTCCAT	Fernández et al. (2006a) Fernández et al. (2006b)
	Hdc2	ACGGTCATACGAAACAATACCATC	
	Gram-negativas		
	KPF2	AAAGCTGGGGGTATGTGACC	Kanki et al. (2002)
	KPR4	GTGATGGAGTTTTTGTTGC	
	Hdc-f	TCHATYARYAACTGYGGTGACTGGRG	Takahashi et al. (2003)
	Hdc-r	CCCACAKCATBARWGGDGTRTGRCC	
	106	AAYTCNTTYGAYTTYGARAARGARG	de las Rivas et al. (2005)
	107	ATNGGNGANCCDATCATYTTRTGNCC	
	HIS2-F	AAYTSNTTYGAYTTYGARAARGARGT	de las Rivas et al. (2006) de las Rivas et al. (2007)
	HIS2-R	TANGGNSANCCDATCATYTTRTGNCC	
	DegF	GGYGGIACIGARGGNAANATG	Morii et al. (2006)
	DegR	GGRAAIACICANGTNWTGGAG	
Tiramina	P2-for	GAYATIATIGGIATIGGIYTIGAYCARG	Lucas y Lonvaud-Funel (2002)
	P1-rev	CCRTARTCIGGIATIGCRAARTCIGTRTG	
	41	CAYGTNGAYGCNGCNTAYGGNGG	Marcobal et al. (2005b)
	42	AYRTANCCCATYTTRTGNGGRTC	
	Pt3	TACACGTAGATGCTGCATATG	Costantini et al. (2006)
	Pt4	ATGGTTGACTATGTTTTAAAAGAA	
	p0303	CCACTGCTGCATCTGTTTG	Lucas et al. (2003)
	TD5	CAAATGGAAGAAGAAGTAGG	Coton et al. (2004)
	TD2	ACATAGTCAACCATRTTGAA	
	57	ATGAGTGAATCATTGTCG	Marcobal et al. (2004)
	58	TTATTTTGCTTCGCTTGCC	
	TDC1	AACTATCGTATGGATATCAACG	Fernandez et al. (2004)
	TDC2	TAGTCAACCATATTGAAATCTGG	
	TDC-F	TGGYTNGTNCCNCARACNAARCAYTA	de las Rivas et al. (2006) de las Rivas et al. (2007)
	TDC-R	ACRTARTCNACCATRTTRAARTCNGG	
Putrescina	3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	Marcobal et al. (2004)
	16	TACRCARAATACTCCNGGNGGRTANGG	
	4	ATNGARTTNAGTTCRCAYTTYTCNGG	Marcobal et al. (2005b)
	15	GGTAYTGTTYGAYCGGAAWAAWCAYAA	
	AODC1	GMTCGTGAAATYAARCKG	Costantini et al. (2006)
	AODC2	KGRGTTCMGCYGGRGTAT	
	PUT1-F	TWYMAYGCNGAYAARACNTAYYYTGT	de las Rivas et al. (2006)

Amina	Cebador	Secuencia 5′ → 3′	Referencia
	PUT1-R	ACRCANAGNACNCCNGNGGRTANGG	de las Rivas et al. (2007)
	PUT2-F	ATHWGNTWYGGNAAYACNATHAARAA	
	PUT2-R	GCNARNCCNCCRAAYTTNCCDARTC	

3.2 Métodos de cuantificación

3.2.1 Métodos de cuantificación de aminas de forma individual:

La mayor parte de los métodos de cuantificación individual de aminas se han centrado en la histamina porque es la amina más tóxica y porque es la única para la que se han establecido límites máximos legales de concentración. Los primeros métodos desarrollados para la cuantificación de AB en vinos fueron los enzimáticos basados en reacciones bioquímicas en las que la amina aparecida era sustrato de una reacción acoplada a otra que generaba NADH. Estos métodos sólo permitían la cuantificación de una única amina, por ejemplo, la histamina (Landete et al. 2004; Somavilla et al. 1986), la tiramina (Ulla et al. 1989) o ambas (Vidal-Carou et al. 1989). La limitación de estos métodos es que para evaluar el contenido en AB de un alimento hay que utilizar tantos ensayos como aminas queramos cuantificar y además, sólo se han desarrollado para la histamina y la tiramina pero no para el resto.

3.2.2 Métodos que permiten la cuantificación simultánea de varias aminas:

La cromatografía de líquidos de alta eficacia (HPLC) con detección mediante ultravioleta (UV) o fluorescencia es el método más utilizado para la determinación de aminas en alimentos. Debido a la ausencia de cromóforos en estos compuestos es necesario formar derivados que absorban en UV o que sean fluorescentes, con el fin de facilitar su detección. Los reactivos derivatizantes más empleados son el ortoftaldehído (OPA), el cloruro de dansilo (DNS-Cl), el (fluorenilmetil)-cloroformato (FMOC) y el 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC). En el caso del DNS-Cl, la reacción se realiza antes de la separación cromatográfica, (Galgano et al. 2003; Romero et al. 2000) en el del OPA se puede hacer antes de la separación en columna (Iñiguez Crespo y Vázquez Lasa 1994; Moreno-Arribas et al. 2003) o después (Vidal-Carou et al. 2003). De todos estos agentes derivatizantes, el más utilizado es el OPA, debido entre otras razones a que la reacción de derivatización es rápida y en un solo paso y a la posibilidad de automatizar la reacción, lo que mejora la reproducibilidad del método de análisis. Debido a la interferencia que puede generar la propia matriz y las bajas cantidades presentes de aminas biógenas, generalmente es necesario aplicar un pretratamiento de separación o limpieza a la muestra. Los métodos más usados son la extracción líquido-líquido y la extracción en fase sólida (Guo et al. 2015). En la cuantificación por HPLC algunos parámetros cromatográficos como el pH, temperatura y

eluyente afectan a los resultados, además hay que tener en cuenta que requiere un largo tiempo para el análisis. Recientemente han surgido procedimientos de HPLC para la detección de AB en vino más rápidos que los anteriormente utilizados (Berbegal et al. 2015). Por otro lado, la técnica de HPLC se puede unir a la de espectrometría de masas (UPLC/Q-TOFMS) para mejorar la cuantificación de AB (Jia et al. 2012), y de esta manera se puede llegar a detectar menos de 15 ng/mL.

La cromatografía de gases se ha aplicado también a la cuantificación de aminas (Fernandes y Ferreira 2000; Önal 2007), pero esta técnica, muy adecuada para el análisis de compuestos volátiles, no lo es tanto para el de los no volátiles, como las AB. Es necesario no sólo extraer las aminas, sino formar derivados volátiles, lo que alarga y complica el análisis.

Se han descrito algunas aplicaciones de la electroforesis capilar para la cuantificación de aminas. En este caso también es necesario formar derivados detectables con detectores de UV (Kovács et al. 1999) o de fluorescencia, (Male y Luong 2001). Esta técnica no se está aplicando aún para análisis de rutina aunque es posible que se imponga en unos años ya que tiene como ventajas un alto poder de separación y un tiempo de análisis inferior al de la técnica de HPLC; el problema es que también necesita de un pretratamiento de la muestra. Esta técnica también se puede acoplar a espectrometría de masas para aumentar su sensibilidad (Simó et al. 2008).

En una resolución de la OIV (RESOLUCIÓN OIV/OENO 348/2010) se ha establecido la cromatografía de capa fina como método de análisis oficial para la cuantificación de AB en vinos. Este método permite separar e identificar histamina, tiramina, putrescina, cadaverina y feniletilamina. Las aminas se transforman en sus derivados fluorescentes de dansilo, se separan en placas de gel de sílice y los compuestos dansilados se separan por migración vertical en una mezcla de cloroformo y trietilamina. Las manchas se observan con luz UV mediante un transiluminador acoplado a un de equipo fotográfico. El límite de detección de las aminas es de 0,01 mg/mL a excepción de la histamina cuyo límite es de 1 mg/mL.

El método ELISA puede ser considerado como una alternativa a la HPLC por su simplicidad, rapidez y bajo coste (en comparación con el HPLC) y puede ser utilizado al menos como método de muestreo en laboratorios en los que no disponen de cromatógrafos o que tienen que analizar un número elevado de vinos. Ya hay kits comerciales para la histamina como el Veratox®, en ellos, la histamina se extrae de la muestra usando un proceso de eliminación rápida del agua, y la histamina libre compite con la enzima marcada (conjugado) por los sitios de unión al anticuerpo. Después de una etapa de lavado, el sustrato reacciona con el conjugado de la enzima unida para producir color que se cuantifica por medida de la densidad óptica en un lector de placas micropocillos.

Varios autores han comparado varios métodos para la cuantificación de aminas, así Landete et al. (2004) compararon un método enzimático desarrollado por ellos con un método basado en HPLC, llegando a la conclusión de que existía una buena correlación entre ellos. Por otro lado, los resultados del método de HPLC de los OPA-derivados de la histamina, se han comparado con los del inmunoanálisis CD-ELISA para esta amina, obteniendo resultados concordantes (Marcobal et al. 2005a).

4. Enzimas que actúan sobre las aminas biógenas:

4.1 Amino oxidasas:

Las amino oxidasas (AO) son enzimas que se encuentran en los organismos vivos (animales, bacterias y plantas) y que catalizan la oxidación de una amplia gama de AB, incluyendo muchas de las que actúan como neurotransmisores, aminas xenobióticas y aminas procedentes de la dieta (Pomilio et al. 2013). Representan una clase heterogénea de enzimas en términos de estructura, cofactor empleado, mecanismo catalítico y sustrato, lo que ha hecho que su nomenclatura sea confusa. En cualquier caso, la reacción que catalizan sobre cualquier amina es de carácter oxidativo y conlleva la desaminación de la misma, generando el aldehído correspondiente y H_2O_2. La reacción es similar independientemente de que la enzima actúe sobre monoaminas, diaminas, poliaminas o derivados de estas aminas, como se puede ver en la siguiente reacción (Yamada et al. 1965b):

$$RCH_2NH_2 + H_2O + O_2 \longleftrightarrow RCHO + NH_3 + H_2O_2$$

Todas las investigaciones sobre AO bacterianas se pueden separar en dos grupos según el artículo de revisión de Yagodina et al. (2002): por un lado estarían aquellos trabajos que se realizaron utilizando métodos clásicos de bioquímica y que se basaron en el estudio de la especificidad de la reacción sustrato-inhibidor de la enzima aislada y purificada hasta cierto grado, permitiendo clasificar la enzima estudiada como perteneciente a una cierta clase de AO. Sin embargo, en algunos trabajos se encontraron AO que aunque pertenecían a las diamina oxidasas de acuerdo con su especificidad sustrato-inhibidor, como por ejemplo la putrescina oxidasa de *Micrococcus rubens*, contenían FAD como cofactor y no cobre (Yamada 1971). Además, la tiramina oxidasa de *Sarcina lutea*, pierde la capacidad de desaminar la tiramina y adquiere propiedades lisina oxidasa (Iakovlev et al. 1971). Por lo tanto, en opinión de algunos autores para atribuir una AO a una clase determinada no se debe determinar solo en función de la especificidad sustrato-inhibidor si no de la estructura química, de su secuencia de aminoácidos y del cofactor empleado (Gorkin y Ovchinnikova 1993).

Así surge la segunda etapa de estudio de las AO bacterianas donde se obtiene la enzima para saber la naturaleza del cofactor, su secuencia y si es posible su estructura, además de los estudios bioquímicos de sustratos e inhibidores. De este modo, se establece la clasificación de este grupo de enzimas de acuerdo con el cofactor implicado en la catálisis enzimática. Siguiendo este criterio las AO se clasifican, como dependientes de cobre (EC 1.4.3.6) o de flavina (EC 1.4.3.4) (Wilmot et al. 1997).

Las amina oxidasas dependientes de FAD (FAD-AO) se pueden dividir en diferentes subclases (Hines et al. 2011): monoamina oxidasas (MAO, EC 1.4.3.4) que, en general, oxidan aminas primarias y son activas frente a una amplia gama de compuestos aromáticos; las poliaminas oxidasas (PAO, EC 1.5.3.11) que oxidan aminas secundarias; las L- y D- amino-ácido oxidasas (EC 1.4.3.2 y 1.4.3.3) y las putrescina oxidasas (PUO, EC 1.4.3.10).

Las AO dependientes de cobre (CAO) incluyen, entre otras, la bencilamina oxidasa (ahora re-clasificada como oxidasa de aminas primarias, EC 1.4.3.21) y la diamina oxidasa (DAO, EC 1.4.3.22) que se descubrió en tejidos de mamíferos en 1929 y que originalmente se llamó histaminasa debido a que catalizaba la desaminación oxidativa de histamina. El nombre diamina oxidasa se introdujo algunos años más tarde, en base a que la histaminasa del riñón de cerdo degrada también diaminas alifáticas cortas, como putrescina y cadaverina. Por lo tanto, el nombre diamina oxidasa sólo se emplea para una sub-clase particular de enzimas CAO.

Las primeras referencias de AOs de origen microbiano datan de 1965, cuando Yamada et al. (1965a) purificaron y caracterizaron una AO obtenida a partir del hongo *Aspergillus niger* (Yamada et al. 1965a), posteriormente en 1981 se purificó y caracterizó una metilamina oxidasa a partir de la levadura *Candida* (Haywood y Large 1981). Yamada et al. en 1965 y 1967 llevaron a cabo las primeras purificaciones y caracterizaciones de AO bacterianas, concretamente de una putrescina oxidasa de *Micrococcus rubens* y de una tiramina oxidasa de *Sarcina lutea* (syn *Micrococcus luteus*), (Yamada et al. 1965c; Yamada et al. 1967). Más tarde, Murooka et al. (1979) describió que la enzima responsable de la oxidación de AB era una monoamino oxidasa (MAO) capaz de degradar la tiramina, octopamina, dopamina y la norepinefrina, y que se encuentra en la membrana de diferentes bacterias Gram- de los géneros *Klebsiella*, *Enterobacter*, *Escherichia coli*, *Salmonella*, *Serratia*, *Proteus* y también en algunas no enterobacterias: *Pseudomonas aeruginosa* IFO 3901, *Micrococcus luteus* IFO 12708, y *Brevibacterium ammoniagenes* IAM 1641 (Murooka et al. 1979). Parte de la información anterior fue compilada y completada en la amplia revisión realizada por Yagodina et al. (2002), los cuales informaron de la existencia de una AO en la arqueobacteria *Methanosarcina barkery*. En 2004, también se purificó y caracterizó una histamina oxidasa termoestable de *Arthrobacter crystallopoietes* (Sekiguchi et al. 2004). Algunas de estas AO se recogen en la Tabla 6.

Tabla 6. Amino oxidasas bacterianas, tipo de sustratos sobre los que actúan y número de acceso correspondiente a la base de datos Swiss prot.

Cu-AO			
Monoaminas (1.4.3.21)		**Diaminas** (1.4.3.22)	
Especie	**Nº Acceso**	**Especie**	**Nº Acceso**
Arthrobacter sp.	(Q07121)	*Arthrobacter crystallopoietes*	(Q33BP9)
E. coli	(P46883)	*Arthrobacter globiformis*	(Q59118)
Enterobacter aerogenes	(P49250)	*Natrinema gari*	-
Klebsiella oxytoca	(P80695)		
Arthrobacter globiformis	(P46881)		

FAD-AO					
Monoaminas (1.4.3.4)		**Diaminas** (1.4.3.10)		**Poliaminas**	
Especie	**Nº Acceso**	**Especie**	**Nº Acceso**	**Especie**	**Nº Acceso**
Enterobacter aerogenes	(Q5KQQ0)	*Kocuria rosea*	(P40974)	*Actinoplanes* sp	(G8SM18)
Kocuria rhizophila	(B2GIQ6)	*Kocuria rhizophila*	(B2GJT4)	*Kocuria rosea*	(P40974)
Sarcinea lutea	(O82865)	*Rodococcus erythropolis*	(B0F9F6)	*Kocuria rhizophila*	(B2GJT4)
		Micrococcus luteus	(C5CB11)	*Rodococcus erythropolis*	(B0F9F6)
		Pseudomonas putida	-	*Micrococcus luteus*	(C5CB11)
		Arthrobacter aurescens	(11ROW1)	*Pseudomonas putida*	-
				Arthrobacter aurescens	(11ROW1)

4.2 Lacasas:

Las lacasas (EC 1.10.3.2) constituyen el grupo de enzimas más numeroso dentro de la familia de las proteínas multicobre oxidasas (MCO). Este grupo también incluye tirosinasas, monooxigenasas y dioxigenasas. Filogenéticamente, estas enzimas han evolucionado a partir de pequeñas azurinas de procariotas hasta dar lugar a proteínas plasmáticas eucariotas como la ceruloplasmina (Claus 2003). Las azurinas bacterianas contienen un centro de cobre de tipo 1. Un ejemplo de ellas es la rusticianina que se cristalizó a partir de *Thiobacillus ferrooxidans*, y que puede ser considerada como la proteína precursora de las lacasas (Hough et al. 2001). Las lacasas por lo general contienen cuatro dominios de unión a cobre ricos en histidina (Messerschmidt y Huber 1990). Estos átomos de cobre se organizan en tres centros cuyas características se determinan utilizando espectroscopía UV-visible y resonancia paramagnética electrónica (EPR). El centro tipo I (T1) es responsable del color azul intenso de este tipo de enzimas, tiene una fuerte absorción electrónica alrededor de 600 nm y es detectable por EPR. El centro II (T2) es incoloro, pero EPR detectable, y el centro III (T3), que contiene un par de átomos de cobre, presenta una absorbancia débil cerca del espectro UV pero no da señal por EPR. Los centros de cobre T2 y T3 están muy juntos y forman un centro de trinuclear donde se produce la unión del dioxígeno y la reducción de cuatro electrones para dar lugar al agua (Figura 5).

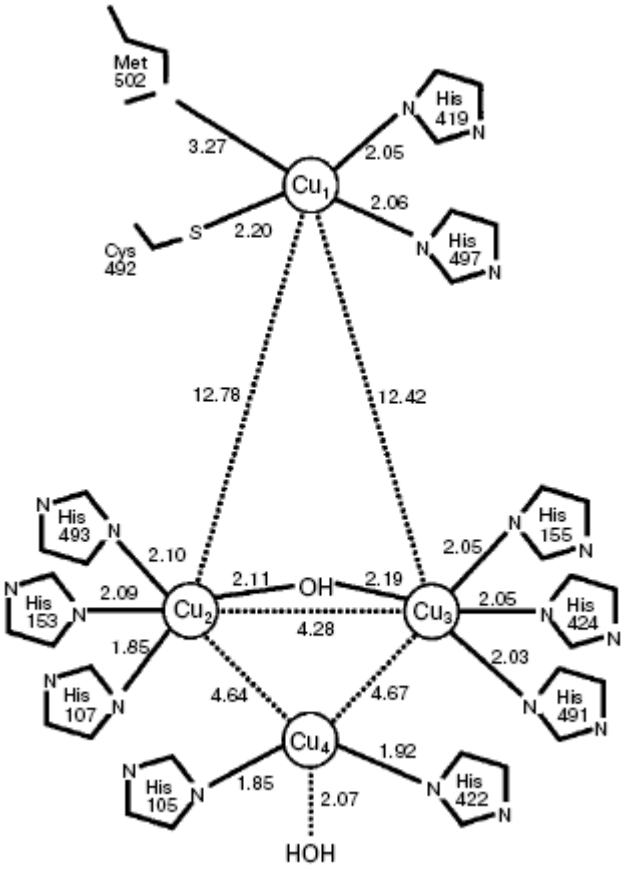

Figura 5. Estructura del centro activo de la lacasa Cot A de *Bacillus subtilis* (Morozova et al. 2007).

Las lacasas purificadas exhiben un color azul característico de su absorción electrónica alrededor de 600 nm. El espectro UV-visible típico de las lacasa (en estado de reposo) muestra dos máximos alrededor de 280 y 600 nm y un hombro cerca de 330 nm. La relación entre las absorbancias a 280 nm y 600 nm (280/600) suele estar en un rango entre 14 y 30, además, este ratio se puede utilizar como indicador del nivel de purificación alcanzado. La relación de la absorbancia a 330 nm y 600 nm (330/600) es de 0,5 a 2 (Xu et al. 2007). Se han descrito lacasas que carecen de algún dominio de Cu como es el caso de SLAC de *Streptomyces coelicolor* (Machczynski et al. 2004), Epo A de *Streptomyces griseus* (Endo et al. 2003) y Ssl1 de *Streptomyces sviceus* (Gunne y Urlacher 2012).

Las lacasas participan en la reticulación de monómeros, la degradación de polímeros, y la escisión del anillo de compuestos aromáticos (Kawai 1988). La lista de sustratos susceptibles de ser oxidados por las lacasas se ha incrementado significativamente en los últimos años. Entre ellos están compuestos metoxi o amino-monofenoles y varios no fenólicos, tales como diaminas aromáticas, 2,2'-azinobis (ácido 3-etilbenzotiazolina-6-sulfónico) o ABTS, 1-naftol, hidroxiindoles y siringaldacina (Cai et al. 1993; Mayer 1987). Además las lacasas pueden catalizar la oxidación de sustratos no fenólicos o sustratos que no son típicos de estos enzimas y para ello requieren la presencia de un mediador en el medio. Un mediador es una molécula pequeña que se comporta como una "lanzadera de electrones"

entre la lacasa y el sustrato y estos compuestos de bajo peso molecular se convierten en radicales estables por medio de la oxidación enzimática. El primer mediador descrito fue el 2,2'-azinobis (ácido 3-etilbenzotiazolina-6-sulfónico) o ABTS (Bourbonnais y Paice 1990). En presencia de éste, la lacasa de *Trametes versicolor* puede oxidar compuestos no fenólicos de la lignina. Las lacasas oxidan fácilmente el ABTS convirtiéndolo en el catión radical coloreado $ABTS^{+}$. La intensidad de la coloración (verde – azul) que se produce en la reacción puede correlacionarse con la actividad del enzima. El radical catiónico puede oxidarse aún más hasta $ABTS^{+2}$.

Otros mediadores de descripción más reciente son de tipo N-OH como el 1-hidroxibenzotriazol (HBT), ácido violúrico (VLA) y N-hidroxiacetanilida (NHA).

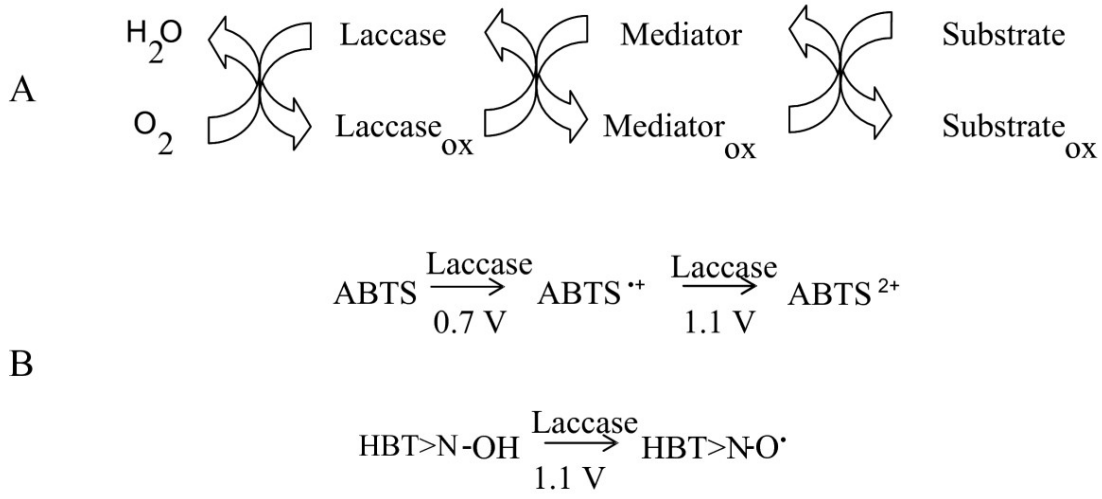

Figura 6. A: Modo de actuación de la lacasa en presencia de mediador. B: incremento del potencial electrónico en presencia de mediador. Figura tomada de Kunamneni et al. (2008)

Se han desarrollado otros mediadores artificiales mediante síntesis química, como el HBT, el VLA y el NHA, nombrados anteriormente, además de la N-hidroxi ftalimida (NHI), y el 2,2,6,6-tetrametilpiperidin-1-iloxi (TEMPO) (Bourbonnais et al. 1997). El uso de estos mediadores han dado lugar a un gran número de estudios sobre los mecanismos de oxidación de sustratos no fenólicos (Baiocco et al. 2003). Sin embargo, también se han descrito mediadores naturales que en comparación con los artificiales presentarían ventajas ambientales y económicas, por tanto las investigaciones actuales se centran en la búsqueda de éstos (Camarero et al. 2005; Cristopher et al. 2014).

En varios sustratos alimentarios existen medidores naturales (Figura 7) que favorecen la degradación de compuestos fenólicos y no fenólicos por parte de las lacasas.

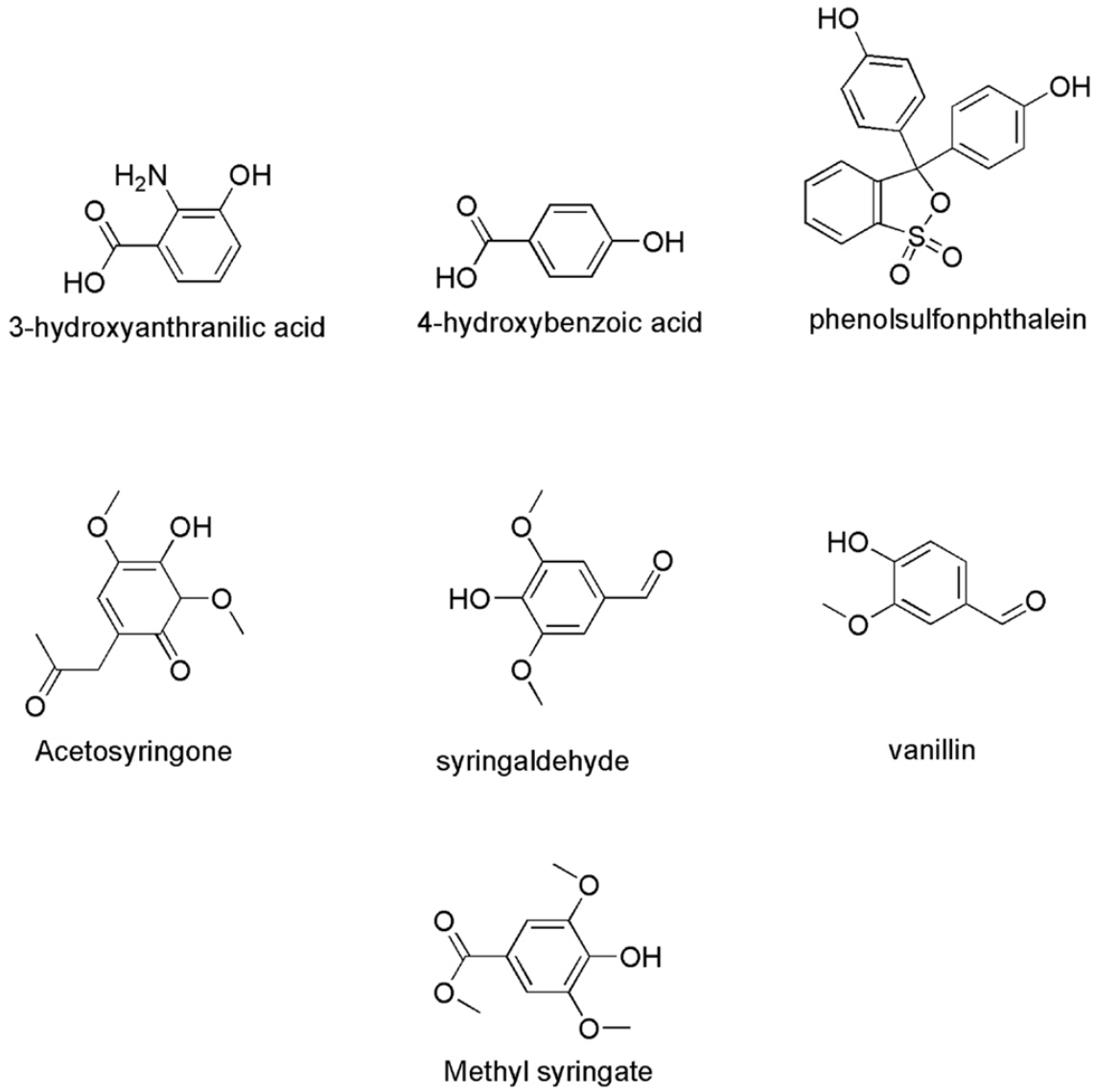

Figura 7. Algunos mediadores naturales de las reacciones de oxidación catalizadas por las lacasas (Christopher et al. 2014).

Las lacasas poseen un gran potencial biotecnológico debido a sus capacidades de reacción, así como amplia especificidad de sustrato. Entre las aplicaciones más prometedoras se incluyen su uso para la construcción de biosensores para el análisis de drogas y para la cuantificación de fenoles en alimentos ricos en ello, como el té (Ghindilis et al. 1992; Peter y Wollenberger 1997), en la síntesis de polímeros (Huttermann et al. 2001) , en los procesos de blanqueo de colorantes textiles y de pasta de papel (Claus et al. 2002; Palonen y Viikari 2004), en estrategias de biorremediación (Murugesan 2003; Wesenberg et al. 2003), como fungicidas, y para la clarificación de zumos y vinos (Sharma et al. 2007).

Las lacasas se han encontrado en los hongos, plantas superiores y también bacterias (Claus 2003; Giardina et al. 2010; Hoegger et al. 2006). En los hongos, estas enzimas están involucradas en la degradación de la lignina, en la producción de pigmentos, y constituyen uno de los mecanismos de ataque del hongo fitopatógeno a la planta (Kunamneni et al. 2007). Las lacasas en las plantas participan en el proceso de lignificación (Giardina et al. 2010; Hoegger et al. 2006; O'Malley et al. 1993; Sharma et al. 2007). Se han investigado las funciones fisiológicas de estas enzimas en microorganismos y se ha descrito que intervienen en la producción de melanina, en los mecanismos de la resistencia de la espora, en morfogénesis, en la división celular, en el transporte de electrones, y en los procesos de desintoxicación de cobre y Mn^{+2} (Tabla 7) (Giardina et al. 2010; Sharma et al. 2007).

Se han estudiado unas pocas lacasas bacterianas en comparación con las de hongos. Sin embargo el rápido progreso en el análisis de genomas sugiere que estas enzimas también están muy extendidas entre las bacterias. Los estudios bioinformáticos sobre las lacasas indican que están presentes en diversas bacterias Gram positivas de alto contenido en G + C y en Gram- de las clases α-, γ- y ε-proteobacteria (Alexandre y Zhulin 2000; Bains et al 2003; Sharma et al 2007). También se han descrito estas enzimas en especies bacterianas tales como *E. coli* (Roberts et al. 2002), diversas especies de *Bacillus* (Hullo et al. 2001; Ruijssenaars y Hartmans 2004; Singh et al. 2014; Zhang et al. 2013, *Thermus thermophilus* (Miyazaki 2005), varios estreptomicetos (Arias et al. 2003; Endo et al. 2003; Gunne y Urlacher 2012). También se han detectado este tipo de enzimas en el metagenoma de muestras de suelos de manglar llegando a la obtención de la enzima (Ye et al. 2010).

La primera lacasa bacteriana que se aisló fue la de *Azospirillum lipoferum,* una bacteria de la rizosfera del arroz (Givaudan et al. 1993). Algunas lacasas de origen bacteriano se han purificado y caracterizado completamente como se puede ver en la Tabla 7.

La mayoría de las lacasas bacterianas son enzimas intracelulares o periplásmicas como ocurre en *A. lipoferum* y *Bacillus subtilis*, sin embargo las fúngicas son extracelulares. Pese a que las lacasas fúngicas son las más usadas en aplicaciones industriales tienen limitaciones que podrían solventarse con las de origen bacteriano. El motivo por el que hasta ahora las aplicaciones existentes se centran exclusivamente en las lacasas fúngicas, se debe al gran número de enzimas de hongos caracterizadas, su alto potencial redox y su eficiencia catalítica hacia una amplia variedad de sustratos. Sin embargo, las lacasas fúngicas están fuertemente glicosiladas, por tanto, no pueden expresarse en huéspedes procariotas. Hasta la fecha, sólo se ha descrito la expresión en *E. coli* de una lacasa fúngica (Salony et al. 2008). Esto hace que los sistemas de expresión utilizados para obtener las enzimas recombinantes sean eucariotas, lo cual hace que la producción y la optimización de las lacasas fúngicas consuma más tiempo debido a que el cultivo y la manipulación genética de estos sistemas de expresión son más complicados (Gunne 2014). Por otra parte, las lacasas fúngicas son activas en

condiciones de reacción ácidas lo que restringe las posibles condiciones de reacción, y su aplicación en procesos industriales a valores de pH neutros o alcalinos como por ejemplo en el tratamiento de ciertas aguas residuales, en el blanqueo de la pulpa de papel, en polvos para lavar, y en la industria de biocombustibles (Baldrian 2006). Algunos autores han tratado de mejorar o ampliar la actividad de las lacasas fúngicas a estos valores de pH, de hecho mediante experimentos de evolución molecular dirigida se consiguió que la lacasa mutante de *Myceliophthora thermophila* tuviera un 80% de su actividad máxima frente al DMP a pH 7, mientras que la enzima de tipo salvaje obtuvo una actividad del 80% a pH 6 (Torres-Salas et al. 2013). Otro obstáculo en la biocatálisis con lacasas fúngicas es su susceptibilidad a varias sustancias. Se ha demostrado que los haluros, iones metálicos, detergentes, y los ácidos grasos comunes entre los residuos industriales, inhiben las lacasas fúngicas incluso en bajas concentraciones (Mate et al. 2013). Por naturaleza, las lacasas bacterianas poseen un rango de pH más amplio e incluso se han descrito lacasas con actividad óptima a pH 9 (Gunne y Urlacher 2012; Machczynski et al. 2004). También se han descrito lacasas en *Bacillus* con actividad a pH neutros y alcalinos (Koschorreck et al. 2008; Ruijssenaars y Hartmans 2004; Zhang et al. 2013). Por otro lado, las lacasas bacterianas generalmente exhiben mayor estabilidad térmica y con frecuencia son más tolerantes a los disolventes orgánicos, alta concentración de sales e inhibidores en comparación con las lacasas fúngicas. A pesar de estas ventajas para las aplicaciones prácticas, la utilización de lacasas bacterianas es insignificante debido a su bajo potencial redox (Singh et al. 2011). Además, aunque las lacasas bacterianas pueden ser expresadas en sistemas de expresión procariotas estándar tales como *E. coli*, su expresión heteróloga a menudo resulta en enzimas que no contienen su centro activo repleto de los iones de cobre, como ocurre en las lacasas Cota de *B. subtilis*, cotA de *Bacillus licheniformis*, SSL1 de *Streptomyces sviceus*, MCOA de *Marinomonas mediterranea*, y ACCV de *Haloferax volcanii* (Enguita et al. 2003; Fernandes et al. 2007; Gunne et al. 2013; Gunne y Urlacher 2012; Koschorreck et al. 2008; Uthandi et al. 2012). En consecuencia, los rendimientos de actividad de las lacasas bacterianas recombinantes son a veces insatisfactorios.

Tabla 7. Posible función de las lacasas bacterianas adaptado de Sharma et al. (2007). *pv (patovar)

Especie	Posible función	Referencia
Leptothrix discophora SS1	Desintoxicación de Mn^{2+} Desintoxicación de especies reactivas del O_2	Adams y Ghiorse (1987)
Pseudomonas maltophilia	Nucleosido oxidasa	Isono y Hoshino (1989)
Pseudomonas syringae *pv. *tomato* (copA)	Resistencia al Cu^{2+}	Cha y Cooksey (1991)
Streptomyces antibioticus	Síntesis fenoxazinona	Freeman et al. (1993)
Azospirillum lipoferum	Pigmentación Oxidación de compuestos fenólicos Transporte de electrones	Givaudan et al. (1993)
Xanthomonas campestris (copA)	Resistencia al Cu^{2+}	Lee et al. (1994)
Bacillus sp. (mnxG)	Esporulación, desintoxicación de Mn^{2+}	van Waasbergen et al. (1996)
Bacillus sphaericus	Esporulación y pigmentación	Claus y Filip (1997)
Phormidium valderianum	Resistencia a altas concentraciones de N_2	Shashirekha et al. (1997)
Pseudomonas fluorescens GB-1	Desintoxicación de Mn^{2+} Desintoxicación de especies reactivas del O_2	Okazaki et al. (1997)
Marinomonas mediterranea (ppoA)	Pigmentación	Sanchez-Amat et al. (2001)
Aquifex aeolicus (suf1)	Proteína de division celular	Deckert et al. (1998)
Pseudomonas putida GB1	Desintoxicación de Mn_2^+	Brouwers et al. (1999) Bromberg y Duran (2001) Senan y Abraham (2004)
Bacillus subtilis (cotA)	Pigmentación de esporas Resistencia a UV y H_2O_2	Hullo et al. (2001)
E. coli (yacK)	Resistencia al Cu^{2+} oxidación de sideróforos actividad ferro-oxidasa	Kim et al. (2001) Roberts et al. (2002)
Pseudomonas sp. (CumA)	Desintoxicación de Mn_2^+	Francis y Tebo (2001)
α-proteobacterium SD21	Desintoxicación de Mn_2^+	Francis y Tebo (2001)
Pyrobaculum aerophilum (pae1888)	Desconocida	Fitz-Gibbon et al. (2002)
γ-proteobacterium JB	Oxidación de compuestos tóxicos	Bains et al. (2003) Malhotra et al. (2004)
Bacillus halodurans C-125(lbh 2082)	Resistencia al Cu^{2+}	Ruijssenaars y Hartmans (2004)
Sinorhizobium meliloti	Termoestabilidad	Rosconi et al. (2005)
Stenotrophomonas maltophilia	Degradación de colorantes sintéticos	Galai et al. (2009)
Rhodococcus sp.	Degradación de polietileno	Santo et al. (2013)

5. Métodos para el control de aminas biógenas:

5.1 Estrategias preventivas para evitar la producción de aminas biógenas:

La mayor parte de las aproximaciones que se han ideado para evitar el problema de las aminas en alimentos se han basado en evitar su producción, lo cual se puede lograr a distintos niveles: reduciendo los niveles de precursores (aminoácidos), limitando el crecimiento de las bacterias alterantes productoras de AB, o usando cultivos iniciadores en los procesos fermentativos que carezcan de los genes responsables de la descarboxilación de los aminoácidos precursores de la mismas.

Tradicionalmente se han usado métodos físicos para reducir las AB en alimentos, su acción se basa en limitar el crecimiento de los microorganismos alterantes o en eliminarlos. La forma más habitual hasta actualidad es el uso de bajas temperaturas, es decir, mantener los alimentos refrigerados o congelados (Duflos 2009; Prester 2009). Sin embargo en algunos productos derivados del pescado y otros alimentos como por ejemplo el vino esto es imposible. Es por ello que ya hay estudios sobre métodos físicos alternativos en alimentos que no puedan mantenerse a baja temperatura como es la aplicación de altas presiones hidrostáticas, irradiación e incluso empaquetado en atmosfera controlada (Naila et al. 2010). El uso de altas presiones hidrostáticas es un método de conservación no térmico que produce daños en las membranas celulares de los microorganismos provocando su inactivación o lesiones subletales (Rivas 2008), esta técnica se ha utilizado en algunos zumos, jamón cocido y guacamole.

La adición de compuestos antimicrobianos o de aditivos que modifiquen las características físico-químicas de los alimentos permite limitar el crecimiento de los microorganismos. Así por ejemplo el uso de ácidos o sus sales, tales como ácido sórbico, cítrico, succínico, málico, ascórbico, sorbato sódico o potásico, hexametafosfato sódico y nitrito sódico han logrado disminuir o retrasar la formación de aminas en diversos alimentos como la caballa (Shalaby 1996; Shalaby y El-Rahman 1995), marisco (Shalini 2001), salchichas (Bozkurt y Erkmen 2004; Kurt y Zorba 2009) o col en vinagre (Yuecel 2008), debido a que producen una disminución del pH.

Por otro lado aditivos como por ejemplo los azúcares y sus derivados se incluyen normalmente en la fabricación industrial de productos cárnicos fermentados, aunque los procesos tradicionales pueden omitir este ingrediente. Durante la fermentación y maduración, las bacterias de ácido láctico convierten la glucosa (la fuente de energía primaria) en ácido láctico, que es el componente principal responsable de la disminución del pH. Esta acidificación tiene un efecto conservante que domina la microbiota competitiva, y contribuye al desarrollo de las típicas características organolépticas de los embutidos fermentados (Hugas y Monfort 1997). Algunos autores han comprobado que la adición de azúcares

disminuye las AB como por ejemplo la delta-glucono–lactona en carne (Maijala 1993a) y la glucosa y lactosa en salchichas (Bover-Cid et al. 2001). También se ha estudiado el efecto de la adición de glicina sobre la producción de AB, ya que inhibe la actividad descarboxilásica de los microorganismos. Algunos autores describen una disminución en la producción de AB cuando se añade glicina al Myeolchi-jeot, un producto fermentado a partir de anchoas saladas (Mah y Hwang 2009a).

También se ha demostrado que el uso de especias y hierbas aromáticas inhiben la formación de AB en alimentos (Komprda et al. 2004). Entre los componentes de estos productos que han demostrado tener estos efectos se incluyen la curcumina (presente en la cúrcuma), la capsaicina (en pimiento rojo), la piperina (en pimienta negra) (Shakila 1995; Wendakoon 1992), el timol (un monoterpeno fenólico que tiene propiedades antioxidantes y antimicrobianas) (Singh 1999), la alicina (proveniente del ajo) (Mah et al. 2009) y el 6-gingerol, (componente picante del jengibre) (Singh 1999). Un componente de la canela (aldehído cinámico) y del clavo (eugenol), también inhibieron eficazmente la formación de AB por *Enterobacter aerogenes* (Wendakoon 1995). La desventaja de estas sustancias es la considerable pérdida de eficacia que se produce durante la cocción (Suresh 2007).

Aunque los estudios han demostrado los efectos inhibitorios de los aditivos, especias y conservantes en la acumulación de AB, algunos autores han destacado sus potenciales efectos negativos tanto en el alimento como en el consumidor. Uno de estos efectos es el hecho de que la curcumina inhibe la DAO que es una de las enzimas implicadas en la desintoxicación natural de las AB en humanos (Bhutani et al. 2009).

En resumen, se requiere más investigación sobre la eficacia de aditivos y conservantes para controlar la producción de AB. Los aditivos alimentarios que retrasan la formación de AB necesitan probarse en gran variedad de sustratos alimentarios antes de determinar su aplicación real. Esta razón y el hecho de que algunas legislaciones no permiten el uso de determinados antimicrobianos y aditivos son factores que limitan su empleo.

En el caso del vino las estrategias usadas para prevenir la síntesis de AB han sido limitar las prácticas enológicas que conlleven un aumento de los aminoácidos precursores como, las maceraciones largas, la crianza sobre lías o la adición de aminoácidos (Ferrer y Pardo 2005) y el uso de sustancias antimicrobianas como el SO_2 o la lisozima. Sin embargo, la imposibilidad de evitar las prácticas enológicas antes nombradas, sobre todo cuando se pretende obtener vinos de alta expresión, y la acción limitada de la lisozima y el SO_2 (Polo et al. 2010) hacen que no sea fácil solventar el problema. La estrategia que se ha revelado como más factible para reducir los niveles de aminas en los vinos es el uso de cultivos iniciadores que carezcan de actividad descarboxilásica para controlar la fermentación maloláctica (López 2008).

5.2 Estrategias curativas para la eliminación de aminas biógenas:

5.2.1. Métodos físico-químicos

La irradiación con rayos γ tiene un doble efecto, ya que produce la degradación por radiólisis de las AB (Mbarki et al. 2008), además de disminuir la cantidad de microorganismos (Kim et al. 2004). Esta técnica se ha aplicado en carne de cerdo, ternera (Min et al. 2007), salchicha (Kim et al. 2005a), pasta de soja (Kim et al. 2005b), pollo (Min 2007) y pescado (Mbarki et al. 2008). En el caso vino es posible reducir la concentración de AB tratando el vino con clarificantes u otros medios enológicos, a pesar de que los ensayos de diferentes investigadores no siempre producen los mismos resultados. Estos productos eliminan las AB porque se adsorben a la superficie de estas y se eliminan de forma conjunta. Las sustancias utilizadas son la bentonita, la cola de pescado (Woller 2005) y la polivinil pirrolidona (PVP) (Moreno-arribas 2007). La bentonita es una arcilla impura formada por la erosión de las cenizas volcánicas, es un material cargado positivamente que es capaz de unir compuestos, las partículas de carga negativa forman agregados de alto peso molecular que precipitan. En los trabajos de Mannino et al. (2006) y de Kally y Body- Szalkai (1996) se describe que la aplicación de 80 g/Hl de bentonita reducía en un 60% la histamina de vinos tintos y que el porcentaje de eliminación era dosis dependiente. De acuerdo con la investigación realizada por Grossmann et al. (2007), la bentonita es más eficaz en el mosto que en el vino, del cual se elimina en menor cantidad. Esto es especialmente evidente para la histamina. La bentonita elimina mejor la histamina y tiramina, y en menor medida la putrescina, espermina y espemidina. La cola de pescado sólo disminuye la concentración de histamina, tiramina y putrescina pero en menor medida que la bentonita (Grossmann et al. 2007).

5.2.2. Métodos biológicos:

Uso de enzimas o extractos capaces de degradar las aminas biógenas:

Varios autores han investigado la eficacia del uso de diversas amino oxidasas para reducir las concentraciones de AB en alimentos. Se han publicado varias patentes acerca de los procedimientos para la obtención de preparados enzimáticos de AO de diversas fuentes, como órganos de animales y microorganismos (Carlos y Georgina, 1985; Hiemenz y Setz, 1942; Underberg y Lembke, 1988; Williams, 1943). En 1985, la patente Nº EP0132674 describe la preparación y utilización de la amino oxidasa de *Aspergillus niger* IMI17454 para eliminar aminas en el queso, cerveza, mosto o extracto de levadura (Hobson y Anderson 1985), sin embargo, aunque los autores mencionaron que se conseguía la reducción de AB en mosto de uva, no proporcionaron datos concretos acerca de su eficacia en este sustrato o en el vino. Otra patente describe la obtención de histaminasas a partir de células de especies de los géneros *Lactobacillus* y *Candida* y su uso para eliminar BA de productos alimenticios, bebidas y forrajes (Underberg y Lembke 1988).

Por otro lado, en el trabajo de Dapkevicius et al. se analizó la eficacia de una diamina oxidasa (DAO) purificada a partir de riñón porcino para la degradación de histamina, tanto en tampón fosfato (pH 7.0) como en ensilado de pescado (pH 4.5). Estos autores constataron que la degradación no se producía a valores bajos de pH (4.5); este hecho limitaba mucho el uso de este enzima en muchos procesos fermentativos ya que en ellos se alcanzan valores de pH inferiores a 4.5 (Dapkevicius et al. 2000).

En el trabajo de Cueva et al. (2012) se describe la capacidad de degradar histamina, tiramina y putrescina en vino, utilizando extractos de una cepa del hongo *Penicillium citrinum* procedente de un viñedo. Los resultados de este trabajo dieron lugar a la patente WO2013050641A1.

Hasta ahora, a excepción de la DAO, ninguna de las enzimas descritas en los trabajos anteriores están caracterizadas ni provienen de microorganismos de origen alimentario.

Uso de microorganismos capaces de degradar las AB

Basándose en el hecho de que las enzimas AO son responsables de la desintoxicación de AB de la dieta, y que dichas enzimas también se han encontrado en bacterias, los primeros trabajos encaminados a encontrar AO se centraron en la prospección de tales actividades en microorganismos aislados de los alimentos. El estudio de la capacidad de los microorganismos para degradar las AB en medios de cultivo o sistemas tampón ha sido objeto de muchos trabajos, que han descrito qué cepas poseían esa capacidad. Una vez identificadas estas bacterias, algunas de ellas se han utilizado para disminuir el contenido de AB en algunos alimentos. Voigt y Eitenmiller (1978) mencionaron la capacidad de las BAL para degradar la histamina y tiramina en algunas cepas de las especies *Streptococcus lactis, Streptococcus liquefaciens*, *Leuconostoc cremoris* y *Microbacteriun lacticum*. Más tarde, Leuschner et al. (1998) encontraron que algunos microorganismos aislados de los alimentos, entre ellos BAL, eran capaces de degradar AB, siendo la actividad degradadora de histamina más frecuente que la de tiramina y que las cepas más degradadoras pertenecían a las especies *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus pentosus* y *Pediococcus acidilactici*. Martuscelli et al. (2000) encontraron que 21 de las 26 cepas de *Staphylococcus xylosus* reducían la histamina, y/o tiramina en tampón fosfato (pH 7). De acuerdo con Dapkevicius et al. algunas cepas de *L. sakei* y *Lactobacillus curvatus* aisladas de pasta de pescado (caballa) eran capaces de degradar la histamina en medio MRS modificado. En 2001, Fadda et al. describieron que algunas cepas de *Lactobacillus casei* y de *L. plantarum* aisladas de carne poseían actividades degradadoras de tiramina. Más tarde, también se describió que una cepa de una arquea halófila perteneciente a la especie *Natrinema gari,* y que fue aislada de salsa de anchoa; presentaba la capacidad de degradar la histamina en medio con alto contenido en sal (Tapingkae et al. 2010). Estos autores constataron que el pH óptimo y la temperatura para

la degradación estaban por encima de 6.5 y 40 ° C, respectivamente. Sin embargo, no comprobaron su actividad en alimentos, por lo que se desconoce su utilidad.

Leuschner y Hammes (1998a) consiguieron reducir en un 70% y en un 55% la histamina y la tiramina, respectivamente, en queso Munster inoculando éste con varias cepas de *Brevibacterium linens*. En el mismo año, los mismos autores utilizaron una cepa de *Micrococcus varians*, que presentaba actividad tiramina oxidasa para degradar la tiramina que se producía durante la maduración de embutidos (Leuschner y Hammes 1998b). En el año 2000, Dapkevicius et al. utilizaron 2 cepas de *L. sakei* para degradar histamina en una pasta de pescado ensilado. En 2002, Gardini et al., consiguieron disminuir las concentraciones de tiramina, espermina y espermidina en embutidos curados inoculados con un cultivo mixto de *L. sakei* G-20 y *Staphylococcus xylosus* S8. Más tarde, en 2007, Yongsawatdigul et al. describieron que la inoculación con *Virgibacillus* sp. SK33 en una salsa de pescado tailandesa, reducía la concentración de histamina en un 50%. Más recientemente Mah y Hwang (2009b) lograron la reducción de aminas biógenas en un 16% en Myeolchi-jeot, una anchoa (*Engraulis japónicas*), salada y fermentada mediante el uso de cultivos iniciadores de *Staphylococcus xylosus* en la maduración. Con respecto al vino, García-Ruiz et al. (2011) demostraron que algunas BAL de origen vínico pertenecientes a los géneros *Lactobacillus* y *Pediococcus* eran capaces de degradar histamina, tiramina y putrescina en una versión modificada del medio descrito por Dapkevicius et al. (2000), sin embargo estas cepas no fueron capaces de reducir la concentración de AB en el vino, solo la cepa *L. casei* IFI-CA52 degradó un 16 % de histamina, un 15 % de tiramina y un 8 % de putrescina en vino. En la tabla 8 se muestra un resumen de las especies descritas en la bibliografía con capacidad de degradar las AB.

Tabla 8. Especies de hongos y bacterias capaces de degradar aminas biógenas. *B. (Bacillus), Br. (Brevibacterium), E. (Epicoccum), G. (Geotrichum), L. (Lactobacillus), O. (Oenococcus), P. (Pediococcus), Pen. (Penicillium), St. (Staphylococcus), U. (Ulocladium)*

Amina biógena	Especies	Sustrato	Referencia
Histamina Tiramina	*L. plantarum*, *L. sakei*, *L. pentosus*, *P. acidilactici*, *Rhodococcus* sp., *Arthrobacter* sp, *Micrococcus* sp., *Br.linens*, *G. candidum*	Tampón	Leuschner et al. (1998)
Histamina Tiramina	*Br. linens*	Queso Munster	Leuschner y Hammes (1998a)
Histamina	*L. sakei*	Pescado ensilado	Dapkevicius et al. (2000)
Histamina	*B. amyloliquefaciens*, *St. carnosus*	Tampón	Zaman et al. (2010)
Putrescina Cadaverina	*B. subtilis*, *St. intermedius*	Tampón	Zaman et al. (2010)

Amina biógena	Especies	Sustrato	Referencia
Histamina	*B. amyloliquefaciens*, *St. carnosus*	Salsa de pescado fermentada	Zaman et al. (2011)
Histamina	*St. xylosus*	Tampón	Martuscelli et al. (2000)
Tiramina	*L. casei*, *L. plantarum*	Tampón	Fadda et al. (2001)
Histamina Tiramina	*L. casei*	Queso Cabrales	Herrero-Fresno et al. (2012)
Histamina Tiramina Putrescina	*L. casei*, *L. hilgardii*, *P. parvulus*, *O. oeni*, *L. plantarum*, *P. pentosaceus*	Medio de cultivo	García-Ruiz et al. (2011)
Histamina Tiramina Putrescina	*L. casei*	Vino	García-Ruiz et al. (2011)
Tiramina Putrescina	*L. plantarum*	Medio de cultivo	Capozzi et al. (2012)
Histamina Tiramina Putrescina	*Pen. citrinum*, *Alternaria* sp., *Phoma* sp., *U. chartarum*, *E. nigrum*	Medio de cultivo Vinos comerciales	Cueva et al. (2012)
Histamina Tiramina Putrescina	*L. plantarum, P. acidilactici*	Medio de cultivo Vinos	Callejón et al. (2014)

6. AB en vinos

6.1 Presencia de AB en vino: Antecedentes históricos

La histamina fue hallada por primera vez en el vino en 1954 por Tarantola (1954). Inicialmente no se atribuyó mucha importancia a este hecho, ya que se había encontrado en otros alimentos en concentraciones más elevadas sin que se detectasen afecciones relacionadas con su consumo. Sin embargo, a partir del trabajo de Marquardt (1968), en el que se relaciona el consumo de vino con histamina y daños en la salud de los consumidores, se exigió la retirada de aquellos vinos que contuviesen concentraciones superiores a 2 mg/L. Poco después, la presencia de otras aminas como la tiramina, la putrescina, la cadaverina, espermina y espermidina suscitó un interés adicional. Woller (1990) determinó la presencia de 22 aminas diferentes en el vino. La presencia de AB en esta bebida es un fenómeno recurrente, recogido en muchos trabajos, como puede verse en el trabajo de revisión de Guo et al. (2015).

6.2 Origen de la formación de aminas en vino, factores implicados y necesidad de control:

Según la Oficina Internacional de la Viña y del Vino (OIV), fundada en 1.924 y constituida hoy por las representaciones de los gobiernos de 45 países: "El vino es la bebida resultante exclusivamente de la fermentación total o parcial de la uva fresca o del mosto".

La concentración de AB depende de los mismos factores que afectan a la presencia de los precursores. En cuanto a la materia prima y factores enológicos que influyen en la concentración de aminas, hay estudios donde se han encontrado AB ya en la uva (Moreno-Arribas et al. 2003) y en los mostos (Wang et al. 2014) y que la concentración inicial depende de la variedad de uva, de su grado de maduración e incluso de la composición y tipo de suelo. En el trabajo de Landete et al. 2005 se demostró que la concentración de putrescina en el vino estaba más relacionada con la región geográfica y la variedad de uva que las prácticas enológicas. Algunos autores también han corroborado la influencia de la añada en la producción de AB (Ortega-Heras et al. 2014), seguramente debida a las diferentes condiciones climáticas.

Por otro lado, a lo largo del proceso de vinificación se presentan diversas especies de hongos, levaduras y bacterias. De entre estos tres grupos de microorganismos, las levaduras y las BAL tienen un papel relevante en la vinificación (Lonvaud-Funel 1999). Además de las BAL, también se han aislado del mosto y del vino bacterias acéticas (BA) cuyo efecto es perjudicial para la calidad del vino. En el proceso de vinificación las levaduras, principalmente las de la especie *Saccharomyces cerevisiae,* se desarrollan en el mosto y van transformando los azúcares del mismo en etanol y anhídrido carbónico mediante la fermentación alcohólica (FA), (Fleet 2006). El metabolismo de los azúcares, ácidos y aminoácidos del mosto por parte de las levaduras da lugar a la producción de una amplia gama de sustancias organolépticas como ácidos orgánicos, glicerol, alcoholes, ésteres, aldehídos, cetonas, aminas y compuestos azufrados volátiles. Éstos compuestos son los que determinan, principalmente, en el carácter del vino (Cocolin et al. 2001; Suárez 1997). Se ha demostrado que la formación de AB también depende de la duración de la FA en presencia de pulpa y hollejos (Marques et al. 2008; Smit et al. 2013), de manera que cuanto más tiempo están en contacto con el vino mayor es la concentración de AB. Algunos autores han observado que las AB pueden aumentar durante la FA como consecuencia de la actividad de ciertas levaduras como por ejemplo Caruso et al. (2002) y Goñi y Azpilicueta (2001), que además observaron que algunas cepas de *Kloeckera apiculata*, *Brettanomyces bruxellensis* y *Metschnikowia pulcherrima* formaban agmatina y ß-feniletilamina, mientras que *Saccharomyces cerevisiae* producía etanolamina. Sin embargo, se ha llegado a la conclusión de que la formación de AB por la levadura durante la FA es mucho menos importante que el contribución de las BAL durante la fermentación maloláctica (Smit et al. 2013).

La mayoría de vinos tintos y algunos vinos blancos se someten a una segunda fermentación llevada a cabo por bacterias llamada fermentación maloláctica (FML). La FML consiste en la conversión del ácido málico, presente de forma natural en el vino, en ácido láctico y CO_2. Esta fermentación pueden llevarla a cabo todas las BAL presentes en el proceso de vinificación. Puede producirse espontáneamente, (Davis et al. 1986), pero es

recomendable inducirla, seleccionando el momento adecuado y el inoculo apropiado para su realización si se desea evitar riesgos derivados de la actuación de cepas desconocidas. *Oenococcus oeni* es la especie que lleva a cabo este proceso más frecuentemente. A pesar de que inicialmente se consideró que esta especie no producía AB, varios autores han demostrado que algunas cepas son capaces de producir histamina y putrescina (Landete et al. 2007b). Es por ello que varios autores que han estudiado el momento de la vinificación en el que éstas se producen, han observado que es tras la fermentación maloláctica y durante la crianza cuando ocurre con mayor intensidad (Jiménez Moreno et al. 2003; Marcobal et al. 2006b; Polo et al. 2010). La explicación puede ser la supervivencia de BAL en la lías que estén en contacto con el vino y también el hecho de que las descarboxilasas pueden continuar activas en el vino incluso cuando la bacteria está muerta (Kanki et al. 2007).

Como en otros alimentos la presencia de AB en el vino supone la coincidencia de tres factores diferentes: la existencia de precursores (aminoácidos); la presencia de microrganismos con la actividad descarboxilasa correspondiente; y la concurrencia de las condiciones ambientales adecuadas (Ferrer y Pardo 2005). Debido al cambio climático actualmente los vinos son menos ácidos y de pH alto, por lo que la proliferación de bacterias capaces de producir AB es mayor. Varios autores han demostrado la correlación existente entre el pH de los vinos y la concentración de AB, entre ellos Landete et al. describieron una menor formación de aminas a valores de pH reducidos (Landete et al. 2005b), por otro lado Wang et al. (2014) describieron que los vinos con valores de pH por encima de 3.7 tenían mayor concentración de aminas.

Además de comprometer la calidad de vino la presencia de AB puede comprometer la salud del consumidor, como ya se ha apuntado para otros alimentos, pero en el caso de las bebidas alcohólicas, como el vino y la cerveza, los efectos tóxicos de las mismas se potencia por el etanol presente en las mismas (Ladero et al. 2010; Maintz y Novak 2007). De las 22 aminas encontradas en los vinos, la más abundante es la putrescina y la más peligrosa la histamina, seguida de la tiramina (Landete 2005).

La putrescina es la amina más abundante en vino ya que se puede encontrar en la propia uva, pero también puede ser sintetizada por los microorganismos asociados a la vinificación. Además, la putrescina puede originarse a partir de varios aminoácidos precursores como son la arginina, la ornitina y la agmatina. La arginina es el aminoácido más abundante en el vino, hasta hace poco solo se conocían dos vías implicadas en el catabolismo de la arginina. Una de ella es la encontrada en cepas de *Lactobacillus fermentum*, en ellas se ha demostrado la producción de óxido nítrico desde arginina. Según Morita et al. (1997) esta reacción es catalizada por la enzima óxido nítrico sintetasa que puede también producir citrulina como un subproducto. Una vía más común para el catabolismo de la arginina en bacterias lácticas es la vía de la arginina deiminasa (ADI) (Cunin et al. 1986; Konings et al.

1995; Liu et al. 1995; Manca de Nadra et al. 1982). Esta vía resulta en la conversión de arginina en el interior celular a citrulina y amonio mediante la enzima ADI, posteriormente la citrulina es transformada en carbamilfosfato y ornitina; la ornitina sale al exterior celular a través de un antiportador arginina/ornitina situado en la membrana plasmática. El carbamilfosfato, da lugar a amonio y dióxido de nitrógeno mediante la actividad de la carbamatoquinasa, con la consiguiente generación de energía en forma de ATP. La ornitina se transforma en putrescina mediante una reacción de descarboxilación llevada a cabo por la ornitina descarboxilasa. El catabolismo de arginina por bacterias lácticas del vino se ha relacionado con la producción de etilcarbamato, una sustancia cancerígena que se forma a partir de la citrulina o de la urea (Liu et al. 1995). Además de estas dos vías se ha descrito una tercera vía de catabolismo de la arginina. Ésta consiste en la trasformación de arginina en agmatina vía la arginina descarboxilasa, la agmatina a su vez puede seguir dos rutas, una de ellas es la trasformación en putrescina directamente mediante la agmatina deiminasa y la otra es la formación de N-carbamoilputrescina y posteriormente en putrescina por la acción secuencial de la agmatinasa y la N-carbamoilputrescina hidrolasa. En cantidades elevadas puede producir un sabor desagradable en los vinos afectando a su calidad organoléptica (Pérez Hernández et al. 2006).

Se ha demostrado que las cepas productoras de histamina en vino pertenecen a las especies Pediococcus parvulus, Lactobacillus hilgardii y O. oeni. Esta última especie es la que presenta un porcentaje más alto de cepas que poseen el gen *hdc* (aproximadamente un 80% de las cepas), que codifica para la histidina descarboxilasa, enzima responsable de la síntesis de histamina (Landete et al. 2005). Afortunadamente, las cepas de esta especie suelen tener actividades de histidina descarboxilasa bajas y no contribuyen demasiado a la cantidad de histamina presente en el vino: raramente producen más de 5 mg/L en el vino. Por el contrario, aunque la incidencia de cepas productoras de histamina es menor en *P. parvulus* y *L. hilgardii,* ésta cepas son capaces de producir entre 40 y 50 mg/L en el vino. Algunas cepas de otras especies de lactobacilos y leuconostocs, también pueden producir cantidades intermedias de histamina. Un 78% de cepas de *Lactobacillus brevis* producen tiramina, y un 25% de las cepas ensayadas de *L. hilgardii* (Landete et al. 2007b). En el trabajo de Landete et al. (2007c), se ha demostrado que existe una correlación entre la síntesis de tiramina y feniletilamina, quizá porque el mismo enzima cataliza ambas actividades. En la Tabla 10 se muestran las especies responsables de la síntesis de histamina, tiramina, y putrescina en vinos.

Tabla 10. Especies de BAL capaces de producir AB en vinos.

AB	Especie	Referencias
Histamina	*Lactobacillus* sp 30 a	(Costantini et al. 2006; Moreno-Arribas et al. 2003)
	L. buchneri	Landete et al. (2005)
	L. hilgardii	(Costantini et al. 2006; Farías et al. 1993; Landete et al. 2005; Lucas et al. 2005)
	L. mali	(Landete et al. 2005)
	L. parabuchneri	(Costantini et al. 2009)
	L. rossiae	(Costantini et al. 2009)
	Lc. mesenteroides	(Landete et al. 2005)
	O. oeni	(Coton et al. 1998b; Guerrini et al. 2002; Landete et al. 2005; Lonvaud-Funel y Joyeux 1994)
	P. damnosus	(Aerny 1985; Delfini 1989)
	P. parvulus	(Landete et al. 2005)
Tiramina	*L. brevis*	(Costantini et al. 2006; Landete et al. 2007b; Lucas et al. 2003; Lucas y Lonvaud-Funel 2002; Moreno-Arribas et al. 2000)
	L. curvatus	(Masson et al. 1996)
	L. hilgardii	(Landete et al. 2007b; Moreno-Arribas et al. 2000)
	L. plantarum	(Arena et al. 2007)
	Lc. mesenteroides	(Moreno-Arribas et al. 2000)
Putrescina	*Lactobacillus* sp 30 a	(Tabor y Tabor 1985)
	O. oeni	(Coton et al. 1999; Guerrini et al. 2002; Mangani et al. 2005; Marcobal et al. 2004)
	L. buchneri	(Moreno-Arribas et al. 2003)
	L. hilgardii	(Arena y Manca de Nadra 2001)
	L. plantarum	(Arena y Manca de Nadra 2001)

La producción de alimentos con una menor concentración de AB es una preocupación en todo el mundo. En el proyecto europeo BiamFood (Comunidad europea, 2011), se especifica lo siguiente: “El control de aminas biogénas en los productos alimenticios reducirá significativamente el riesgo de salud para el consumidor y, por lo tanto, aumentará la competitividad de las industrias locales”. Además, la Organización Internacional de la Viña y el Vino (OIV, 2011) publicó el "Código de buenas prácticas vitivinícolas” con el fin de reducir los AB en vino, ya que además de los problemas de la salud, la presencia de elevadas concentraciones de aminas en vinos puede suponer una barrera a la exportación. Varios países han establecido límites recomendados a los contenidos de histamina de los vinos. No existe legislación europea acerca de límites máximos permisibles de AB en vinos, aunque en varios países existen distintos valores aceptados como niveles máximos así, por ejemplo, el nivel máximo de histamina en Alemania es de 2 mg/L, en Bélgica 5-6 mg/L, en Suiza y Austria 10 mg/L, en Francia 8 mg/L y en Holanda 3 mg/L. Ello supone barreras comerciales para la exportación de vinos a estos y otros países. Por ello, es importante conocer la evolución de las AB durante el proceso de vinificación ya que la concentración final de las mismas podría condicionar su destino en los mercados europeos.

Ninguna de las estrategias citadas en el apartado 5.2 de este trabajo ha proporcionado una metodología eficaz en la eliminación de AB de vino. Las condiciones físico-químicas del vino previenen el desarrollo y las actividades de la mayoría de las enzimas o microorganismos que se han usado para eliminar las AB en otros tipos de alimentos. Por tanto, el uso de microorganismos capaces de desarrollarse en vino y con capacidad degradadora de AB o de sus enzimas podría paliar el problema que suponen estos metabolitos a nivel de calidad, seguridad alimentaria y competitividad en el mercado.

Objetivos

1. Búsqueda de cepas bacterianas de origen alimentario, capaces de degradar aminas biógenas.
2. Aislamiento, purificación e identificación de las enzimas responsables de la degradación de aminas biógenas. Determinación y caracterización de la actividad de la enzima purificada de *K. varians* LTH 1540
3. Clonaje y la caracterización de las enzimas degradadoras de aminas biógenas de *Lactobacillus plantarum* J16 CECT 8944 y de *Pediococcus acidilactici* CECT 5930.
4. Aplicación de sistemas biológicos para degradar aminas biógenas en vinos.

Resultados

Los resultados correspondientes a los objetivos especificados para este trabajo se exponen en los capítulos y en las publicaciones siguientes:

Objetivo 1: Capítulo 1 y artículos 1 y 2

Objetivo 2: Capítulo 2 y artículos 1 y 2

Objetivo 3: Capítulo 3 y artículos 3 y 4

Objetivo 4: Capítulo 1 y artículos 1 y 2

Artículo 1: S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2014. Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine. Appl. Microbiol. Biotechnol., 98:185-198.

Artículo 2: S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2015. Ability of *Kocuria varians* LTH 1540 to degrade putrescine: identification and characterization of a novel amine oxidase. J. Agric. Food Chem., 63: 4170−4178.

Artículo 3: S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2016. Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation. Appl. Microbiol. Biotechnol., 100: 3113-3124.

Artículo 4: Recombinant laccase from *Pediococcus acidilactici* CECT 5930 with ability to degrade tyramine. Enviado a Biotechnology journal.

En la presente Tesis Doctoral se ha realizado una extensa búsqueda de bacterias de origen alimentario capaces de degradar histamina, putrescina y tiramina. Se ha estudiado la capacidad de células enteras para degradar histamina, putrescina y tiramina, en distintos sustratos: tampón, medios de cultivo y vino. También se ha ensayado la capacidad de los extractos celulares de degradar AB en gel de poliacrilamida, mediante zimograma. Además, se han aislado, purificado, identificado y caracterizado a nivel bioquímico las enzimas responsables de dicha degradación; en dos de los casos la caracterización bioquímica ha requerido del clonaje del gen en *E. coli* para obtener suficiente cantidad de proteína.

En cuanto a los experimentos con extractos, de los 77 extractos probados 40 extractos de BAL (el 52,6 % de las mismos) y el de *Kocuria varians* dieron lugar a la aparición una única banda de color marrón en el ensayo en gel cuando en la mezcla de reacción se pusieron las tres aminas. Las cepas que presentaron una reacción positiva frente a las aminas pertenecían a las especies: *Enterococcus faecium, Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus hilgardii, Lactobacillus mali, Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus vini, Pediococcus acidilactici, Pediococcus parvulus y Pediococcus pentosaceus,* además de *Kocuria varians*.

En los experimentos con células enteras se ensayaron un total de 14 cepas y todas ellas degradaban una o más aminas tanto en medio sintético como en vino aunque con diferentes eficiencias. Las cepas degradadoras pertenecían a las especies *Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus paracasei, L. plantarum, P. acidilactici, Enterococcus faecium* y *K. varians*.

En cuanto a la identificación de las enzimas responsables de la degradación de AB, se han identificado mediante análisis proteómicos las enzimas en las cepas *L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540. En los dos primeros casos se trataba de un tipo de enzimas que no se había relacionado previamente con esta función, eran lacasas (EC 1.10.3.2) pertenecientes a las MCO. La enzima responsable de la degradación de aminas en *K. varians* era una putrescina oxidasa (EC 1.4.3.10). La actividad de este último tipo de enzimas ya se conocía anteriormente pero es la primera vez que se describe la presencia de esta enzima en la especie *K. varians.* La lacasa de *L. plantarum* degrada principalmente la tiramina y en menor grado histamina y putrescina, la de *P. acidilactici* sólo degrada la tiramina y la putrescina oxidasa de *K. varians* degrada principalmente diaminas, concretamente, putrescina y cadaverina y, en menor grado poliaminas, como espermidina, aunque es incapaz de degradar monoaminas.

Las enzimas identificadas en *L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540 se han caracterizado bioquímicamente. La baja cantidad de proteína obtenida a partir de la purificación de los extractos celulares de las dos primeras cepas requirió del clonaje de genes codificantes de las proteínas en *E. coli*. Se ha demostrado que, a pesar de tratarse de enzimas pertenecientes al mismo grupo, las lacasas de *L. plantarum* y de *P. acidilactici* difieren en sus masas moleculares, en su rango de sustratos, en sus constantes cinéticas y en sus pH y temperaturas óptimas de actuación. Las principales diferencias entre las lacasas de bacterias lácticas y la putrescina oxidasa de *Kocuria varians* es, además de la preferencia de esta última por las diaminas, el que tiene un pH óptimo de actuación neutro o ligeramente alcalino, y una estabilidad térmica mucho más baja.

Los logros más importantes que se derivan de este trabajo son:

Es la primera vez que se describe la degradación de aminas en vino de forma eficaz por parte de las bacterias lácticas de las especies: *Lactobacillus farciminis, Lactobacillus plantarum* y *Pediococcus acidilactici.* Así como de la cepa *Kocuria varians* de origen alimentario.

Es el primer trabajo donde se demuestra que las enzimas lacasa (EC 1.10.3.2) son capaces de degradar las AB.

Es la primera vez que se han identificado las enzimas responsables de la degradación de tiramina, histamina y putrescina en las especies *L. plantarum* y *P. acidilactici*.

Se ha identificado una putrescina oxidasa (EC 1.4.3.10) en la especie *K. varians* que no se había descrito previamente.

Es la primera vez que se describe que la enzima lacasa del hongo filamentoso *T. versicolor* es capaz de degradar tiramina, histamina y putrescina en tampón, y las dos primeras también en vino.

Capítulo 1

Búsqueda de cepas bacterianas de origen alimentario, capaces de degradar aminas biógenas

Los resultados correspondientes al objetivo 1 "*Búsqueda de cepas de origen alimentario capaces de degradar AB en concreto histamina, tiramina y putrescina"* se resumen a continuación:

Se realizaron 77 extractos celulares de cultivos bacterianos, pertenecientes a los géneros *Enterococcus*, *Leuconostoc, Lactobacillus*, y *Pediococcus,* todos ellos pertenecientes al grupo de las bacterias lácticas, y de 1 cepa de la especie *Kocuria varians*. Todas las cepas procedían de distintos sustratos alimentarios (Tabla 11).

Tabla 11. Especies de bacterias utilizadas en esta Tesis.

BAL	**Cepa**	**Aislada de / Colección de cultivo**
E. faecium	C1	Pasta fermentada/ENOLAB
E. faecium	C2	Pasta fermentada/ENOLAB
K. varians	LTH 1540	Sachicha fermentada/ Dr Hammes
L. brevis	Lb 131, Lb 250	Vino tinto/ENOLAB
L. brevis	Lb 67	Mosto de uva/ENOLAB
L. casei	CECT 475^{T}	Queso/CECT
L. collinoides	Lb 373, Lb 404	Vino tinto /ENOLAB
L. curvatus	C9-19C [a] , C13-48[a]	Salchichas / Roig-Sagués
L. delbrueckii	CECT 286	Puré de grano/CECT
L. farciminis	CRL 678[b]	Salchichas/CERELA
L. fermentum	CHMDW 5A	Vino tinto /ENOLAB
L. hilgardii	L6, L21, L27, L41, L44, L56	Mosto de uva /ENOLAB
L. mali	C46, Lb 44, 45, 47, 52, 53, 75, 110, 197, 206, 334	Vino tinto /ENOLAB
L. paracasei	L51 , Lb 54	Mosto de uva /ENOLAB
L. paracasei	Lb 309, 340, 362, 365, 380, 444, 446L, 446R, 451	Vino/ENOLAB
L. pentosus	Lb 445, Lb 453	Vino/ENOLAB
L. plantarum	CECT 748^{T}	Repollo en vinagre/CECT
L. plantarum	C24, C51,C145, J16, J33, J39, Lb 98, 132, 135, 140	Mosto de uva /ENOLAB
L. plantarum	Lb 102, Lb 153, Lb 291	Vino /ENOLAB
L. plantarum	MRS 6, MRS 69A	Pasta fermentada/ENOLAB
L. sakei	CECT 906^{T}	Sake/CECT
L. vini	CECT 7072^{T}	Vino tinto /CECT
L. vini	Lb 154, Lb 209P	Vino tinto /ENOLAB
P. acidilactici	CECT 5765^{T}	Cebada/CECT
P. acidilactici	CECT 5930	Cebada/CECT
P. parvulus	P 205, P486BL, P487, R210 1A, R210 1B, R211 A, R211 B	Vino /ENOLAB
P. pentosaceus	MRS 12, 14, 45, 77	Vino /ENOLAB

Los extractos se sometieron a una electroforesis no desnaturalizante en gel de poliacrilamida para separar las proteínas presentes. Este gel se incubó en una mezcla de reacción que contenía un tampón fosfato con una mezcla de histamina, tiramina, y putrescina; tras eliminar esta solución, el gel se incubó con la misma solución anterior a la que se añadió peroxidasa de rábano (HRP) y diaminobenzidina (DAB). En el caso de que los extractos enzimáticos hubiesen degradado alguna de las aminas, uno de los productos de la reacción era H_2O_2, la cual oxida la DAB en presencia de HRP, dando lugar a la aparición de un precipitado marrón (Figura 8).

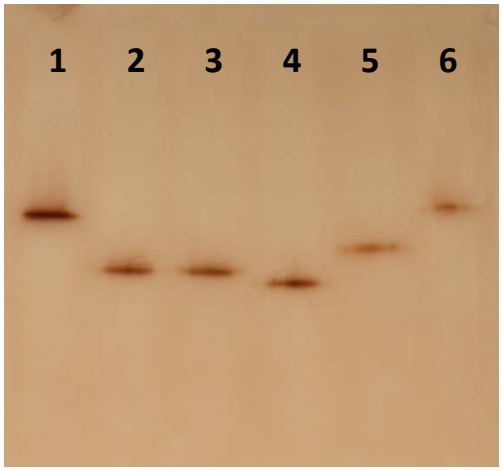

Figura 8. Actividad degradadora de aminas de extractos celulares en gel de poliacrilamida no desnaturalizante revelado con DAB. Carrera 1: *K. varians* LTH 1540; Carrera 2-4: *L. plantarum* J16, Lb 132 y Lb 291, respectivamente; Carrera 5: *L. farciminis* CRL 678; Carrera 6: *P. acidilactici* CECT 5930.

De los 77 extractos probados, 40 extractos de BAL (el 52,6 % de las mismos) y el de *K. varians* dieron lugar a la aparición una única banda de color marrón en el ensayo en gel cuando en la mezcla de reacción se pusieron las tres aminas. Las cepas que presentaron una reacción positiva frente a las aminas pertenecían a las especies: *E. faecium, L. brevis, L. collinoides, L. delbrueckii, L. farciminis, L. hilgardii, L. mali, L. paracasei, L. pentosus, L. plantarum, L. vini, P. acidilactici, P. parvulus* y *P. pentosaceus,* además de *K. varians.* Cuando en el tampón de reacción del ensayo en gel se sustituyeron las aminas biógenas por 2,6-dimetoxifenol (DMP), un sustrato típico de las lacasas, el número de extractos que mostraron una banda marrón fue de 47, 7 extractos más que aquellos que dieron reacción con las aminas. Todos los extractos que mostraron actividad sobre las aminas también lo hicieron sobre el DMP, excepto los de *E. faecium* y el de *K. varians*. Los resultados del ensayo de las actividades de los extractos sobre el DMP y sobre las AB pueden verse ver en la Tabla 12. Los extractos que presentaban mayor intensidad y rapidez en la reacción de degradación de las AB pertenecían a las especies *K. varians*, *L. plantarum y P. acidilactici.* Cuando se ensayaron los extractos en una mezcla de reacción que contenía cada una de las tres aminas

por separado, se observó, que todos los extractos dieron reacción positiva frente a varias aminas a excepción de *K. varians* que solo dio una reacción positiva con la putrescina.

Tabla 12. Porcentajes de cepas bacterianas cuyos extractos mostraron actividades enzimáticas sobre AB y/o sobre DMP.

Especie	Nº de cepas ensayadas	BA+DMP+	BA-DMP+	BA-DMP-	BA+DMP-
E. faecium	2	0	0	0	2
K. varians	1	0	0	0	1
L. brevis	2	100	0	0	0
L. casei	1	0	0	100	0
L. collinoides	2	50	0	50	0
L. curvatus	2	0	0	100	0
L. delbrueckii	1	100	0	0	0
L. farciminis	1	100	0	0	0
L. fermentum	1	0	0	100	0
L. hilgardii	6	33.4	50	16	0
L. mali	11	45.5	9.0	45.5	0
L. paracasei	11	18.2	27.3	54.5	0
L. pentosus	2	100	0	0	0
L. plantarum	16	100	0	0	0
L. sakei	1	0	0	100	0
L. vini	3	33.3	33.3	33.3	0
P. acidilactici	2	100	0	0	0
P. parvulus	7	14.3	14.3	71.4	0
P. pentosaceus	4	50	0	50	0

Se ensayó también la capacidad degradadora de AB por parte de las células enteras en medio de cultivo y en vino. Sólo se ensayaron aquellas cepas cuyos extractos dieron una reacción positiva más rápida frente a las mismas en el ensayo en gel. Doce cepas fueron capaces de degradar parcialmente la histamina, en medio sintético suplementado con 150 mg/L de cada amina por separado, dos de ellas degradaron hasta un 34% (Tabla 13). La tiramina fue degradada por 8 cepas, de las cuales 6 redujeron la concentración inicial en un tercio. La putrescina fue degradada por trece cepas, pero en menor grado que las otras aminas, a excepción de *K. varians* que la degradó totalmente. Siete cepas fueron capaces de degradar las tres aminas (5 de ellas pertenecían a la especie *L. plantarum*, una a *L. delbrueckii* y una a *P. acidilactici*). En los ensayos de degradación de estas aminas en vino, al que se adicionó 40 mg/L de cada amina por separado, se observó que las cepas de *L. plantarum* J16 y Lb 98 degradaban la histamina en mayor porcentaje que en medio sintético. Lo mismo ocurrió con la putrescina en todos los casos, excepto en el de *K. varians,* que degradó esta amina en menor proporción en vino que en medio sintético. En la totalidad de los casos, la degradación de tiramina fue siempre menor en vino que en el medio sintético (Tabla 13).

Tabla 13. Porcentaje de degradación de AB por células enteras en medio sintético (150 mg/L de AB, 28ºC, 48 h) y en vino (40 mg/L de AB, 28ºC, 7 días); n.d: no determinado, n.e: no se observó ningún efecto en las condiciones ensayadas.

		% degradación					
		Histamina		Tiramina		Putrescina	
BAL	Cepa	Medio	Vino	Medio	Vino	Medio	Vino
K. varians	LTH 1540	n.e	n.e	n.e	n.e	100	30.1
L. delbrueckii	CECT 286	33	n.d	6.3	n.d	18.0	n.d
L. farciminis	CRL 678	n.e	n.e	33.7	16.2	25.2	44
L. paracasei	ENOLAB Lb 444	11.3	n.d	n.e	n.d	n.e	n.d
L. plantarum	ENOLAB J16	4.7	13.4	33	22.5	26.2	26.5
L. plantarum	ENOLAB Lb 98	7.3	27.8	41.7	25	13.8	41.1
L. plantarum	ENOLAB Lb 132	15.3	14.7	42.9	28.4	14.5	35.5
L. plantarum	ENOLAB Lb 291	18.6	15.6	39	17.8	26	29.8
L. plantarum	ENOLAB J33	6.4	n.d	n.e	n.d	14.8	n.d
L. plantarum	ENOLAB J39	16.4	n.d	n.e	n.d	5.8	n.d
L. plantarum	ENOLAB Lb 140	33.9	n.d	8.6	n.d	15.7	n.d
L. plantarum	ENOLAB C145	14.6	n.d	n.e	n.d	6.2	n.d
P. acidilactici	CECT 5930	13.8	13.5	40	18.8	19.3	31.7
E. faecium	C1	3.6	n.d	n.e	n.d	16.8	n.d

Dado que las células de *K. varians* LTH 1540 fueron las más efectivas en la degradación de putrescina, se investigó la rapidez con la que se producía esta degradación en distintos sistemas de ensayo. Se observó que la cepa de *K. varians* era capaz de degradar 100 mg/L de putrescina en tampón fosfato en menos de 24 h, mientras que si la concentración de la misma se aumentaba a 1000 mg/L se degradaba más de la mitad tras 4 h. Cuando el ensayo se realizó en el medio sintético descrito por Dapkevicius et al. (2000), *K. varians* eliminó el 100% de la concentración inicial de putrescina (150 mg/L) después de 48 h, mientras que en vino fue capaz de eliminar el 30% de una concentración inicial de 40 mg/L tras 7 días de incubación.

Los resultados completos correspondientes a este objetivo se recogen en los artículos:

S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2014. Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine. Appl. Microbiol. Biotechnol., 98:185-198.

S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2015. Ability of *Kocuria varians* LTH 1540 to degrade putrescine: identification and characterization of a novel amine oxidase. J. Agric. Food Chem., 63: 4170−4178.

Capítulo 2

Aislamiento, purificación e identificación de las enzimas responsables de la degradación de aminas biógenas. Determinación de la actividad de la enzima purificada de K. varians LTH 1540

Los resultados correspondientes al objetivo 2 *"Aislamiento, purificación e identificación de las enzimas responsables de la degradación de aminas biógenas. Determinación de la actividad de la enzima purificada de* K. varians *LTH 1540"* se resumen a continuación.

El aislamiento y la purificación de las enzimas responsables de la degradación de AB en los microorganismos *K. varians* LTH 1540, *L. plantarum* J16 CECT 8944 y *P. acidilactici* CECT 5930 se llevó a cabo mediante sucesivas etapas de purificación. Inicialmente se obtuvieron extractos celulares de estas cepas mediante ruptura mecánica con perlas de vidrio. Posteriormente, se llevó a cabo una precipitación fraccionada con sulfato de amonio, seguida de cromatografía de intercambio iónico. Las proteínas de las fracciones que mostraron actividad se separaron mediante electroforesis nativa. La banda que mostró actividad frente a las aminas en el ensayo en gel (color marrón) se recortó y se sometió a una purificación adicional mediante SDS-PAGE. Las proteínas purificadas de *L. plantarum J16, P. acidilactici* CECT 5930 y *K. varians* LTH 1540 se obtuvieron a partir de la banda de SDS–PAGE (Figuras 9, 10 y 11).

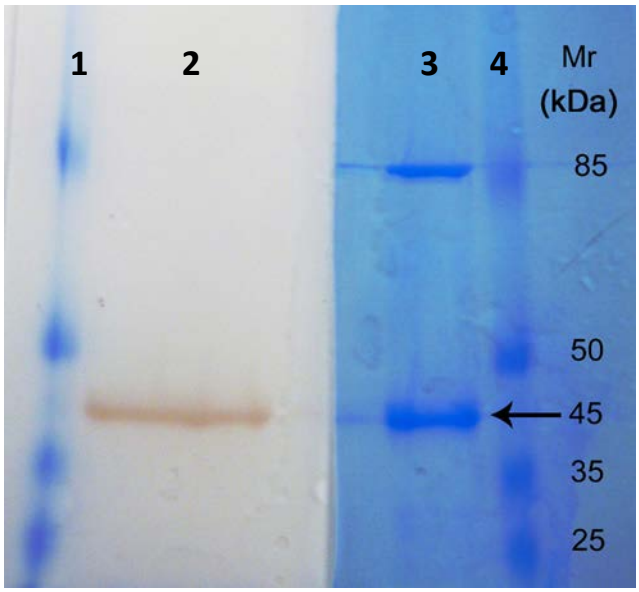

Figura 9. 10% de SDS-PAGE de la proteína purificada a partir de *L. plantarum* J16. Carrera 1: marcador de peso molecular (Pierce™ Prestained Protein MW Marker, Fisher); Carrera 2: proteína purificada teñida con DAB; Carrera 3: proteína purificada teñida con azul de Coomassie; Carrera 4: marcador de peso molecular teñido con azul de Comassie (Pierce™ Prestained Protein MW Marker, Fisher).

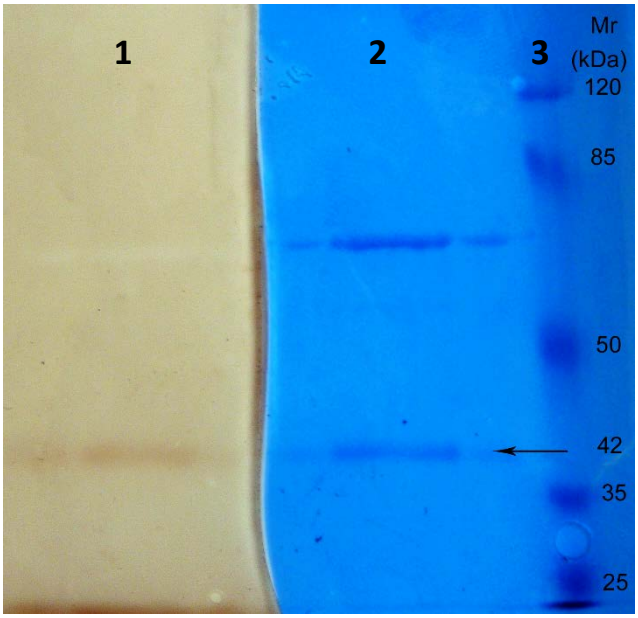

Figura 10. 8% de SDS-PAGE de la proteína purificada a partir de *P. acidilactici* CECT 5930 J16. Carrera 1: proteína purificada teñida con DAB; Carrera 2: proteína purificada teñida con azul de Coomassie; Carrera 3: marcador de peso molecular teñido con azul de Coomassie (Pierce™ Prestained Protein MW Marker, Fisher).

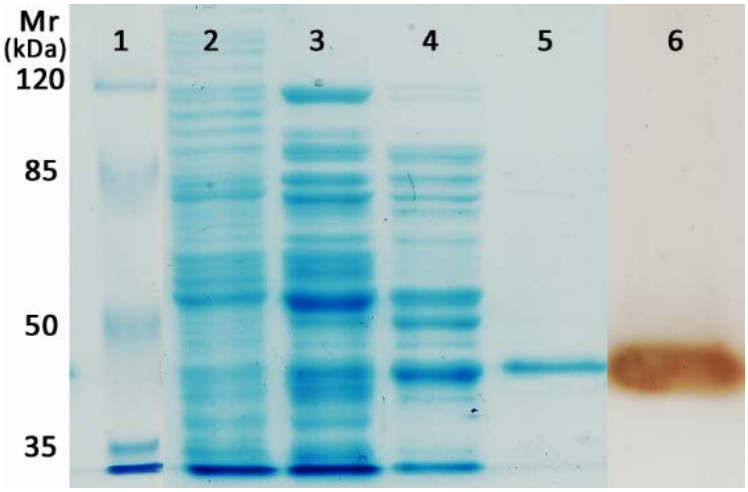

Figura 11. Análisis electroforético de las proteínas en diferentes etapas de la purificación de la PuO de *K.varians* en gel de 8% SDS-PAGE. Carrera 1: marcadores de peso molecular (Pierce™ Prestained Protein MW Marker, Fisher); Carrera 2: extracto crudo; Carrera 3: fracción precipitada con sulfato de amonio al 70%; Carrera 4: fracción eluida de la cromatografía Macro-Prep Q; Carreras 5 y 6: proteína purificada resultante de recortar la proteína cargada de la carrera 4 en un gel nativo. Las carreras 1-5 se tiñeron con colorante azul de Coomasie, y la carrera 6 fue cortada y sometida al ensayo de actividad PuO en gel con DAB. Las masas moleculares de las proteínas marcadoras se indican a la izquierda (kDa).

Para la identificación de las proteínas purificadas, se realizaron digestiones con tripsina y los péptidos resultantes se identificaron mediante MALDI-TOF MS en el servicio de proteómica del SCSIE (Servei Central de Support a la Investigació Experimental, Universitat de València).

El análisis de los péptidos significativos, llevado a cabo por el programa MASCOT, mostró que los péptidos obtenidos de la proteína aislada de *L. plantarum* J16 CECT 8944 coincidían con los de la proteína de división celular SufIa, cuyo número de acceso es C6VK53. La secuencia de los péptidos cubría el 54 % de la secuencia de dicha proteína (Figura 12). La proteína SufI está clasificada en la base de datos "The laccase engineering database" (LccED) como una multicobre oxidasa (MCO) perteneciente a la subfamilia J (CueO bacteriana).

MATRIX SCIENCE **Mascot Search Results**

Protein View

Match to: **gi|28377271** Score: **1237**
cell division protein SufI [Lactobacillus plantarum WCFS1]

Nominal mass (M_r): **57095**; Calculated pI value: **4.93**
NCBI BLAST search of gi|28377271 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Lactobacillus plantarum WCFS1
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|227897722 from Lactobacillus plantarum subsp. plantarum ATCC 14917
gi|254555487 from Lactobacillus plantarum JDM1
gi|28270102 from Lactobacillus plantarum WCFS1
gi|227854057 from Lactobacillus plantarum subsp. plantarum ATCC 14917
gi|254044414 from Lactobacillus plantarum JDM1

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Glu->pyro-Glu (N-term E),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **54%**

Matched peptides shown in **Bold Red**

```
    1 MAKKVYTDYF FDEPAYNTHD GGYIPLVTPK VEPQPLAIPP LLKPDRQTDT
   51 DDYYTVTAQE SETQFLPGKK TKTWGYNAGF LGQTIVFRNG KQTHIDLENK
  101 LPELTTFHWH GLNVPGPITD GGCHAPVYPG ETNHIDFKVH QPAATTWLHA
  151 HPCPSTATQV WKGLATMVII KDDVEDQLPL PRNYGVDDIP LVLQDREFHD
  201 DNQFDYRADY DPDGVQGHTA LVNGTVNPYF DVTTQRVRLR ILDGSNRREW
  251 RLHFNDDLEF AQVASDGGIL PAPVYMTKVM MTCAERDEIV VDFGQYQPGD
  301 EVTLMTDDTP LCRFRIKSFV PDDTKLPEHL VDIPDETPTP DLPVRTITMD
  351 GMDDEVALDG KKFDMSRIDA RQKVGDVAIW EIRNTNSTEN GMVHPFHVHG
  401 TQFRVLARND GPVYPNEHGL KDTVGVNPGE TVRIKVKFEL TGVYMYHCHI
  451 IEHEDGGMMA QIESYDPQHP QTYHLMDMDT LRNAFAKEQG IKPEDVWMPG
  501 M
```

Figura 12. Resultados del análisis de los péptidos obtenidos de *L. plantarum* J16 por MALDI-TOF mediante el programa de búsqueda MASCOT. Los péptidos coincidentes se muestran en color rojo.

Los péptidos obtenidos por digestión tripsica de la proteína aislada de *P. acidilactici* CECT 5930 cubrían un 36 % de la secuencia completa de una proteína identificada como una presunta MCO cuyo número de acceso es D2EK17 (Figura 13).

Mascot Search Results

Protein View

Match to: **gi|270290946** Score: **199** Expect: **1.7e-13**
conserved hypothetical protein [Pediococcus acidilactici 7_4]
Found in search of D:\SERVEI\MASES\CONVENI VALL HEBRON\Valld'Hebron\Sara\PuoP\0_J13\1\1SRef\pdata\1\peaklist.xml

Nominal mass (M_r): **54555**; Calculated pI value: **5.41**
NCBI BLAST search of gi|270290946 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Pediococcus acidilactici 7_4
Links to retrieve other entries containing this sequence from NCBI Entrez: gi|270280342 from Pediococcus acidilactici 7_4

Fixed modifications:
Carbamidomethyl (C) Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P Number of mass values searched: **71**
Number of mass values matched: **19**
Sequence Coverage: **36%**

Matched peptides shown in **Bold Red**

```
    1 MITKYLYDEN AYDYHDGGYR PLKKAPGEEH PLNVPAFLKP DRIEGNEIYY
   51 TVTAQAGETK ILPGKPTHTW GYNGSILGPA IQFETGKTYH VTLKNELDEV
  101 TTFHWHGLNI VGPYEDGGPH APVYPHGERK ITFTVDQPAA NIWLHPHCP
  151 ETARQVWNGL AAPVIITDGH EQSLKLPRRW GVNDFPVVLQ DRSYHDNQLD
  201 YKADYDVDGT LGDYALVNGT VNPVVNVTKP IVRLRFLNGS NRREWRLHFA
  251 DYHPFTQIGS DGGLLPEAVE MDRIMLTCAE RADVLVNFSD YQPGQEVILQ
  301 TDDFNLIKFK IGDIKKENML LPSPLAEIPA LSVDENTPVF KTVMSGMDDQ
  351 VRLDGKLFDM QRIDTRQQVD QTQIWEVSNT NDMEGGMIHP FHIHGCQFQL
  401 IDRNGHAVNP NEHGWKDTIG VNPNETVRIK VKFTKLGIFM YHCHILEHED
  451 TGMMAQIEIF DPDHPIEYHL MPMNHKM
```

Figura 13. Resultados del análisis de los péptidos obtenidos de *P. acidilactici* CECT 5930 por MALDI-TOF mediante el programa de búsqueda MASCOT. Los péptidos coincidentes se muestran en color rojo.

El análisis por MALDI-TOF MS de los péptidos de la proteína de *K. varians* mostró que se trataba de una proteína ortóloga de la putrescina oxidasa (PuO) de las especies *K. rosea* y *K. rhizophila*, cercanas filogenéticamente a *K. varians* (Figura 14). Por lo tanto, la PuO de *K. varians* se clasificaría en el grupo de enzimas EC 1.4.3.10.

Search ResultInfo

Search Result	Location	Search Engine	Database
ESI_NCBInr_Actinobacteria_Mascot_ -03-18 12:03:38	/UV/110318_MaoP/ 110317_MaoP1_1-A,8_01_2789.d	Mascot	NCBInr

Protein 1: putrescine oxidase [Kocuria rhizophila DC2201]

Accession:	gi|184199732	**Score:**	325.4
Database:	NCBInr(NCBInr_20100430.fasta)	**MW [kDa]:**	53.4
Database Date:	2010-05-06	**pI:**	4.7
		Sequence Coverage [%]:	11.5
		No. of unique Peptides:	6

10	20	30	40	50	60	70	80
MTEQRTVGSV	DAIERDVVVV	GAGPAGLMAA	RRLSEAGRSV	AVLEARDRVG	GRTWSNVIDG	AFLEIGGQWI	SPDQTELLGL
90	**100**	**110**	**120**	**130**	**140**	**150**	**160**
VEELGLTTFE	RYREGQSVYI	APDGSRHLYD	GHMFPTSEGT	EKEMDRLIHL	MDELAAEIGP	TEPWAHPKAR	ELDTVSFRDW
170	**180**	**190**	**200**	**210**	**220**	**230**	**240**
LRQNSDDEEA	CNNIGIFIAG	GMLTKPSHAF	SALQAVLMAA	SAGSFSNLVD	EDFILDRRVV	GGMQSVSTTM	AEQLGVDAGT
250	**260**	**270**	**280**	**290**	**300**	**310**	**320**
DVVTRDPGSV	VFLNSPVRTI	QWAGEDQTYE	EHDPDFTVRV	LSDRVTVKAK	DVVVAVPPNL	YSRISFEPPL	PRAQHQMHQH
330	**340**	**350**	**360**	**370**	**380**	**390**	**400**
QSLGIVIKIH	AVYETPFWRE	KGLSGTGFCA	HELSQEVYDN	TNHEDERGTL	VGFVSDEKAD	EVFRLPAEER	KRQVLESLSH
410	**420**	**430**	**440**	**450**	**460**	**470**	**480**
YLGEEAKNPV	VYYESDFGSE	EWTRGAYAAS	YDLGGLHRYG	AIQSEPVGPI	RWACSDLAAE	GYQHVDGALR	QGALAAAQIN
490							
QKRGA							

Cmpd.	No. of Cmpds	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
121	2	422.780	75.7	2	39.6	54.9	0	39-46	R.SVAVLEAR.D	
239	2	814.530	83.5	2	44.8	59.7	1	289-303	K.AKDVVVAVPPNLYSR.I	
287	2	715.410	719.1	2	46.9	38.2	0	291-303	K.DVVVAVPPNLYSR.I	
278	1	528.370	136.5	2	46.5	39.6	0	304-312	R.ISFEPPLPR.A	
258	2	576.380	137.4	2	45.6	58.9	0	368-378	R.GTLVGFVSDEK.A	
218	2	693.870	-	2	43.9	76.7	0	439-451	R.YGAIQSEPVGPIR.W	

Continúa en página siguiente

Protein 2: **RecName: Full=Putrescine oxidase**

Accession:	gi|730425	**Score:**	229.3
Database:	NCBInr(NCBInr_20100430.fasta)	**MW [kDa]:**	51.9
Database Date:	2010-05-06	**pI:**	4.9
		Sequence Coverage [%]:	8.2
		No. of unique Peptides:	5

10	20	30	40	50	60	70	80
MTDQRTLGSE	TAIERDVVVV	GAGPAGLMAA	RTLVAAGRTV	AVLEARDRVG	GRTWSKTVDG	AFLEIGGQWI	SPDQTELLAL
90	100	110	120	130	140	150	160
VDELGLETYQ	RYREGESVYL	APDGTRHTYT	GSMFPAGEST	IVEMEKLVAL	LDGLVAEIGA	TEPWAHPAAR	ELDTISFHHW
170	180	190	200	210	220	230	240
LRQHSDDEAA	CSNIGIFVAG	GMLTKPAHAF	SVLQAVLMAA	SAGSFSNLVD	EDFILDRRVV	GGMQSVSETM	AAELGEDVVF
250	260	270	280	290	300	310	320
LDTPVRTIRW	AGDGGTYAEH	VPGTPVTVWS	DRLTVRAKDV	VVAVPPNLYS	RISFEPPLPR	LQHQMHQHQS	LGLVIKVHAV
330	340	350	360	370	380	390	400
YETPFWRDKG	LSGTGFGAHE	LSQEVYDNTN	HGDPRGTLVG	FVSDERADEL	FGLPAEERRR	LILESLSHYL	GEEALHPVVY
410	420	430	440	450	460	470	480
YESDFGSEEW	TRGAYAASYD	LGGLHRYGAH	QRTPVGPIRW	ACSDLAAEGY	QHVDGALRQG	RLAAAEVLGA	GSLTGAER

Cmpd.	No. of Cmpds	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
136	1	571.700	152.5	3	40.3	34.7	1	92-106	R.YREGESVYLAPDGTR.H	
160	3	697.380	66.9	2	41.4	57.0	0	94-106	R.EGESVYLAPDGTR.H	
239	2	814.530	83.5	2	44.8	59.7	1	277-291	R.AKDVVVAVPPNLYSR.I	
287	2	715.410	719.1	2	46.9	38.2	0	279-291	K.DVVVAVPPNLYSR.I	
278	1	528.370	136.5	2	46.5	39.6	0	292-300	R.ISFEPPLPR.L	

Figura 14. Resultados del análisis de los péptidos obtenidos de *K. varians* LTH 1540 por MALDI-TOF mediante el programa de búsqueda Bruker. Los péptidos coincidentes se muestran en color rojo.

En este caso, además, se corroboró la identidad de la proteína mediante identificación, por espectrometría de masas en el SCSIE, del producto de reacción de la putrescina oxidasa: la Δ^1-pirrolina. La aparición de este compuesto indica que la enzima que cataliza la reacción es una putrescina oxidasa dependiente de flavina (PuO) (Figura 15).

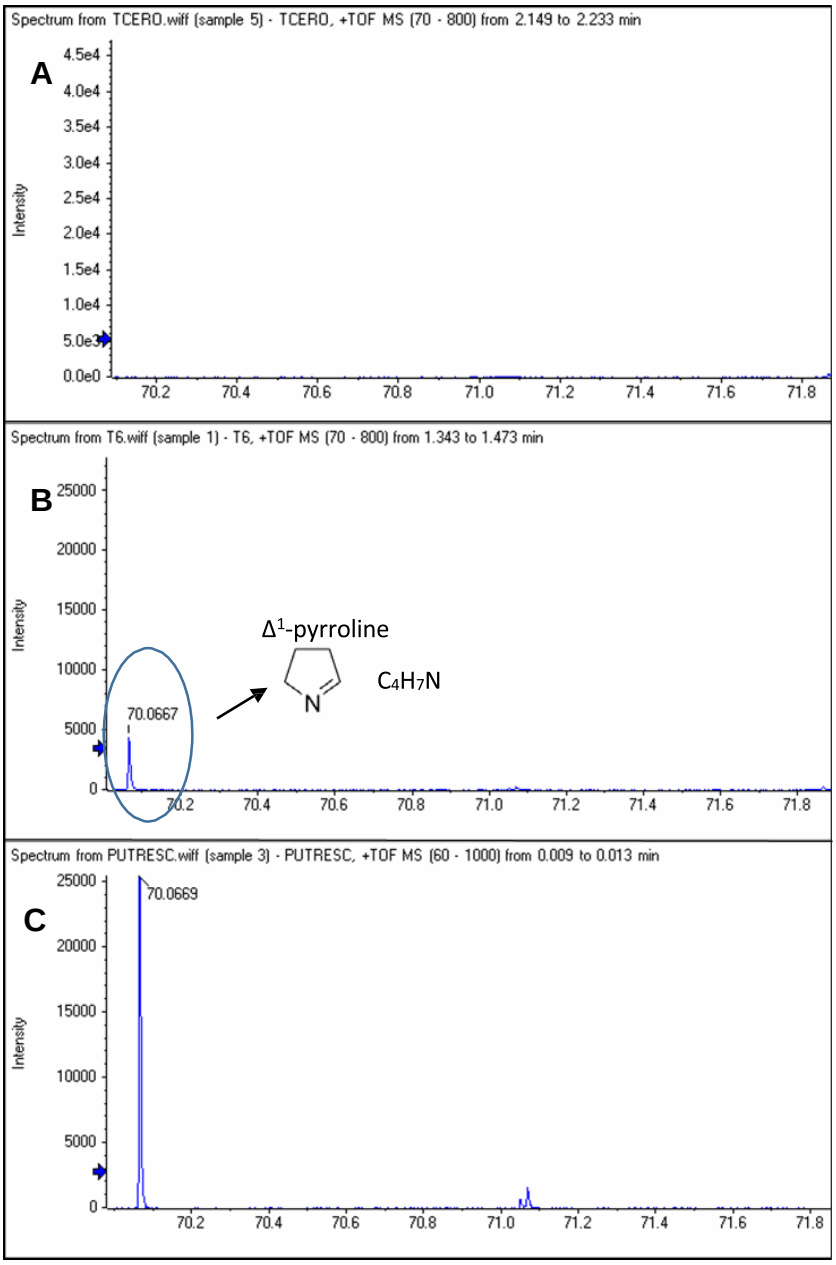

Figura 15. Análisis QTof de los productos finales de la degradación de la putrescina por las células y por la enzima parcialmente purificada en tampón fosfato de sodio con putrescina. A: espectro que muestra los picos obtenidos a tiempo cero (control) incubado con células de *K. varians*. B: espectro que muestra los picos obtenidos con la incubación de células de *K. varians* durante 6 horas. C: espectro de los picos obtenidos tras la incubación con la PuO parcialmente purificada durante 1 hora.

Para corroborar las identificaciones realizadas por técnicas proteómicas, se abordó la búsqueda de los genes que codificaban para esos enzimas en los genomas de las cepas *K. varians* LTH 1540, *L. plantarum* J16 CECT 8944 y *P. acidilactici* CECT 5930. Para ello se procedió a diseñar cebadores específicos a partir de las secuencias publicadas de proteínas semejantes a las identificadas. Con los cebadores descritos en la Tabla 14 se amplificaron parcialmente los genes de todas las cepas que pertenecían a las especies *L. plantarum*, *P. acidilactici* y *K. varians*.

Tabla 14. Cebadores diseñados para la obtención de la secuencia parcial de las proteínas identificadas

Oligonucleotido	**Secuencia (5'-3')**	**Tamaño banda**
Lac Lp1	CCCAGAATTGACGACTTTCC	765 pb
Lac Lp2	GGATGGGATGGATGATGAAGT	
Lac Pa 1	CAAACAACTTGCCATCCAAC	485 pb
Lac Pa 2	GTCGGCTTTGTAATCTAGTTGA	
Puod3	CCGTGGCGGTCCTCGAGGCCCG	773 pb
Puod4r	AGGGGCGGCTCGAAGGAGAT	

En todos los casos ensayados se obtuvieron bandas de amplificación del tamaño y secuencias esperadas 765 pb y 485 pb en cepas de *L. plantarum* y *P. acidilactici,* respectivamente (Figura 16) y 773 pb en el caso de *K. varians* (Figura 17). La secuenciación de estos fragmentos de amplificación en el servicio de secuenciación del SCSIE, mostró que correspondían a las secuencias nucleotídicas de un fragmento interno del gen que codifica cada enzima.

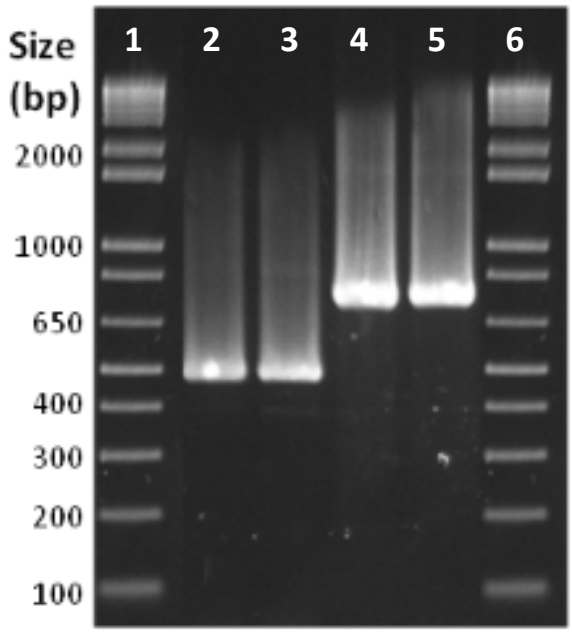

Figura 16. Resultados de las reacciones de amplificación de PCR obtenidos a partir de cepas de *L. plantarum* y *P. acidilactici*. Carrera 1 y 6: Marcador de peso molecular 1 Kb Plus (Invitrogen); Carreras 2 y 3: fragmentos de amplificación obtenidos de *P. acidilactici* CECT 5930 y *P. acidilactici* CECT 5765^{T} con Lac Pa 1 / Pa 2; Carreras 4-5: fragmentos de amplificación obtenidos de *L. plantarum* J16 y *L. plantarum* CECT 748^{T} con Lac Lp 1 / Lp 2.

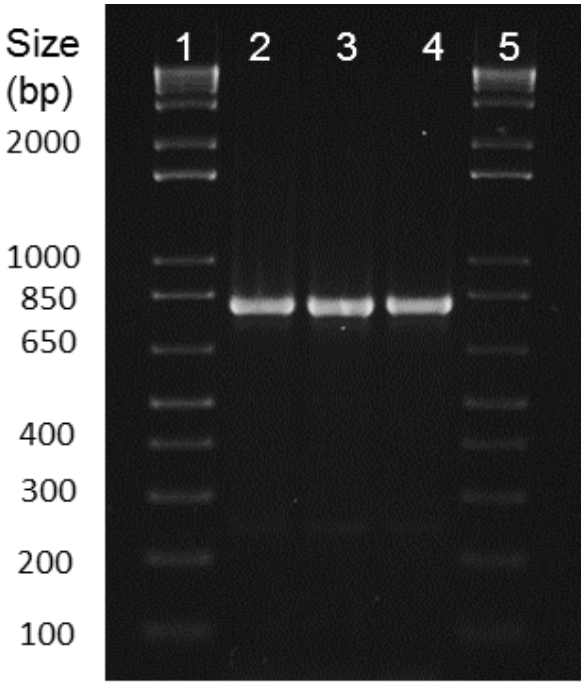

Figura 17. Resultados de las reacciones de amplificación de PCR obtenidos a partir de cepas de *K. varians* y *K. rosea* (control positivo). Carrera 1 y 5: Marcador de peso molecular 1 Kb Plus (Invitrogen); Carreras 2 y 3: fragmento de amplificación de *K. varians* obtenidos con Puod3/Puod4r; Carrera 4: fragmento de amplificación obtenido de *K. rosea* con Puod3/Puod4r.

Tras corroborar la presencia del gen responsable de las proteínas identificadas en nuestras cepas, se procedió a comprobar que las enzimas MCO y la PuO eran capaces de degradar aminas biógenas en tampón y vino. Para ello se usó como control de la actividad de las MCO la enzima comercial del hongo *T. versicolor*. Para analizar la capacidad de degradar aminas por las enzimas MCO purificadas de *T. versicolor* y *L. plantarum* se estudió la influencia del mediador ABTS y del sustrato en el que se realizaba la degradación de las aminas: tampón o vino. En el caso del enzima nativo purificado de *P. acidilactici* no se pudieron realizar los experimentos debido a la escasa cantidad de enzima, ya que fue necesaria su totalidad para llevar a cabo la identificación. Cuando los ensayos se realizaron en tampón fosfato al que se le añadió 1 g/L de cada amina por separado, se observó que la enzima de *T. versicolor* fue capaz de degradar el 100% de la tiramina añadida tras 24 h de incubación en ausencia de ABTS; este enzima fue menos eficaz en la degradación de la histamina ya que sólo degradó un 42,2 % de la misma en 24 h aunque, únicamente, en presencia del mediador ABTS y finalmente, se observó, que la enzima sólo degradó el 10 % de la putrescina tras el mismo periodo de tiempo tanto en presencia como en ausencia del mediador. Cuando esta enzima se ensayó en vino al cual se le añadieron 40 mg/L de cada amina, separadamente, la enzima comercial de *T. versicolor* degradó el 21% de la tiramina, el 8% histamina y el 5% de putrescina con o sin ABTS. La enzima MCO de *L. plantarum* ensayada en tampón fosfato con 150 mg/L de cada amina por separado degradó el 36 % de histamina, el 80 % de tiramina y el 17 % de putrescina en presencia de ABTS y tras 48 h. de incubación. En ausencia de mediador la degradación fue mucho menos efectiva. Cuando la proteína se ensayó en vino se produjo la precipitación de la misma y no se observó ningún tipo de degradación.

La baja cantidad de enzimas MCO obtenido a partir del aislamiento y la purificación a partir de los extractos celulares sólo nos permitió obtener la cantidad adecuada para la identificación pero impidieron la caracterización física y bioquímica. La concentración de proteína obtenida de la purificación de *L. plantarum* J16 fue 0.25 mg/mL, en el caso de *P. acidilactici* fue 11.5 mg/mL y 2.8 mg/mL en el caso de la proteína de *K. varians.* Por ello, se requirió la obtención de las proteínas heterólogas, expresadas en *E.coli* BL21 DE3 de las dos primeras, los resultados de este trabajo se detallaran en el siguiente capítulo. Sin embargo, sí que se obtuvo cantidad suficiente para la caracterización de la proteína obtenida directamente de *K. varians* cuyas características se resumen a continuación.

La enzima PuO purificada de *K. varians* LTH 1540 era capaz de oxidar las diaminas putrescina y cadaverina, y, en menor medida, poliaminas, tales como espermidina, pero no monoaminas. Las constantes cinéticas (K_m y $V_{máx}$) se determinaron para la putrescina y la cadaverina. La estabilidad térmica, temperatura y pH óptimos se determinaron para la putrescina. Los resultados de estos experimentos se resumen en la Tabla 15. La enzima purificada fue capaz de degradar tan sólo un 5 % de la putrescina presente en el vino.

Tabla 15. Características físicas y bioquímicas de la PuO de *Kocuria varians* LTH 1540.

	K_m (µM)	$V_{máx}$ (µmol/min mg)	Temperatura óptima (ºC)	Estabilidad térmica (ºC)	Ph óptimo
Putrescina	94±10	2.3±01	45	50	8.5
Cadaverina	75±5	0.15±0.02	-	-	-

Los resultados completos correspondientes a este objetivo se recogen en los artículos:

S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2014. Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine. Appl. Microbiol. Biotechnol., 98:185-198.

S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2015. Ability of *Kocuria varians* LTH 1540 to degrade putrescine: identification and characterization of a novel amine oxidase. J. Agric. Food Chem., 63: 4170−4178.

Capítulo 3

Clonaje y caracterización de las enzimas degradadoras de aminas biógenas de Lactobacillus plantarum J16 CECT 8944 *y* Pediococcus acidilactici CECT 5930.

Como ya se ha mencionado en el capítulo anterior, debido a las pequeñas cantidades de proteínas degradadoras de AB obtenidas de forma nativa de las cepas *L. plantarum* CECT 8944 *y P. acidilactici* CECT 5930, éstas se tuvieron que obtener de forma recombinante para conseguir suficiente cantidad de enzima para abordar su caracterización bioquímica.

Para conseguir la secuencia completa del gen codificante de la proteína homologa de SufI de *L. plantarum* J16 (en adelante denominada SufI J16) y la de la homóloga de D2EK17 de *P. acidilactici* CECT 5930 (en adelante denominada Lpa 5930) se diseñaron parejas de cebadores homólogos del inicio y final de las regiones codificantes de esos genes. El diseño de los cebadores se realizó a partir de las secuencias publicadas de enzimas MCO de mayor homología con SufI J16 y Lpa 5930. Una vez amplificadas estas secuencias codificantes de los genes correspondientes, se clonaron en *E. coli* DH5α y se expresaron en *E. coli* BL21 DE3. En ambos casos hubo problemas iniciales para obtener proteína soluble suficiente para llevar a cabo la caracterización física y bioquímica de las enzimas. En el caso de *L. plantarum* J16 no se obtuvo proteína soluble cuando se realizó la inducción 37 ºC, y además fue necesaria la presencia de cobre en el momento de la inducción. La obtención de la proteína Lpa 5930 requirió la incorporación del cobre en el medio de cultivo antes de la inducción. En ambos casos la expresión se llevó a cabo a una temperatura de 20ºC y en el caso de la proteína Lpa 5930 se requirió la introducción de un plásmido accesorio que contenía chaperonas (pGr07) para garantizar el correcto plegamiento de la proteína. Las proteínas recombinantes se purificaron mediante el sistema Ni-NTA, diseñado para purificar proteínas marcadas con colas polihistidínicas (His-tag). La eficiencia del procedimiento de purificación de las proteínas SufI J16 y Lpa 5930 en un único paso se muestra en las Figura 18 y 19.

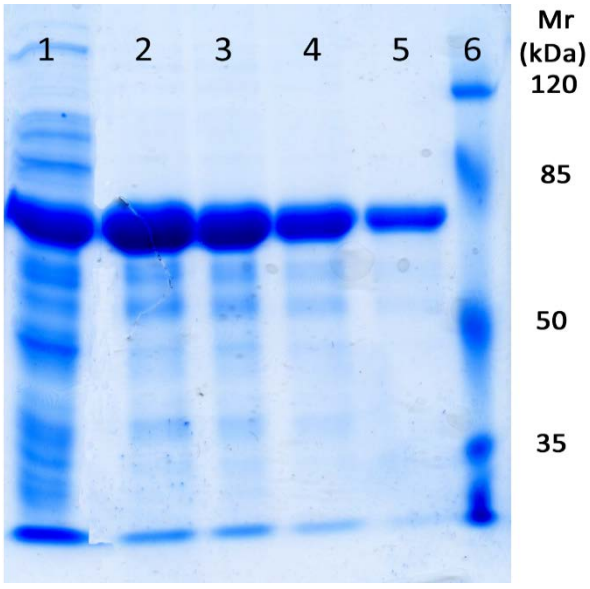

Figura 18. Análisis electroforético en gel 8% SDS-PAGE de la lacasa recombinante de *L. plantarum* J16 antes y después del procedimiento de purificación. Carrera 1: extracto crudo; Carreras 2-5: fracciones 1 a 4, respectivamente, obtenidas después de la elución con imidazol 250 mM de las proteínas unidas al gel de agarosa Ni-NTA-; Carrera 6: marcador de peso molecular (Pierce™ Prestained Protein MW Marker, Fisher).

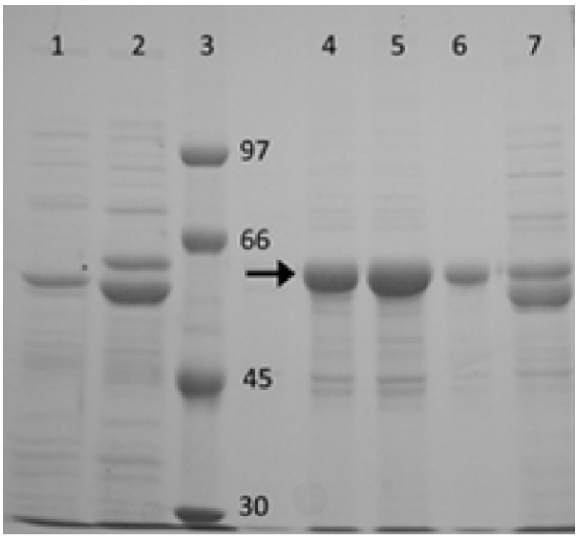

Figura 19. Análisis en gel 8% SDS-PAGE de las fracciones correspondientes al procedimiento de purificación de lacasa recombinante de *P. acidilactici* CECT 5930. Carrera 1: control no inducido; Carrera 2: Control inducido; Carrera 3: LMW marcador (Amersham); Carreras 4-6: fracciones 1 a 4 obtenidas tras la elución con imidazol 250 mM; Carrera 7: extracto crudo antes de la purificación.

Después de la purificación, se corroboró que se trataba de enzimas MCO por sus espectros de absorción, por las masas moleculares de las mismas y por su caracterización bioquímica. Se obtuvieron las constantes cinéticas de estos enzimas para el sustrato canónico de las lacasas (ABTS), y se estudiaron los efectos de la temperatura, el pH y la adición de Cu^{+2} sobre sus actividades enzimáticas. Igualmente, se estudió el efecto de sustancias potencialmente inhibidoras. Además se estudió el comportamiento de estos enzimas sobre las AB histamina, tiramina y putrescina. También se estudió si el sustrato ABTS actuaba como mediador

Se observó que las proteínas SufI J16 y Lpa 5930 recombinantes purificadas exhibían un intenso color azul, típico de las lacasas, corroborando este hecho que se trataba de enzimas multicobre oxidasas del tipo lacasa EC 1.10.3.2.(Figura 20 A y B).

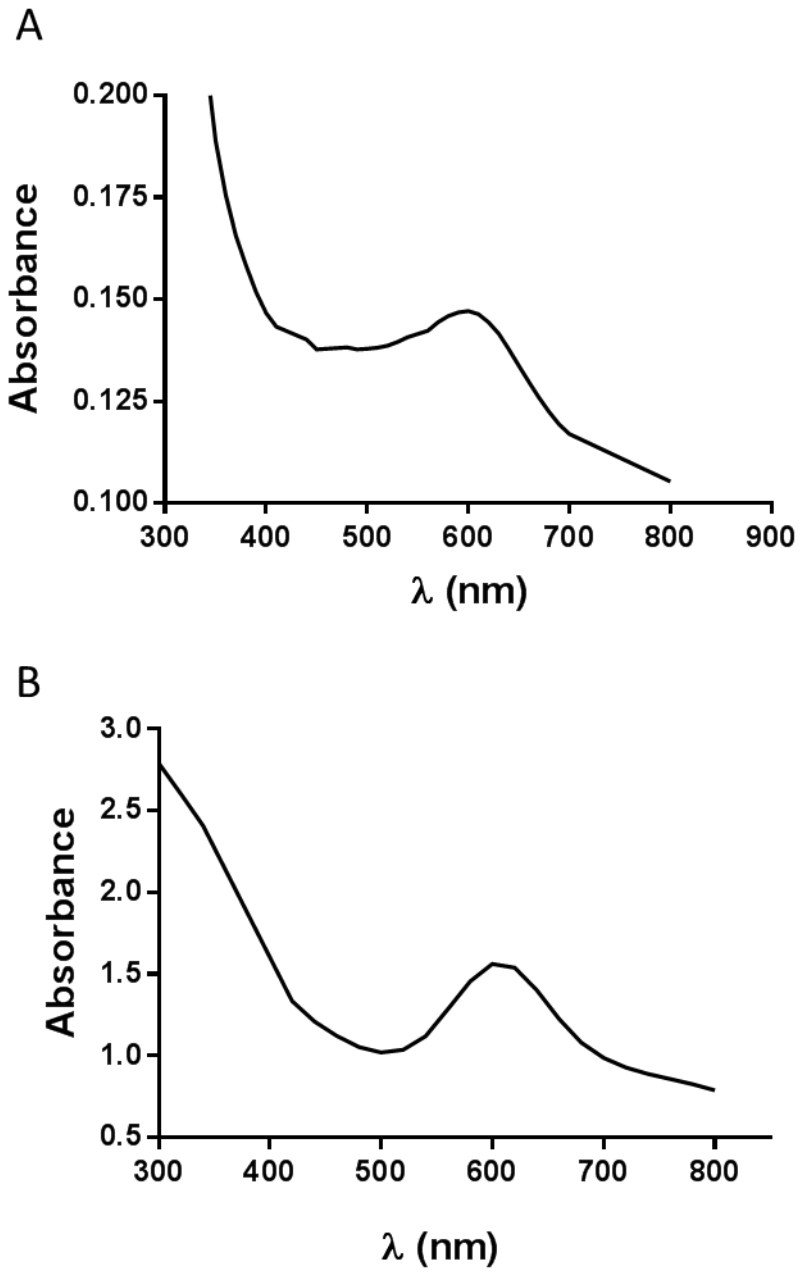

Figura 20. Espectro de absorción obtenidos para las proteínas recombinantes SufI J16 y Lpa 5930 en tampón fosfato sódico 50 mM; pH 6.5. A: Espectro de absorción de la proteína SufI J16 (3.5 mg/mL). B: Espectro de absorción de la proteína Lpa 5930 (14.16 mg/mL).

Las masas moleculares obtenidas para SufI J16 y Lpa 5930 fueron ~62.5 y 60 kDa, respectivamente. Los parámetros cinéticos de las proteínas SufI J16 y Lpa 5930 para el sustrato ABTS se muestran en la Tabla 16. También se muestran en esa Tabla las características físico-químicas de las enzimas, tales como temperaturas y pH óptimos de actuación sobre el ABTS y la tiramina, y la termorresistencia. La temperatura óptima de actuación de SufI J16 fue de 60°C mientras que de Lpa 5930 fue de 28ºC.

La proteína SufI J16 fue capaz de mantener el 70% de su actividad tras un tratamiento térmico a 70ºC durante 10 min, mientras que la Lpa 5930 fue algo menos termoresistente, ya que mantuvo el mismo porcentaje de actividad pero tras el tratamiento a 65ºC. Los ensayos realizados para conocer la influencia del Cu^{+2} sobre la actividad de estas proteínas mostraron que la adición de Cu^{+2} en un rango de 50 a1000 µM aumentaba ligeramente (un 5%) la actividad de ambas enzimas. El pH óptimo de actuación sobre el ABTS fue 3,5 para SufI J16 y 4 para Lpa 5930. En cuanto al efecto de productos considerados potencialmente inhibidores, se encontró que la semicarbazida y el EDC inhibieron la actividad de la proteína SufI J16 en un 20% y un 99,5 %, respectivamente, mientras que la de Lpa 5930 lo hizo en un 60% y un 15%, respectivamente. Ninguno de los dos quelantes de metales, fenantrolina y bipiridilo, inhibía significativamente la actividad de la proteína Lpa 5930, pero sí la de SufI J16, llegando a observarse inhibiciones de la actividad del 90 y 95 %, respectivamente (Figura 21).

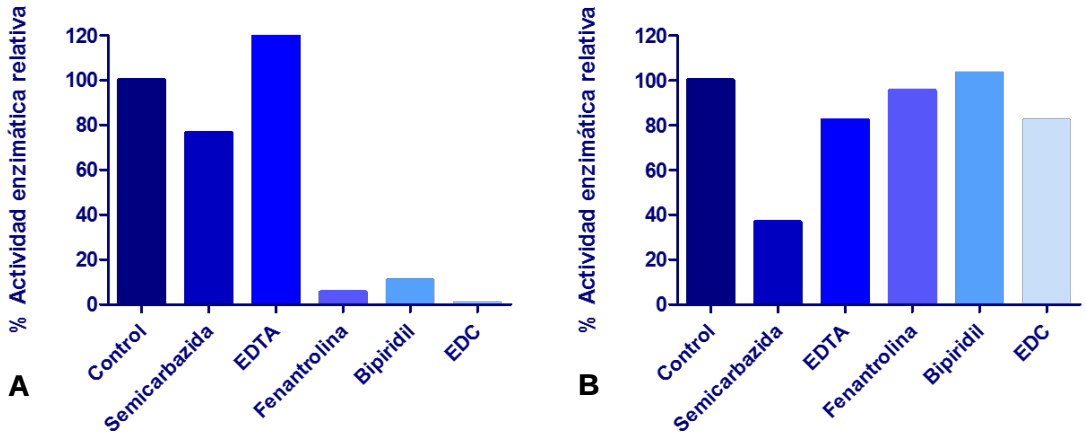

Figura 21. Efecto de los potenciales inhibidores sobre la lacasas recombinantes de *L. plantarum* J16 (A) y *P. acidilactici* CECT 5930. Los ensayos se realizaron en tampón fosfato sódico 50 mM, pH 6.5, utilizando DMP como sustrato en el caso de la lacasa de *L. plantarum* y ABTS en el caso de la de *P. acidilactici*, ambos se llevaron a cabo en presencia de 100 mM de los compuestos indicados. Los controles se corresponden con ensayos de las enzimas en ausencia de inhibidores.

Cuando se ensayó la actividad de estas dos enzimas sobre la histamina, tiramina y putrescina, añadidas a una concentración de 150 mg/L a un tampón fosfato, se llegó a la conclusión de que el mejor sustrato para ambas era la tiramina. Se observó, además, que la eficiencia de la oxidación dependía de la concentración de ABTS y de Cu^{+2}. Así, se observó que la enzima SufI J16 degradaba casi el 100% de la tiramina, un 36% de la histamina y un 17% de la putrescina en presencia de 5 mM de ABTS. Sin embargo cuando la concentración de ABTS era de 1 mM la degradación de tiramina se redujo al 85 % y cuando se omitió de la reacción la degradación de tiramina, histamina y putrescina se reducía a un 30, 10 y un 5% respectivamente.

La adición de Cu^{+2} (50-1000 µM) aumentó la actividad de las proteínas SufI J16 y Lpa 5930 en un 5%.

La enzima Lpa 5930 tenía como único sustrato a la tiramina a la que degradó en un 70% en tampón acetato sódico pH 4, con 1 mM del mediador ABTS y 100 µM de $CuCl_2$. En ausencia del mediador la enzima fue capaz de degradar aproximadamente un 20%.

Curiosamente, las dos proteínas mostraron dos pH óptimos de actuación sobre la tiramina: 4 y 9,5 (Tabla 16)

Tabla 16. Características físicas y bioquímicas de las proteínas recombinantes obtenidas de *L. plantarum* J16 y *P. acidilactici* CECT 5930.

Enzima	Sustrato	K_m (µM)	$V_{máx}$ (U/mg)	Temperatura óptima (°C)	Estabilidad térmica (°C)	pH óptimo
SufI J16	ABTS	0.21	0.54	60	70	3.5
Lpa 5930	ABTS	1.70	24	28	65	4
SufI J16	Tiramina	n.d	n.d	28	n.d	4, 9.5
Lpa 5930	Tiramina	n.d	n.d	28	n.d	4, 9.5

Los resultados completos correspondientes a este objetivo se recogen en los artículos:

Artículo 3: S. Callejón, R. Sendra, S. Ferrer, I. Pardo. 2016. Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation. Appl. Microbiol. Biotechnol., 100: 3113-3124.

Artículo 4: Recombinant laccase from *Pediococcus acidilactici* CECT 5930 with ability to degrade tyramine. Enviado a Biotechnology Journal.

Discusión

Aunque los métodos más habituales y adecuados para el control de AB en los alimentos son los que implican la prevención, no siempre es posible aplicar este tipo de medidas. En el caso de que la producción de AB sea consecuencia del desarrollo de bacterias alterantes de alimentos frescos, el método más extendido para su control es la refrigeración (Naila et al. 2010). Sin embargo, algunas bacterias pueden crecer y producir AB por debajo de 5 °C, lo cual demuestra que la refrigeración por sí sola no siempre es eficaz (Emborg y Dalgaard 2006). Otras medidas que han demostrado su eficacia en el control de los niveles de AB han sido el uso de altas presiones hidrostáticas, la irradiación, nuevos envasados con gases que retrasen el crecimiento microbiano, sistemas predictivos del crecimiento de microorganismos, y el uso de aditivos alimentarios o conservantes. Estos métodos se basan principalmente en la inhibición y el retraso del crecimiento de las bacterias alterantes o en la inhibición de la actividad de la enzima descarboxilasa responsable de la producción de AB (Naila et al. 2010). Sin embargo, estas estrategias no se pueden aplicar a los productos fermentados ya que afectarían no sólo a los agentes alterantes sino también a las especies responsables de las fermentaciones. Otra estrategia sería limitar la cantidad de precursores en la materia prima que da origen a los alimentos fermentados, pero esto es imposible en el caso de alimentos con un gran contenido proteico y aminoacídico como son la carne, el pescado o la leche. En el caso del vino la limitación del aporte de precursores se puede lograr evitando prácticas tales como largas maceraciones del mosto con los hollejos, la crianza sobre lías, o la adición externa de N_2 en forma de aminoácidos. Sin embargo, evitar estas prácticas puede comprometer la obtención de vinos de alta expresión o el desarrollo de la FA por escasez de N_2 asimilable por las levaduras. Por otra parte, la prevención del crecimiento bacteriano durante la vinificación es difícil debido a la acción limitada de los conservantes aprobados para uso enológico, que son la lisozima, el SO_2 y el dicarbonato de dimetilo (Velcorín®) (Costa et al. 2008; Polo et al. 2010).

Por tanto, se requiere el desarrollo de estrategias paliativas, que permitan la reducción de AB en alimentos fermentados cuando los tratamientos preventivos no han sido 100% eficaces. Una de las que resulta más atractiva es la degradación de estas aminas por bacterias

propias del entorno alimentario no implicadas en alteraciones. La descripción previa de la existencia de bacterias degradadoras de AB procedentes de alimentos fermentados (Dapkevicius et al. 2000; Fadda et al. 2001; García-Ruiz et al. 2011; Leuschner et al. 1998; Martuscelli et al. 2000; Voigt y Eitenmiller 1978), y la constatación de que se había logrado reducir los niveles de AB mediante la inoculación de las mismas en alimentos (Dapkevicius et al. 2000; Gardini et al. 2002; Leuschner y Hammes 1998a; Leuschner y Hammes 1998b; Mah y Hwang 2009b; Yongsawatdigul et al. 2007) fueron el origen de esta Tesis doctoral cuyo objetivo principal es la búsqueda de bacterias degradadoras de aminas, la identificación de las enzimas responsables de su degradación y la evaluación de su uso para la eliminación de las principales AB del vino.

En la mayoría de los trabajos previos la búsqueda de actividades degradadoras de AB se ha realizado utilizando células enteras, que se ensayaban en tampón, medios sintéticos o en alimentos, sin embargo en esta Tesis se abordó ese objetivo utilizando inicialmente extractos celulares. El trabajo con extractos evitaba los falsos negativos que se podían registrar en el caso de que existiesen problemas de transporte de las aminas al interior celular. Dado que uno de los objetivos del trabajo pretendía la degradación de AB en vino, la mayor parte de las bacterias ensayadas en esta Tesis provenían del entorno enológico y pertenecían al grupo de las BAL, sin embargo también se trabajó con bacterias procedentes de otras fermentaciones alimentarias o de alimentos crudos. El 52,6 % de los extractos de BAL y el de *Kocuria varians* dieron lugar a una reacción positiva en el ensayo en gel cuando en la mezcla de reacción se pusieron las tres aminas. Cuando las células enteras de los extractos más activos se ensayaron frente a histamina, putrescina y tiramina en medio sintético y en vino, se observó que los mayores porcentajes de degradación de histamina y tiramina lo exhibieron varias cepas de *L. plantarum* y la cepa *P. acidilactici* CECT 5930, mientras que la mayor degradación de putrescina se consiguió con la cepa *K. varians* LTH 1540 y en menor medida varias cepas de *L. plantarum* y la cepa *L. farciminis* CRL 678. La descripción de que varias cepas de BAL pertenecientes a *L. plantarum y P. acidilactici* eran degradadoras de histamina ya fue descrita por Leuschner et al. (1998) aunque ellos encontraron un porcentaje menor de

cepas positivas y actividades bastante más bajas que las que nosotros hemos encontrado. Las diferencias en los porcentajes de cepas capaces de degradar las aminas entre el estudio de Leuschner et al. (1998) y este trabajo se pueden atribuir a los diferentes enfoques utilizados para poner en evidencia la presencia de actividades degradadoras de aminas: la microbiológica frente a la bioquímica. En ambos trabajos se corrobora que, entre las bacterias lácticas, *L. plantarum* y, *P. acidilatici* son las especias con mayor capacidad de degradar AB respecto a las otras especies ensayadas. De entre las no lácticas, la única especie común analizada en ambos trabajos es *Kocuria varians* (syn. *Micrococcus varians*). Leuschner et al. (1998) describieron la capacidad degradadora de tiramina en la misma cepa de *Micrococcus varians* LTH 1540 (syn. *Kocuria varians* LTH 1540) que se ha utilizado en esta Tesis, sin embargo, en las condiciones experimentales utilizadas en este trabajo no se ha puesto de manifiesto esa actividad. Por el contrario, si se ha demostrado que la cepa posee la capacidad de degradar putrescina, capacidad que no fue analizada por los anteriores autores. En esta Tesis se demuestra que la capacidad de degradar aminas biogénas es dependiente de la cepa en la mayoría de las especies, con la excepción de *L. plantarum* en la que todos los extractos ensayados dieron reacción positiva en la mezcla de reacción que contenía histamina, putrescina y tiramina. Por el contrario, Leuschner et al. (1998) observaron que sólo el 58% de las cepas de *L. plantarum* daban reacción positiva frente a la histamina, lo cual podría significar que esta actividad no es un rasgo general de esta especie o que la utilización de células en lugar de extractos celulares impidió poner de manifiesto la actividad en algunas cepas que pudiesen tener una actividad demasiado baja. En algunos casos, el bajo número de extractos de cepas ensayadas que pertenecen a la misma especie no es suficiente para poder afirmar que la actividad degradadora es dependiente de la especie, como ocurre en los casos de *E. faecium*, *L. delbrueckii*, *L. farciminis*, *L. pentosus* y *P. acidilactici*. Lo mismo ocurre con la especie *L. sakei*, ya que aunque la cepa analizada en esta Tesis *L. sakei* CECT 906^T no degradó ninguna amina biógena, en el trabajo de Dapkevicius et al. (2000) se describen cepas de esta especie capaces de degradar la histamina.

En esta Tesis se ha demostrado que el 50% de las células enteras de *L. plantarum* fueron capaces de degradar las tres aminas tanto en medio como en vino, y el otro 50 % fue capaz de degradar al menos dos de las tres aminas en medio (en vino no se determinó), sin embargo las cepas ensayadas por Leuschner et al. (1998) exhibían una menor versatilidad degradadora, tan sólo una de las 33 cepas *de L. plantarum* analizadas por ellos fue capaz de degradar dos aminas, concretamente histamina y tiramina. Tampoco ninguna de las dos cepas de *P. acidilactici* degradaron otra amina diferente de la histamina, mientras que la utilizada en este trabajo *P. acidilactici* CECT 5930 degradó histamina, tiramina y putrescina, tanto en medio como en vino. Recientemente, en el trabajo de Capozzi et al. (2012) se describen cinco cepas de *L. plantarum* capaces de degradar las tres aminas anteriormente citadas, además de cadaverina, o al menos dos de ellas, corroborando estos resultados la versatilidad de *L. plantarum* frente a las aminas. Los porcentajes de degradación de estas las cepas de *L. plantarum* utilizadas por Capozzi et al. (2012) son menores a los obtenidos en esta Tesis para la histamina y la tiramina 8 y 22 % respectivamente, pero semejantes para la putrescina (31%). Como nosotros, García-Ruiz et al. (2011) describen que las cepas de BAL más activas sobre la histamina, tiramina y putrescina en medio de cultivo pertenecían a los géneros *Lactobacillus* y *Pediococcus* y los resultados que obtienen con las cepas de *L. plantarum* son similares a los nuestros. De todos los autores nombrados, los únicos que ensayaron la actividad de las cepas en vino fueron García-Ruiz et al., encontrando que tan solo una cepa de *L. casei* degradó pequeños porcentajes de histamina, de tiramina y putrescina, todos ellos inferiores de 2 a 5 veces a los que se han encontrado en esta Tesis. Los resultados más espectaculares en cuanto a la degradación de aminas fueron los conseguidos con células de la bacteria *K. varians* LTH 1540, esta bacteria utilizada como cultivo iniciador en la fermentación de productos cárnicos, es capaz de degradar además de la putrescina, la cadaverina y la espermidina. Fue capaz de degradar el 100% de putrescina en tampón, pero cuando la degradación se llevó a cabo en el vino, el porcentaje de degradación de esta amina fue menor (30%). La misma disminución de la actividad degradadora de tiramina observaron Leuschner y Hammes (1998b) cuando utilizaron esta cepa para disminuir las AB durante fermentación de carne para salchichas. La disminución de actividad de las células de

K. varians LTH 1540 en vino puede deberse a que las condiciones físico-químicas hacían que las células muriesen rápidamente. Sin embargo, lo contrario ocurría cuando se utilizaban BAL para eliminar las AB en vino: los porcentajes de degradación obtenidos en este sustrato superaban a los obtenidos en medio de cultivo, lo cual puede apuntar a la existencia de compuestos mediadores en el vino que colaboran en la degradación de aminas. Otros autores como Dapkevicius et al. (2000), también observaron mayor degradación de histamina en el alimento (suspensión de pescado ensilado) que en medio sintético.

Hasta ahora, las actividades degradadoras de AB se han atribuido exclusivamente a las amino oxidasas, sin embargo, en ninguno de los trabajos anteriores se ha logrado identificar este tipo de enzimas en bacterias lácticas, aunque sí en otras bacterias y levaduras como *Arthrobacter crystallopoietes*, *Candida boidinii*, *Klebsiella aerogenes*, *Micrococcus rubens*, *Sarcinea lutea* y *Rhodococcus erythropolis* (Cooper 1997; DeSa 1972; Haywood y Large 1981; Ishizuka et al. 1993; Okamura et al. 1976; Ota et al. 2008; Sekiguchi et al. 2004; Van Hellemond et al. 2008; Yamada et al. 1965; Yamashita et al. 1993). Si las responsables de la degradación de AB fuesen amino oxidasas se requeriría la presencia de varias de ellas para explicar que una misma cepa fuese capaz de degradar varias aminas. Sin embargo, este no parecía ser el caso de las bacterias *L. plantarum* J16 y *P. acidilactici* CECT 5930, ya que cuando las proteínas de sus extractos enzimáticos se separaban en gel se obtenía reacción positiva frente a cada una de las aminas de una enzima que migraba a la misma distancia, es decir, la banda que indicaba la reacción positiva se situaba a la misma altura del gel. Nuestros resultados demuestran que la capacidad de degradar AB en al menos las dos cepas de BAL antes nombradas, está vinculada a la presencia de una única enzima. Por el contrario, el análisis de proteínas de los extractos de *K. varians* LTH 1540 en gel sólo mostró reacción positiva cuando se reveló con putrescina, apuntando este hecho a que no se trataba del mismo tipo de enzima que en los casos anteriores. El hecho de que la actividad de las proteínas degradadoras de AB resistiera las condiciones desnaturalizantes de la electroforesis en gel SDS-PAGE, nos permitió identificar y recortar la banda que mostraba actividad directamente desde el gel. El grado de purificación conseguido mediante este tipo

de electroforesis permitió su identificación por MALDI-TOF MS/MS. Los resultados de la identificación de las proteínas aisladas de BAL y de *K. varians* LTH 1540 por MALDI-TOF MS/MS mostraron que efectivamente se trataba de enzimas distintas, hecho que era esperable debido al diferente comportamiento de los extractos en el gel. Tras la exhaustiva caracterización de estas enzimas se llegó a la conclusión de que las de *L. plantarum* J16 y *P. acidilactici* CECT 5930 eran multicobre oxidasas y la de *K. varians* LTH 1540 era una putrescina oxidasa.

La enzima purificada de *L. plantarum* J16, denominada SufI J16, posee dos dominios conservados presentes en la proteína SufI de la cepa *L. plantarum* JDM, (una proteína anotada como de division celular de *L. plantarum),* y en otras MCO recogidas en la base de datos de ingeniería de lacasas y multicobre oxidasas (base de datos LccED, https://lcced.biocatnet.de/). La proteína aislada de *P. acidilactici* CECT 5930, denominada Lpa 5930, era homóloga de la proteína D2EK17 de *P. acidilactici* 7_4. A diferencia de SufI, D2EK17 no está incluida en esta base de datos LccED, a pesar de ello, esta enzima posee un dominio PRK10965 que es común a otras MCO, por lo que ha sido descrita como una presunta MCO. La enzima obtenida de *K. varians* LTH 1540 se identificó como una putrescina oxidasa (PuO) (EC 1.4.3.10) por homología de secuencia aminoácidica con otras enzimas de especies cercanas, en concreto con *K. rhizophila* (gi|184199732) y *K. rosea* (gi|730425).

Para corroborar los resultados de las identificaciones de las enzimas degradadoras de AB de *L. plantarum* J16, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540, fue necesario demostrar que dos primeras proteínas poseían actividad sobre el DMP, uno de los sustratos canónicos de las MCO, y que la presunta PuO de *K. varians* LTH 1540 catalizaba una reacción en la que se formaba Δ^1-pirrolina. Este compuesto se forma espontáneamente a partir del 4-aminobutanal, que es producto de la degradación de la putrescina por parte de amino oxidasas que contienen FAD como cofactor (DeSa 1972). La caracterización bioquímica también apoyó la identificación de la enzima de *K. varians* como una PuO. El hecho de que en los geles de proteínas de SDS de los extractos de *L. plantarum* J16 y *P. acidilactici* CECT 5930 se observara que la misma banda daba reacción positiva frente al DMP y frente a la

histamina, putrescina y tiramina demostró que todos ellos son sustratos de la misma enzima. Muy recientemente, Guarcello et al. (2016) han confirmado la capacidad para degradar varias aminas biógenas de una MCO aislada de una cepa *L. paracasei*. Además, en la base de datos LccED se describe que varias cepas pertenecientes a las mismas especies que las cepas que actúan sobre AB y DMP analizadas en esta Tesis (*L. brevis, L. delbrueckii*, *L. paracasei*, *L. plantarum* y *P. pentosaceus*) poseen enzimas clasificadas como MCOs en la misma subfamilia J (Bacterial CueO) que la proteína SufI J16, lo que sugiere que otras cepas de estas especies puedan poseer el mismo tipo de enzimas y que éstas enzimas están bastante extendidas entre las BAL.

Sin embargo, la identificación de las enzimas MCO como responsables de la degradación de AB en *L. plantarum* J16 y *P. acidilactici* CECT 5930 es altamente novedosa ya que antes no se había descrito esta actividad para este tipo de enzimas y la presencia de las mismas en bacterias lácticas sólo se había demostrado mediante estudios genómicos, pero no proteómicos. Aunque, se ha descrito la existencia de MCO en genomas de distintas especies de los dominios Eukarya, Bacteria y Archaea, las MCO descritas y estudiadas desde hace más tiempo son las de hongos, principalmente las de aquellos degradadores de la lignina de la madera (Baldrian 2006; Thurston 1994). Las descripciones de las MCO bacterianas son mucho más recientes. En el trabajo de revisión de Sharma et al. (2007) se describen algunas de sus características, tales como su organización estructural, secuencia, localización celular y propiedades catalíticas. Algunas de ellas se han caracterizado, a otras se les ha asignado una función y en algunas otras se han encontrado secuencias señal. El descubrimiento de nuevas MCO bacterianas es un objetivo de interés preferente, ya que la capacidad de estas enzimas para actuar sobre un amplio rango de sustratos las convierte en biocatalizadores muy útiles para diversas aplicaciones biotecnológicas (Reiss et al. 2013). Las MCO pueden actuar sobre diversos sustratos aromáticos, tales como difenoles, monofenoles metoxi sustituidos y aminas, además este rango de sustratos puede extenderse gracias a la acción de los agentes mediadores (Quintanar et al. 2007; Riva 2006; Solomon et al. 1996). Estas enzimas pueden participar en diversas reacciones, como reticulación de monómeros,

degradación de polímeros y escisión de anillos de los compuestos aromáticos, entre otras (Sharma et al. 2007). Estas características las convierte en enzimas de gran interés biotecnológico (Riva 2006).

Entre las MCO se encuentran las lacasas que presentan un color azul típico y son las más estudiadas por sus aplicaciones biotecnológicas. Además, las lacasas bacterianas pueden expresarse en sistemas de expresión procariotas estándar, tales como *E. coli*, aunque su expresión heteróloga a menudo da lugar a enzimas que carecen de los centros de cobre completos dando lugar a enzimas inactivas o con poca actividad. Estos problemas de expresión se han descrito en la obtención heteróloga de las lacasas CotA de *B. subtilis*, CotA de *B. licheniformis*, SSL1 de *S. sviceus*, McoA de *M. mediterranea*, y LccA de *H. volcanii* (Gunne 2014). Como consecuencia de ello, las actividades de las lacasas bacterianas recombinantes son bajas. En esta Tesis las enzimas MCO obtenidas de forma recombinante presentaron problemas de incorporación del cobre, ya que presentaban una baja o nula actividad cuando no se adicionaba Cu durante el crecimiento de *E. coli* BL21 DE3. En el caso de la lacasa de *P. acidilactici* CECT 5930 se requirió la adición de Cu al el medio de cultivo, mientras que en el caso de *L. plantarum* J16 sólo se requirió en el momento de la inducción. Además en ambos casos fue necesario realizar la inducción a baja temperatura (20 ºC) y en condiciones microaeróbicas para asegurar la difusión del cobre hacia el interior de la proteína (Mohammadian et al. 2010), ya que en las condiciones de crecimiento e inducción estándar (37 ºC y agitación) no se obtenía suficiente proteína activa. El efecto de la modificación de las condiciones de cultivo estándar sobre la actividad de estas enzimas ya había sido descrita en la expresión heteróloga de otras lacasas, como la CotA de *B. subtilis* (Durão et al. 2008; Mohammadian et al. 2010). Además, en el caso de la lacasa de *P. acidilactici* CECT 5930, fue necesaria la coexpresión del plásmido PGrO7 que contenía genes codificantes de chaperonas que ayudaron al correcto plegamiento de la proteína. Esta estrategia del uso de chaperonas también fue necesaria para conseguir la expresión de la lacasa de CotA de *B. licheniformis* completamente funcional (Gunne et al. 2013).

Las enzimas SufI J16 y Lpa 5930 clonadas y purificadas exhibieron un intenso color azul, indicando este hecho el que contenían Cu2+, lo cual se corroboró porque presentaban un pico de absorbancia a 600 nm, correspondiente a la presencia del centro de cobre de Tipo 1. Además, la relación de absorbancia 280/600 nm se encontraba dentro del rango típico para las lacasas bacterianas (Xu et al. 2007), así como sus actividades enzimáticas sobre los sustratos canónicos de las lacasas (ABTS y DMP), confirmaron la identificación por MALDI-TOF MS/MS de estas enzimas como multicobre oxidasas del subtipo lacasa. El análisis por SDS -PAGE nos permitió estimar que la enzima SufI J16 tenía un peso molecular de ~62,5 kDa mientras que la Lpa 5930 era de ~60 kDa. Estos valores están en el rango de pesos que presentan la mayoría de las lacasas bacterianas (de 50 a 100 kDa) (Sharma et al. 2007). El análisis de las constantes cinéticas de la lacasa recombinante SufI J16 sobre los sustratos ABTS y DMP proporcionó valores de K_m ligeramente más altos que los obtenidos para CotA de *Bacillus sp.* y para otras lacasas bacterianas (Koschorreck et al. 2008; Reiss et al. 2011). La K_m de la lacasa Lpa 5930 para el ABTS también es más alta que las de las enzimas citadas anteriormente e incluso mayor que la de *L. plantarum* J16. La $V_{máx}$ de la lacasa SufI J16 es mayor para el ABTS que para el DMP, en concordancia con lo que se ha descrito para otras lacasas bacterianas (Gunne y Urlacher 2012; Wu et al. 2010). Los datos cinéticos de SufI J16 proporcionan un valor relativo de preferencia de sustrato (en base a la relación $V_{máx}$/Km) de aproximadamente 46 veces más alta para el ABTS que para el DMP. En el caso de la lacasa Lpa 5930 sólo se obtuvieron las constantes cinéticas para el ABTS, debido a que cuando se ensayó la actividad sobre el DMP se producía un precipitado marrón que interfería la lectura de la D.O en la microplaca. El pH óptimo de las lacasas recombinantes SufI J16 y Lpa 5930 depende en gran medida del tipo de sustrato que se ensaya, esto es una característica general de las lacasas de bacterias y de hongos. Los valores óptimos de pH para la reacción de oxidación de ABTS y DMP de las dos nuevas lacasas eran análogos a los descritos por otros autores para otras lacasas fúngicas y bacterianas (Kiiskinen et al. 2002; Martins et al. 2002). La temperatura óptima de la enzima SufI J16, alrededor de 60 ºC, está dentro del rango de las descritas para otras lacasas bacterianas, como CotA de *Bacillus* (55–75 °C) (Durão et al. 2008; Koschorreck et al. 2008; Reiss et al. 2011) y CueO de *E. coli* (55 °C) (Roberts et al.

2002). Sin embargo, la temperatura óptima de actuación de Lpa 5930 es muy inferior a las de las lacasas citadas anteriormente (28ºC). La estabilidad térmica de las dos nuevas lacasas era similar a la de la proteína CueO de *E.coli* (Martins et al. 2002), pero menor que la de CotA de *B. subtilis* (Durão et al. 2008), que retenía el 50 % de actividad después de 100 min de incubación a 80 °C, y que la de la lacasa de *Bacillus tequilensis* que retuvo el 80 % de actividad a 70 °C después de 24 h de incubación (Sondhi et al. 2014). En el caso de la lacasa SufI J16 se produce una activación de la enzima al preincubarla a temperaturas entre 45–60°C, a estas temperaturas la enzima dobla su actividad respecto a cuándo se preincuba a 28 ºC; este fenómeno no se da en el caso de la proteína de Lpa 5930, sin embargo se ha descrito anteriormente en lacasas de origen bacteriano y fúngico (Mohammadian et al. 2010). La explicación a este fenómeno es que esas temperaturas relativamente altas podrían producir pequeños cambios de conformación que podrían dar lugar a la reorganización de los centros de cobre y a la recuperación de algunas moléculas inactivas (Sendra, comunicación personal). En cuanto al comportamiento de las proteínas clonadas frente a diversos compuestos potencialmente inhibidores, la lacasa SufI J16 se comportó de forma similar a otras lacasas descritas en diversos organismos (Endo et al. 2003), la enzima fue inhibida por los agentes quelantes de metales bipiridilo, fenantrolina, aunque no por EDTA. Posiblemente, los dos primeros quelantes son más eficientes que EDTA en la eliminación de los iones de cobre unidos al enzima, como resultado de su mayor afinidad para este metal. La ausencia de inhibición por EDTA se ha descrito en otras lacasas, como la de *Streptomyces coelicolor* (Machczynski et al. 2004), e incluso se ha observado un efecto de activación por este compuesto en las lacasas de *Haloferax volcanii* y *Trametes hirsuta* (Haibo et al. 2009; Uthandi et al. 2010). Sin embargo estos dos quelantes de metales no afectaron significativamente a la enzima Lpa 5930 que, por el contrario, fue ligeramente inhibida por el EDTA. Curiosamente, hemos encontrado una fuerte inhibición de la lacasa SufI J16 por el EDC, un agente modificador de grupos carboxilo, lo que sugiere la participación de un grupo carboxilo en el proceso catalítico o en la unión del sustrato. Esta hipótesis ya fue propuesta anteriormente por algunos investigadores para explicar el comportamiento de algunas lacasas fúngicas frente a este compuesto (Salony et al. 2008). Por el contrario el EDC sólo afectó ligeramente

la actividad de la lacasa Lpa 5930. La semicarbazida inhibía en mayor medida a esta enzima que a SufI J16. Este compuesto es un modificador de grupos carbonilo que, aunque es un típico inhibidor de enzimas amino oxidasas de cobre, también inhibe a la lacasa CueO de *E.coli* (Roberts et al. 2002).

Una característica interesante que presentan las enzimas bacterianas SufI J16 y Lpa 5930 es su capacidad para degradar AB. La actividad de las MCO sobre las aminas se ha estudiado en muy pocos trabajos. En 2003, Arias et al. encontraron una actividad muy baja hacia la tiramina de una MCO purificada de *Streptomyces cyaneus* CECT 3335 (Arias et al. 2003). En 2008 y 2009, Kudanga et al., trabajando en la funcionalización de superficies de madera para unir fungicidas, describieron la capacidad de las lacasas de mediar en la unión covalente de aminas aromáticas (incluyendo la tiramina) a una molécula modelo de lignina (Kudanga et al. 2009; Kudanga et al. 2008). Sin embargo, en dichos trabajos describieron que la tiramina no era sustrato para la lacasa de Bacillus (CotA) ni para la del hongo *Trametes hirsuta.* Por contra, en el presente trabajo se ha demostrado, por primera vez, la capacidad para degradar tiramina, histamina y putrescina de la lacasa de *T. versicolor*, aunque las dos últimas con una menor eficiencia. Este hallazgo corrobora que algunas lacasas fúngicas y bacterianas son capaces de degradar las AB. De entre las tres AB ensayadas en esta Tesis, la tiramina fue, sin duda, mejor sustrato para las proteínas SufIJ16 y Lpa5930 que la histamina y la putrescina, de hecho, fue el único sustrato de la segunda. Esta mayor capacidad de degradación de la tiramina puede deberse a que, de las tres, es la única que tiene estructura fenólica. Puesto que los fenoles son sustratos naturales de las lacasas, parece lógico que este sustrato se una con mayor afinidad a estas enzimas que la histamina y la putrescina. Es por esta razón, que la caracterización bioquímica de las enzimas SufI J16 y Lpa 5930 se ha realizado con esta amina. Se demostró que la actividad degradadora de tiramina por estos enzimas se incrementaba notablemente con el uso de ABTS en el medio de reacción, de manera dependiente de la dosis. El ABTS actúa como mediador redox facilitando o posibilitando la degradación de ciertos sustratos (Morozova et al. 2007b).

Por otro lado, el hecho de que la proteína de *K. varians* LTH 1540, responsable de la degradación de putrescina, cadaverina y espermidina, sea una PuO no es sorprendente, ya que otras PuO y diferentes amino oxidasas se han descrito en especies filogenéticamente cercanas a esta especie (Okada et al. 1979; Yagodina et al. 2002; Yamada et al. 1967). La caracterización bioquímica de la PuO de *Kocuria varians* LTH 1540 demostró que la masa molecular de la forma nativa es notablemente superior a la del polipéptido catalítico obtenido por SDS–PAGE (43 kDa), lo que indica que la enzima nativa debe de ser un oligómero. Es bastante probable que se trate de un dímero de idénticas subunidades, semejante al homodímero de 88 kDa descrito en *K. rosea* (DeSa 1972). La alta homología de secuencia entre las PuO de las dos cepas, la similar masa molecular de ambas enzimas y la cercanía filogenética entre ambas especies apoyarían esta hipótesis. En cuanto a las constantes cinéticas, la K_m de la PuO de *K. varians* LTH 1540 para la putrescina está en el mismo rango de valores descritos para su homóloga en *K. rosea*, ligeramente superior a la PuO obtenida por (DeSa 1972), y un poco más baja respecto a la obtenida por (Okada et al. (1979). Es, sin embargo, sustancialmente mayor que la PuO de *R. erythropolis* (Van Hellemond et al. 2008). Los resultados de los valores de $V_{máx}$ muestran que la cadaverina (el análogo de cinco carbonos de putrescina) es peor sustrato que la putrescina. Sin embargo, el valor de K_m para la cadaverina es ligeramente inferior al de la putrescina, lo que sugiere, según lo propuesto para la PuO de *K. rosea* (Okada et al. 1979), que la interacción hidrófoba entre la enzima y el sustrato debe aumentar con la longitud de la cadena metilada. El pH óptimo de actuación de la PuO de *K. varians* LTH 1540 fue de 8.5, y su temperatura óptima fue de 45 ºC, los ensayos de estabilidad térmica revelaron que era relativamente termoestable comparada con otras amino oxidasas, ya que retuvo más del 50% de su actividad relativa a 55 ºC, al contrario que la PuO homóloga en *K. rosea* que se desnaturaliza rápidamente con temperaturas superiores a 50 ºC. El EDC inhibió en gran medida la PuO de *K. varians* LTH 1540 incluso a la menor concentración; se ha demostrado que este compuesto también inhibe la actividad de su enzima homóloga de *K. rosea*, aunque en menor grado. Los compuestos rasagilina, pargilina y deprenil también inhiben a este enzima. Estos compuestos afectan al cofactor de la enzima MAO humana formando aductos que inactivan su función (Youdim et al. 2001), la

PuO de *K. varians* LTH 1540 usa el cofactor FAD al igual que la MAO humana, por lo que parece razonable que la enzima bacteriana también se vea inhibida. Sin embargo, la ciclopropilamina, no la inhibe. Este compuesto ejerce inhibición sobre la MAO humana mediante la alquilación del centro activo. Los quelantes metálicos bipiridil y fenantrolina no inhibieron a la enzima, lo cual es lógico dado que el cofactor de esta enzima no es de naturaleza metálica. Sin embargo, la semicarbazida inhibió moderadamente a la enzima, a la mayor concentración ensayada, a diferencia de lo que ocurre con su homóloga de *K. rosea.*

Se desconoce el papel fisiológico de las lacasas SufI J16 y Lpa 5930 por el momento, por lo que no se pueden correlacionar las diferencias en sus propiedades bioquímicas respecto a las lacasas procedentes de otros organismos, su papel debe estar relacionado con su función biológica principal. Una característica interesante que presentan las lacasas SufI J16 y Lpa 5930 bacterianas es su capacidad para degradar AB, aunque desconocemos si esta es o no su función principal, una hipótesis para esclarecerlo sería obtener mutantes en estos genes y analizar el comportamiento, así como el de los correspondientes mutantes complementados con los genes funcionales correspondientes. Su función principal podría tener que ver con la homeostasis del Cu^{+2} como se ha descrito para *E.coli* (Roberts et al. 2002). En el vino la función de estos enzimas podría estar relacionada con la eliminación de los polifenoles (sustrato principal de las lacasas) que tienen efecto antimicrobiano sobre las bacterias (Chung et al. 1993). Así las bacterias de origen vínico con actividad lacasa tendrían una mayor capacidad de supervivencia. En el caso de las AB, la degradación de éstas por las lacasas podría constituir un mecanismo de obtención de energía para las células, aunque este hecho esta por comprobar.

Una vez identificadas las bacterias de origen alimentario con capacidad para eliminar AB e identificadas y caracterizadas las enzimas responsables, se plantea la elección del sistema más adecuado para llevar a cabo la degradación biológica de las AB en vino. Las posibilidades existentes son: utilización de células enteras, utilización de extractos celulares y utilización de enzimas purificadas.

La primera estrategia resultó la más adecuada en nuestro caso, ya que varias de las cepas de *L. plantarum*, *P. acidilactici* CECT 5930, *L. farciminis* CRL 678, y *Kocuria varians* LTH 1540 demostraron ser capaces de degradar al menos una de las tres aminas ensayadas (histamina, putrescina o tiramina) en vino. La estrategia de utilizar células enteras para disminuir las AB de pescados, carnes, quesos o salsas fermentadas ya ha sido utilizada con éxito por otros autores (Dapkevicius et al. 2000; Leuschner y Hammes 1998a; Leuschner y Hammes 1998b; Yongsawatdigul et al. 2007), sin embargo en el vino tan sólo se ha publicado un trabajo, en el cual se describe la degradación de histamina, tiramina y putrescina por *Lactobacillus casei* (García-Ruiz et al. 2011). Sin embargo, los porcentajes de degradación conseguidos por estos autores son muchísimo más bajos que los encontrados en esta tesis y que han quedado reflejados en la publicación Callejón et al. (2014). La utilización de las bacterias vínicas de la especie *L. plantarum* o de las aisladas a partir de cebada (*P. acidilactici* CECT 5930), o de salchichas fermentadas (*K. varians* LTH 1540, *L. farciminis* CRL 678) no supondría ningún problema legal, ya que no son bacterias patógenas, ni alterantes y no producen toxinas, por lo que pueden considerarse cepas seguras o “GRAS” (Generally Recognized As Safe).

La segunda estrategia aplicable para eliminar AB del vino consiste en la utilización de extractos celulares de las cepas capaces de degradar aminas. En nuestro caso esta estrategia no proporcionó niveles de degradación adecuados cuando se añadían al vino. Sin embargo, otros autores han conseguido eliminar histamina, tiramina y putrescina de vinos tintos y blancos utilizando la fracción extracelular del hongo filamentoso *Penicillium citrinum* (Cuevas et al. 2014). Sin embargo, el uso industrial de este procedimiento está seriamente comprometido ya que para llevar a cabo la degradación se requería una gran cantidad de extracto: se debía añadir en una proporción 1/1 al vino; ello supone que el vino se diluye considerablemente lo que lo hace no apto para el consumo. Por otra parte, la utilización de extractos de éste u otros hongos filamentosos no parece la estrategia más adecuada para eliminar AB del vino, ya que algunos autores han descrito que estas cepas de *P. citrinum* son productores de micotoxinas (Rundberget et al. 2004). Además, hay muchos aspectos que

deben evaluarse antes de que pueda vislumbrarse su aplicación práctica, por ejemplo determinar la naturaleza de la enzima responsable, comprobar que la cepa capaz de llevar a cabo la eliminación de AB no produce micotoxinas, producir la enzima a gran escala y conseguir la degradación de las AB con cantidades de enzima razonables. Finalmente también se requeriría que las legislaciones que regulan la producción de vino admitiese su uso. García-Ruiz et al. (2011) también lograron la degradación de histamina usando extractos de una cepa de *L. casei* de origen vínico pero no en vino si no en soluciones tampón a las que añadieron diferentes compuestos propios del vino, como etanol, polifenoles o SO_2.

La última estrategia que se puede utilizar es la utilización de enzimas purificadas. Este procedimiento ha sido usado por Dapkevicius et al. (2000) con el fin de disminuir el contenido de AB en ensilado de pescado. Para ello utilizaron una diamino oxidasa (DAO) comercial de origen porcino purificada. Este enzima fue capaz de degradar un 20 % de la histamina durante la fermentación del pescado pero cuando el ensilado alcanzó un pH de 4.5 la enzima se inactivó por desnaturalización. En nuestro caso, ni la enzima SufI J16 ni la Lpa 5930 fueron capaces de degradar aminas en vino, y aunque la PuO de *Kocuria varians* LTH 1540 fue capaz de eliminar putrescina en este medio, el porcentaje fue demasiado bajo (5%) para considerar posible su aplicación industrial en la actualidad. Los experimentos en vino mostraron que el etanol presente no era el factor más limitante, ya que la enzima mantuvo el 82% la actividad en presencia de 15% de etanol (una concentración más alta que la que normalmente se encuentra en el vino). El factor más limitante parece ser el bajo pH del vino, cuyos valores más habituales están en el rango 3-3,9 (Beelman y Gallander 1979; Schaeffer et al. 2000), un valor al que la PuO está desprovista casi completamente de actividad. En los tres casos se producía una precipitación de la enzima que derivó en una desnaturalización. Por tanto, la estrategia que se ha revelado más prometedora, según se demuestra en esta tesis es la utilización de células enteras. Aunque las células enteras de *K. varians* LTH 1540 fueron capaces de eliminar putrescina en porcentajes similares a los de algunas cepas de *L. plantarum,* esta cepa es incapaz de crecer en vino, un hábitat en el que normalmente no se encuentra. Sin embargo, podrían usarse altas densidades de células no proliferantes para

eliminar la putrescina. Una estrategia similar se ha utilizado para realizar la fermentación maloláctica en vinos (Maicas et al. 2000). Sin embargo, la aproximación más realista es la utilización de células de bacterias lácticas de origen enológico, concretamente de la especie *L. plantarum* que es la que más adaptada está a las condiciones propias del vino.

Como resumen final, por lo que sabemos, los resultados de este trabajo son los primeros que demuestran que varias cepas de BAL son capaces de degradar histamina, tiramina y putrescina, y que la mayor parte de las cepas de *L. plantarum* de origen vínico degradan AB en el vino. También se ha demostrado que *K. varians* LTH 1540 degrada la putrescina en vino. Se han identificado las enzimas responsables de la degradación de estas aminas. En el caso de *L. plantarum* y *P. acidilactici* son MCOs de tipo lacasa, mientras que en el caso de *K. varians* LTH 1540 es una putrescina oxidasa, no descrita anteriormente. Además, hemos demostrado que la lacasa del hongo *T. versicolor* es capaz de reducir la histamina, tiramina y putrescina en el vino.

Este trabajo abre las puertas a nuevos estudios que permitan ampliar el conocimiento de las enzimas capaces de degradar AB en vinos u otros alimentos, y mejorar la actividad de las enzimas identificadas en vino, mediante ingeniería de proteínas o por evolución molecular dirigida.

Conclusiones

1. De los 77 extractos celulares de bacterias procedentes de alimentos analizados en esta Tesis, 41 fueron capaces de degradar aminas biógenas (AB) y 47 el 2,6-dimetoxifenol (DMP). Todos los extractos que mostraron actividad sobre las AB también lo hicieron sobre el DMP, excepto los dos de *Enterococcus faecium* y *Kocuria varians* LTH 1540.

2. Las cepas degradadoras de AB pertenecen a las especies: *Enterococcus faecium*, *Kocuria varians, Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus delbrueckii, Lactobacillus farciminis, Lactobacillus hilgardii, Lactobacillus mali, Lactobacillus paracasei, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus vini, Pediococcus acidilactici, Pediococcus parvulus y Pediococcus pentosaceus*.

3. La capacidad para degradar AB es un carácter dependiente de cepa en las especies *Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus hilgardii*, *Lactobacillus mali, Lactobacillus paracasei, Lactobacillus vini, Pediococcus parvulus y Pediococcus pentosaceus,* mientras que todas las cepas de *Lactobacillus plantarum* presentan capacidad para degradarlas.

4. Las células enteras de las 14 cepas cuyos extractos mostraron mayor actividad sobre las AB en gel, fueron capaces de degradarlas en medio sintético y en vino. En ambos medios todas las cepas de *Lactobacillus plantarum*, la cepa *Pediococcus acidilactici* CECT 5930 y *Lactobacillus delbrueckii* CECT 286 degradaron histamina, tiramina y putrescina, mientras que *Lactobacillus farciminis* CRL 678 sólo degradó tiramina y putrescina y *Kocuria varians* LTH 1540 sólo putrescina. *Enterococcus faecium* C1 degradó histamina y putrescina y *Lactobacillus paracasei* Lb 444 histamina, pero sólo en medio sintético.

5. La cepa que mostró los mayores porcentajes de degradación de histamina en vino fue *Lactobacillus plantarum* Lb 98, mientras que la degradó más tiramina fue *Lactobacillus plantarum* Lb 132 y la que degradó más putrescina fue *L. farciminis* CRL 678.

6. Se han identificado las enzimas responsables de la degradación de aminas en las cepas *L. plantarum* J16 CECT 8944, *P. acidilactici* CECT 5930 y *K. varians* LTH 1540. Las enzimas de las dos primeras cepas son Multicobre oxidasas (MCO) de tipo lacasa (EC: 1.10.3.2). La enzima responsable de la degradación de aminas *en K. varians* LTH 1540 es una Putrescina oxidasa (PuO) (EC 1.4.3.10).

7. La lacasa recombinante de *L. plantarum* J16 degrada principalmente la tiramina y en menor grado histamina y putrescina, la de *P. acidilactici* CECT 5930 degrada únicamente tiramina, mientras que PuO nativa de *K. varians* LTH 1540 degrada principalmente diaminas, concretamente, putrescina y cadaverina y, en menor grado poliaminas, como espermidina, pero no monoaminas.

8. Las lacasas recombinantes SufI J16, Lpa 5930 y la PuO difieren entre sí, además de en sus sustratos, en sus condiciones óptimas de actuación para la degradación de aminas. De entre las 3 aminas estudiadas el mejor sustrato de las lacasas fue la tiramina, mientras que el de la PuO fue la putrescina. Las primeras tienen una temperatura óptima de actuación más baja que la putrescina oxidasa y son más termoestables. La PuO tiene un pH óptimo de actuación sobre la putrescina alcalino, mientras que las lacasas tienen dos valores óptimos de funcionamiento sobre la tiramina, uno ácido y otro alcalino.

9. Las lacasas recombinantes SufI J16 y Lpa 5930 presentan distintos parámetros de actuación frente a los sustratos típicos de las lacasas (DMP y ABTS). Difieren entre sí en sus temperaturas óptimas de actuación, termoestabilidad, constantes cinéticas y susceptibilidad a inhibidores. Sin embargo, presentan similares valores de temperatura y pH óptimos frente a la tiramina. El ABTS actúa como mediador para las dos enzimas recombinantes aumentando considerablemente su actividad degradadora de tiramina.

10. Es la primera vez que se describe la presencia de una putrescina oxidasa en *K. varians* LTH 1540.

11. Es la primera vez que se demuestra la presencia de lacasas en bacterias lácticas.

12. Es la primera vez que se demuestra la capacidad de degradar AB por lacasas de BAL y del hongo *Trametes versicolor*.

13. Es la primera vez que se describe la degradación de aminas en vino por parte de las bacterias de las especies: *Lactobacillus farciminis*, *Lactobacillus plantarum* y *Pediococcus acidilactici*. Así como de la cepa *Kocuria varians* de origen alimentario.

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Anexo 1

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine

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Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine

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Abstract The main objectives of this study were the search for enzymatic activities responsible for biogenic amine (BA) degradation in lactic acid bacteria (LAB) strains isolated from wine, their identification, and the evaluation of their applicability for reducing BAs in wine. Fifty-three percent of the 76 LAB cell extracts showed activity against a mixture of histamine, tyramine, and putrescine when analyzed in-gel. The quantification of the degrading ability for each individual amine was tested in a synthetic medium and wine. Most of the bacteria analyzed were able to degrade the three amines in both conditions. The highest percentages of degradation in wine were those of putrescine: up to 41 % diminution in 1 week. Enzymes responsible for amine degradation were isolated and purified from *Lactobacillus plantarum* J16 and *Pediococcus acidilactici* CECT 5930 strains and were identified as multicopper oxidases. This is the first report of an efficient BA reduction in wine by LAB. Furthermore, the identity of the enzymes involved has been revealed.

Keywords Biogenic amines · Histamine · Laccase · Lactic acid bacteria · Multicopper oxidase · Putrescine · Tyramine · Wine · *Lactobacillus plantarum* · *Pediococcus acidilactici* · *Trametes versicolor*

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Introduction

Biogenic amines (BAs) are low molecular weight, organic bases, frequently occurring in fermented food and beverages (Silla Santos 1996; Ten Brink et al. 1990). If they are present in high concentrations, these compounds can cause human health problems such as headaches, respiratory distress, heart palpitations, hyper- or hypotension, nausea, hot flush, sweating, bright red rash, and oral burning (Ten Brink et al. 1990). BAs can be present in raw food or be formed by microorganisms in fermented products, like wine. Twenty-four different BAs have been identified in wines, with putrescine being the most abundant and histamine the most dangerous (Lehtonen 1996; Vázquez-Lasa et al. 1998). In Europe, there are no regulations limiting the amount of histamine in wines, but the presence of this and other amines is considered a marker of poor quality and bad winemaking practices. In addition to the health concern, a high BA concentration in wine affects its organoleptical properties (Palacios et al. 2005). For these reasons, some European countries have recommended maximum levels for histamine, hampering the commercialization of wines that do not meet these recommendations.

Strategies that could be adopted to diminish BAs formation in wine are mainly (a) reducing precursor levels (amino acids), (b) limiting the growth of spoilage bacteria, and (c) inoculating starter cultures with no amino acid decarboxylases. When the aforementioned strategies fail or cannot be carried out, another strategy is to eliminate the BAs already produced through the use of microorganisms or enzymes. This strategy has been used in some food, but in the case of wine very little literature using this approach has been published. The ability to degrade biogenic amines in culture media or buffer systems by microorganisms has been reported in many papers. Some of these microorganisms were isolated from food. Thus, in 1978, Voigt and Eitenmiller mentioned the ability of some cheese lactic acid bacteria (LAB) to degrade

histamine and tyramine in culture media. Later, Leuschner et al. (1998) also found that some microorganisms isolated from food, LAB among them, degraded histamine and/or tyramine in buffer system. In 2000, Martuscelli et al. found that 21 out of 26 strains of *Staphylococcus xylosus* decreased histamine and/or tyramine in phosphate buffer (pH7). According to the results of Dapkevicius et al. (2000), some strains of *Lactobacillus sakei* and *Lactobacillus curvatus* isolated from mackerel fish paste were able to degrade histamine in MRS broth supplemented with this BA. In 2001, Fadda et al. described some *Lactobacillus casei* and *Lactobacillus plantarum* strains from meat possessing tyramine-degrading activities. Later, an extremely halophilic archaea *Natrinema gari* strain, isolated from anchovy fish sauce (Tapingkae et al. 2010), showed ability to degrade histamine in high-salt media. Recently, García-Ruiz et al. (2011) and Capozzi et al. (2012) reported that some wine LAB belonging to *Lactobacillus* and *Pediococcus* genera were able to degrade histamine, tyramine, and putrescine in culture media.

Some authors have used BA-degrading bacteria in order to diminish BAs in foods. Thus, Leuschner and Hammes (1998a) used three different strains of *Brevibacterium linens* as inocula to eliminate tyramine and histamine in cheese. Later, the same authors used one strain of *Micrococcus varians* in order to degrade tyramine during sausage ripening (Leuschner and Hammes 1998b). In 2000, Dapkevicius et al. used two strains of *L. sakei* for degrading histamine in fish slurry. In 2002, Gardini et al. managed to reduce concentrations of tyramine, spermine, and spermidine in dry sausages by inoculating a mixed starter of *L. sakei* G20 and *S. xylosus* S81. Later, in 2007, Yongsawatdigul et al. described that the inoculation of *Virgibacillus* sp. SK33 in Thai fish sauce fermentation halved the histamine content.

More recently, Mah and Wang (2009) reduced biogenic amine in Myeolchijeot, a salted and fermented anchovy (*Engraulis japonicas*), by using starter cultures of *S. xylosus* during ripening. Finally, García-Ruiz et al. (2011) attempted to reduce BAs in wine by using wine LAB but were unsuccessful.

In addition to microorganisms, amine oxidases (AOs) have been also used to reduce the content of BAs in foods. There are several patented procedures for obtaining enzymatic preparations of AOs from different sources, like animal organs and microorganisms, which could be used to degrade BAs in foods (Charles and Georgina 1985; Hiemenz and Setz 1942; Underberg and Lembke 1988; Williams 1943). In 1985, patent number EP0132674 described the preparation and use of an amino oxidase of *Aspergillus niger* IMI17454 to remove amines in cheese, beer, must, or yeast extract. However, although the authors mentioned that their procedure reduced BAs in grape must, they did not provide data on the efficiency of the method in must or in wine (Charles and Georgina 1985). Another patent reported the preparation of histaminases from bacterial and yeast cells of the genera *Lactobacillus* and *Candida* and their use to eliminate histamine from foodstuffs, beverages, and forages (Underberg and Lembke 1988). The use of a commercial diamine oxidase purified from porcine kidney is another option for histamine degradation. The activity of this enzyme was studied in phosphate buffer (pH7.0) and ensiled fish slurry (pH4.5) by Dapkevicius et al. (2000). Results reported no degradation at pH4.5, thereby limiting its use to food with higher pH values.

Microbial BA-degrading activities have been attributed to AOs. Thus, in 1965, one AO from *A. niger* was purified and characterized by Yamada et al (1965b). The first references of bacterial AOs were those of Yamada et al. (1965c) and Yamada et al. (1967) in which a putrescine oxidase of *Micrococcus rubens* (syn. *Kocuria rosea*) and a tyramine oxidase of *Sarcina lutea* (syn. *Micrococcus luteus*) were characterized. Later, Murooka et al. (1979) described that the enzyme responsible for BA oxidation in Gram-negative bacteria was a membrane-bound monoamine oxidase able to degrade tyramine, octopamine, dopamine, and norepinephrine. Afterwards, one methylamine oxidase was purified and characterized in 1981 from the yeast *Candida boidinii* (Haywood and Large 1981). Part of the above information was compiled and completed in the broad review by Yagodina et al. (2002) which also reported the existence of an AO in the archeobacterium *Methanosarcina barkery* and in the fungus *A. niger*. In 2004, a thermostable histamine oxidase was purified and characterized from the actinobacteria *Arthrobacter crystallopoietes* KAIT-B-007 (Sekiguchi et al. 2004).

No previous work has reported the successful removal of BAs from wine using purified and characterized enzymes or bacteria, even if the bacteria were of wine origin. The physicochemical conditions of wine, primarily low pH, prevent the development and activity of most bacteria, as well as the optimal performance of most described AOs.

So far as we know, the only work reporting successful degradation of BAs in wine is the study by Cueva et al. (2012). In this paper, the authors found an extracellular fraction of a vineyard *Penicillium citrinum* able to degrade histamine, tyramine, and putrescine in wine. However, many fungi, including some *P. citrinum* produce mycotoxins (Rundberget et al. 2004) or extracellular enzymes that can cause the browning of wine (Kassemeyer and Berkelmann-Löhnertz 2009). In contrast, LAB do not produce this kind of toxins and many of them have GRAS status; hence, their use in food fermentations is preferred. We aimed to detect wine LAB strains able to remove BAs of wines and identify the enzyme responsible for histamine, tyramine, and putrescine degradation. The use of LAB or their enzymes could solve the problem of high amine concentrations in wine, thereby improving the quality, safety, and competitiveness of wines in the market.

Materials and methods

Strains and growth conditions

Strains used belonged to our research ENOLAB collection, Spanish Type Culture Collection (CECT) and to the Reference Center for Lactobacilli (CERELA, Tucumán, Argentina). The majority of them were isolated from the winemaking process, but also reference strains from other habitats were included (Table 1). All strains were routinely grown overnight at 28 °C on modified MRS medium supplemented with L-cysteine 0.5 g/L and BAs (histamine, tyramine, and putrescine) at 10 mg/L each.

Obtaining of cell-free extracts and enzymatic assays

Cell-free extracts were obtained from cultures grown overnight in 50 mL of modified MRS (Scharlau) medium under conditions previously described. Cells were collected by centrifugation at 10,000 rpm for 10 min (Multifuge 1 S-R, Heraeus), washed twice with 25 mL of sodium phosphate buffer 50 mM pH7.4 and resuspended in 500 μL of the same buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. Then, cells were disrupted with 1 g of 106 μM diameter glass beads in a Mikrodismenbrator® Sartorius: 10 cycles of 40 s, alternating 5 cycles of disruption with a cooling step of 5 min in ice. Whole-cell extracts were centrifuged at 13,000 rpm for 15 min (PrismR, Labnet), and supernatants were saved at −20 °C until use.

A volume of 25 μL of each cell-free extract was mixed with loading buffer containing 10 % glycerol, 50 mM Tris–HCl, pH6.8, bromophenol blue (0.02 % final concentration), loaded on a stacking 4 % polyacrylamide gel and resolved in a non-denaturing 8 % polyacrylamide gel, using Tris–glycine as electrophoretic buffer (25 mM Tris base, 192 mM glycine). Samples were electrophoresed for an hour at 30 mA. After protein separation, the gel was used for in-gel amine-degrading activity or multicopper oxidase (MCO) activity detection. In the first case, amine oxidase assay was performed following the procedure described by Leuschner et al. (1998), with some modifications: gel was placed in sodium phosphate buffer (50 mM, pH7.4) containing 1 mM of histamine, tyramine, and putrescine for 15 min, and then the solution was discarded and replaced by a new one of the same buffer containing horseradish peroxidase (1,000 U/L) and diaminobenzidine (DAB, 0.25 mM) as chromogenic substrate. The presence of amine-degrading activity was revealed by the apparition of a brown color on the active band after 1–2 h. Brown color was due to the precipitated product of DAB oxidation. In the case of MCO detection, after electrophoresis, gel was placed in sodium acetate buffer 100 mM at pH4 with 10 mM 2,6-dimethoxyphenol (DMP) for 5 min, and then the solution was discarded and replaced with a new one of the same buffer containing 1 mM $CuSO_4$. MCO activity was revealed after 10 min by the presence of an orange-yellow band.

Assays for quantifying amine degradation of cells in synthetic medium and wine

A volume of 100 μL of culture grown overnight on MRS supplemented with L-cysteine and BAs (as described above) was used to inoculate the medium described by Dapkevicius et al. (2000) with some modifications: 0.15 g/L of histamine, tyramine, and putrescine were added separately to the medium and the pH was adjusted to 5.5 (García-Ruiz et al. 2011). After 48-h incubation at 28 °C, the reaction was stopped by adding 1 M HCl. Then, samples were centrifuged at 13,500 rpm for 5 min and filtered through 0.22-μm nylon membranes (Fisher). Amine concentrations were measured by HPLC in an Agilent 1200SL HPLC system. The HPLC system was equipped with an in-line degasser, autosampler, column heater, and a fluorescence detector. Chromatographic separation was performed on HPLC Luna C18 silica Phenomenex column (250×4.6 mm) with a guard column (20×4.6 mm) of the same type. A solution of methanol was used as mobile phase A and a solution of 140 mM sodium acetate trihydrate and 17 mM TEA adjusted to pH5.05 as mobile phase B. Gradient conditions used for separation were described by Hernández-Orte et al.(2006). A sample volume of 10 μL was buffered with 25 μL of a solution containing 0.2 M sodium borate buffer (pH8.8) and 5 mM disodium EDTA. The derivatization reaction was performed by adding 15 μL of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC solution Waters) according to the optimized procedure described by Cohen and De Antonis (1994). Excitation and emission wavelengths of the fluorescence detector were set at 250 and 395 mm, respectively. A volume of 5 μL of the derivatized sample was injected into HPLC system. HPLC column temperature was kept at 65 °C. Flow rate was set at 2 mL/min. Total elution time was 70 min. Degradation ratio relative to the uninoculated medium was calculated after 48 h of incubation.

In order to quantify the degradation of BAs in wine, cells were grown in the semisynthetic must-wine medium number 7 (Pardo et al. 1992) to stationary phase, recovered by centrifugation, washed twice with 50 mM sodium phosphate buffer, pH7.4, and concentrated to obtain a cellular suspension of 10 $O.D_{600}$, which was placed in a red wine at pH3.5 supplemented with 40 mg/L of histamine, tyramine, and putrescine separately. After incubation with mild agitation at 28 °C for 7 days, the resulting reactions for the three amines were mixed in equal amounts, centrifuged, and filtered as described above. Amines from wine and medium samples were extracted with Oasis® MCX 1 cc extraction cartridges (Waters) as described by Peña-Gallego et al. (2009) and quantified by HPLC as described above.

Table 1 Lactic acid bacteria used in this work

LAB	Strains	Isolated from/culture collection
L. brevis	Lb 131, Lb 250	Red wine/ENOLAB
L. brevis	Lb 67	Grape must/ENOLAB
L. collinoides	Lb 373, Lb 404	Red wine/ENOLAB
L. casei	CECT 475^T	Cheese/CECT
L. curvatus	C9-19C [a], C13-48[a]	Sausages/Roig-Sagués
L. fermentum	CHMDW 5A	Red wine/ENOLAB
L. delbrueckii	CECT 286	Grain mash/CECT
L. hilgardii	L6, L21, L27, L41, L44, L56	Grape must/ENOLAB
L. farciminis	CRL 678[b]	Sausages/CERELA
L. mali	C46, Lb 44, 45, 47, 52, 53, 75, 110, 197, 206, 334	Red wine/ENOLAB
L. paracasei	L51 , Lb 54	Grape must/ENOLAB
L. paracasei	Lb 309, 340, 362, 365, 380, 444, 446L, 446R, 451	Wine/ENOLAB
L. plantarum	CECT 748^T	Pickled cabbage/CECT
L. plantarum	C24, C51,C145, J16, J33, J39, Lb 98, 132, 135, 140	Grape must/ENOLAB
L. plantarum	Lb 102, Lb 153, Lb 291	Wine/ENOLAB
L. plantarum	MRS 6, MRS 69A	Fermented pasta/ENOLAB
L. sakei	CECT 906^T	Sake/CECT
L. vini	CECT 7072^T	Red wine/CECT
L. vini	Lb 154, Lb 209P	Red wine/ENOLAB
P. acidilactici	CECT 5765^T	Barley/CECT
P. acidilactici	CECT 5930	Barley/CECT
P. parvulus	P205, P486BL, P487	Wine/ENOLAB
P. pentosaceus	MRS 12, 14, 44, 77	Fermented pasta/ENOLAB
E. faecium	C1	Wine/ENOLAB
E. faecium	C2	Fermented pasta/ENOLAB

[a]These strains were kindly supplied by Roig-Sagués

[b]This strain was received from CERELA as *L. casei* but it was renamed as *L. farciminis* after identification by 16S rDNA gene sequencing

Purification and identification of enzymes

Amine-degrading enzyme from Pediococcus acidilactici CECT 5930

The cell-free extract, obtained from 8 L of culture cells, was ultracentrifuged at 47,000 rpm for an hour in a Beckman L-70 Ultracentrifuge, with a SW55TI rotor and the resulting supernatant fractionated by ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ was added to obtain 50 % saturation, and after standing at 4 °C for 30 min, precipitated proteins were eliminated by centrifugation at 10,000 rpm for 30 min at 4 °C (Multifuge 1 S-R, Heraeus). The supernatant was brought to 75 % saturation, stirred for 30 min at 4 °C, and the precipitate, containing the majority of the BAs degrading activity as revealed in-gel assay, was collected by centrifuging as before. Precipitated proteins were then dissolved, dialyzed overnight against equilibration buffer (50 mM Tris–HCl, pH8, 0.01 M of NaCl, 0.05 % Tween 20), and loaded onto an anion exchange column (3.1×0. 75 cm) of Macro-Prep Q support (Bio-Rad), previously equilibrated in the same buffer. After washing, retained proteins were eluted with 20 mL of a linear gradient of NaCl concentration from 0.1 to 0.6 M in equilibration buffer. Fractions of 500 μL were collected and tested for protein content, by monitoring the A_{280} in a calibrated NanoDrop 2000c spectrophotometer (Desjardins et al. 2001), and for BA-degrading activity by in-gel staining with DAB. Fractions exhibiting the highest enzyme activity (more rapid color onset and more intense colored bands) were pooled and further purified by electrophoresis using a semipreparative native 8 % polyacrylamide gel and run for 1 h at 30 mA. After BA-degrading activity staining, brown bands from several identical lanes were cut, put together, fragmented into small pieces by passing them throughout a perforated Eppendorf tube, and resuspended in loading buffer (50 mM Tris–HCl, pH6.8, 10 % glycerol, 2 % sodium dodecyl sulfate (SDS), 1 % β-mercaptoethanol, and 0.02 % bromophenol blue). The suspension was loaded on an 8 % SDS-polyacrylamide gel which was run under similar conditions as described for native polyacrylamide gel electrophoresis (PAGE), but adding 0.1 % SDS to the electrophoresis buffer. The prestained SDS-PAGE MW Fermentas marker composed of 20 to 120 kDa blue-colored proteins was used. This second gel was also revealed by in-gel assay with DAB, and the resulting brown band was cut and sent for protein

identification to Central Support Service for Experimental Research of the University of Valencia (SCSIE). Quality of the purification was checked by Coomassie blue staining of a parallel lane loaded with the same sample. Tryptic digests of excised band gel were analyzed by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) on an Autoflex speed instrument (Bruker). Samples were prepared using 4-cyano-4-hydroxycinnamic acid as matrix on prespotted anchorchip targets (Bruker). Calibration was performed in an external mode using a peptide calibration standard kit (Bruker Daltonics). The spectra were processed using Flex Analysis 3.3 software (Bruker Daltonics). MS analysis peak lists were generated using the signals in the *m/z* 800 to 4,000 region, with a signal-to-noise threshold greater than 3. The SNAP algorithm included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. After removing *m/z* values corresponding to usually observed matrix cluster ions, an internal statistical calibration was applied. Peaks corresponding to keratin and trypsin autolysis peptides were then removed. The resulting final peak list was used for identification of the proteins by peptide mass fingerprint. The most significant peptides were studied by MS/MS analysis. Mascot 2.2 program (Matrix Science Ltd, London UK) was used to search for homologies of these peptides in the NCBInr_20100430 database (13655082 sequences). Search parameters were trypsin cleavages excluding N-terminal to P, one or two missed cleavages allowed, cysteine carbamidomethylation set as fixed modification, methionine oxidation as variable modification, mass tolerance less than 50 ppm, and monoisotopic mass values. Criteria for positive identification were a significant Mascot probability score >84 ($p<0.05$).

Amine-degrading enzyme from L. plantarum ENOLAB J16

The procedure used was essentially similar to that described for *P. acidilactici* but using 10 L of culture and including an additional purification step necessary for final high-quality protein isolation. Seventy-five percent saturation ammonium sulfate precipitate was dissolved in equilibration buffer (50 mM sodium acetate, pH4.5, 5 mM NaCl, and 0.05 % Tween 20) and then dialyzed overnight at 4 °C against the same buffer. Dialyzed sample was centrifuged (13,500 rpm, 5 min, PrismR, Labnet) to eliminate the precipitate generated during dialysis, and the resulting supernatant was loaded onto a cation exchange column (3.1×0.75 cm) of sulfopropyl Sepharose FF (GE Healthcare) pre-equilibrated with the dialysis buffer. The column was then washed and retained proteins eluted with a linear gradient of NaCl concentration ranging from 5 to 500 mM, in the same buffer (total volume 20 mL). Fractions of 500 μL were collected and analyzed for protein content by spectrophotometry and for BA-degrading activity by in-gel assay. Fractions showing enzymatic activity were pooled, dialyzed against equilibration buffer (50 mM Tris–HCl, pH8.0, 0.1 M NaCl, 0.05 % Tween 20), and loaded onto an anion exchange chromatography on Macro-Prep Q, similarly as described above. After further purification by native and 10 % SDS-PAGE, the purified protein was finally sent to SCSIE for identification, following the procedure described above for *P. acidilactici* protein.

Amine degradation by the purified enzyme SufI and a commercial MCO (laccase) from *Trametes versicolor*

Purified protein from *L. plantarum* J16 was tested for amine degradation in a buffer model system containing 80 μL of 50 mM sodium phosphate buffer, pH6.5+150 mg/L of each BA separately. Twenty microliters of purified enzyme were added to the buffer and incubated at 37 °C for 48 h. A parallel assay adding the laccase mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 10 mM was performed because most laccases are able to degrade some compounds only in the presence of a laccase mediator system. Laccase mediators can be natural or synthetic compounds that enhance enzyme activity by redox mechanisms, and ABTS is the most studied mediator (Morozova et al. 2007). Samples were subsequently analyzed by HPLC.

To test the ability of a known MCO to degrade amines, the activity of commercial *T. versicolor* laccase (Sigma) against BAs was tested by HPLC. Five units of this laccase were added to 2 mL of succinate buffer 0.2 M (pH4.5) plus histamine, tyramine, and putrescine, 1 g/L each final concentration and incubated at 37 °C for 24 h. As in the previous case, a parallel assay adding the laccase mediator ABTS at 5 mM was performed. In both cases, samples were taken at 0 and 24 h, and amine quantification was performed by HPLC analysis.

Primers design and amplification conditions for the detection of genes encoding the multicopper oxidases SufI and D2EK17

Specific PCR primers were designed and used for the detection of genes encoding MCOs from *L. plantarum* and *P. acidilactici*. From gene sequences encoding SufI and D2EK17 proteins, primers were designed for amplification of internal fragments of these genes in *L. plantarum* and *P. acidilactici* strains. The couple of primers Lac Lp1 (5′-CCCAGAATTGACGACTTTCC-3′) and Lac Lp2 (5′-GGATGGGATGGATGATGAAGT-3′) were designed to amplify an internal fragment of *sufI* gene of *L. plantarum*. Primers developed to amplify the gene encoding protein D2EK17 from *P. acidilactici* were Lac Pa1 (5′-CAAACAACTTGCCATCCAAC-3′) and Lac Pa2 (5′-GTCGGCTTTGTAATCTAGTTGA-3′). Fragment amplifications were performed in an Eppendorf thermocycler

programmed with the following thermal profile setup: initial denaturation (95 °C for 5 min), followed by 35 cycles of denaturation (94 °C for 1 min), primer annealing (55 °C for 1 min), and extension (72 °C for 1 min). Reactions were completed with 5-min elongation time at 72 °C followed by cooling to 10 °C. Template DNA was obtained from picked colonies dissolved in 10 μL of mili-U water. DNA from different strains belonging to the species *L. plantarum* and *P. acidilactici* were amplified using the primers and the amplification conditions described above, in order to detect the presence of the gene fragment encoding for MCO proteins. PCR products were resolved by electrophoresis in 1.2 % (*w*/*v*) SeaKem® LE agarose (FMC, Rockland, ME, USA) 0.5× TBE (45 mM Tris–HCl, pH8.0, 45 mM boric acid, and 1 mM EDTA) gels stained with ethidium bromide (0.5 μg/mL). Images were digitalized with a GelPrinter Plus from TDI (Madrid, Spain). The PCR product mass was evaluated with a DNA Ladder 1 KB plus™ High Range (Invitrogen). The partial nucleotide sequence of the amplified gene was determined by sequencing with the primers. The resulting sequences were submitted to the Basic Local Alignment Search Tool program available at the National Center for Biotechnology Information (NCBI, Bethesda, USA; http://www.ncbi.nlm.nih.gov) to search for similarities in the GenBank database. Mega5 alignment software was also used in the case of *P. acidilactici* sequences.

Results

Detection of amine-degrading activities in cell-free extracts by in-gel staining

Forty of the 76 extracts of LAB tested in-gel (52.6 %) showed a single brown band revealed with a mixture of histamine, tyramine, and putrescine in DAB assay. These results show that some LAB strains have enzymes able to degrade amines (Fig. 1a). Positive reactions were found in strains belonging to the species *Enterococcus faecium*, *Lactobacillus brevis*, *Lactobacillus collinoides*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis*, *Lactobacillus hilgardii*, *Lactobacillus mali*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *L. plantarum*, *Lactobacillus vini*, *P. acidilactici*, *Pediococcus parvulus*, and *Pediococcus pentosaceus* (Table 2). Brown bands of different species or even strains appeared at different heights in the gel, suggesting that different enzymes or a number of subunits of the same enzyme could be responsible for amine-degrading activities (Fig. 1a). Table 2 shows that the species with the highest number of positive extracts was *L. plantarum*: all the extracts were positive, suggesting that the ability to degrade BAs could be a general trait for this species. *L. plantarum* and *P. acidilactici* strains exhibited higher intensity and quicker onset of brown color than the other extracts in-gel staining.

For the positive extracts of *L. plantarum* and *P. acidilactici*, additional tests in-gel were performed for each single BA. Results revealed that all of them degraded the three amines (results not shown). The fact that brown marks developed for each amine appeared at the same height in the gels leads us to infer that only one protein was responsible for the three activities.

BA degradation by cells in synthetic medium and wine

Thirteen LAB strains bearing amine-degrading enzymes were tested to quantify their histamine-, tyramine-, and putrescine-degrading ability in Dapkevicius' medium, as described above. Twelve strains were able to degrade histamine, four of them degraded up to 34 % (Table 3). Tyramine was degraded by eight strains, of which six of them reduced the initial concentration by a third. Putrescine was degraded by 12 strains but to a lesser extent than the other amines. Seven strains degraded the three amines (five of them belonged to *L. plantarum*, one to *L. delbrueckii*, and one to *P. acidilactici*).

Strains showing the highest degrading activity towards histamine were *L. plantarum* Lb 140 and *L. delbrueckii* CECT 286. Tyramine was better degraded by four strains of *L. plantarum*: Lb 132, Lb 98, Lb 291, and J16 (in decreasing order), *P. acidilactici* CECT 5930, and *L. farciminis* CRL 678 (positive control for tyramine degradation). The three best degraders for putrescine were two strains of *L. plantarum* (J16, Lb 291) and *L. farciminis* CRL 678 (Table 3).

The most degrading strains in Dapkevicius' medium were tested in wine. All *L. plantarum* and *P. acidilactici* CECT 5930 strains were able to degrade the three amines in wine although with different efficiencies. However, *L. farciminis* only degraded tyramine and putrescine but degraded the latter to a high degree (Table 4). In wine, degradation percentages reached 28 % for histamine (strain Lb 98), 28 % for tyramine (strain Lb 132), and more than 40 % for putrescine (strain Lb 98, *L. farciminis* CRL 678). Histamine was degraded in wine at a higher percentage than in synthetic medium by two strains of *L. plantarum* (J16 and Lb 98). The same phenomenon occurred in all cases for putrescine; however, tyramine degradation in wine was lower than in the medium.

Purification and identification of enzymes responsible for amine degradation in *P. acidilactici* CECT 5930 and *L. plantarum* J16

Amine-degrading enzyme from P. acidilactici CECT 5930

The BA-degrading activity present in whole-cell extract from *P. acidilactici* CECT 5930, revealed by in-gel assay

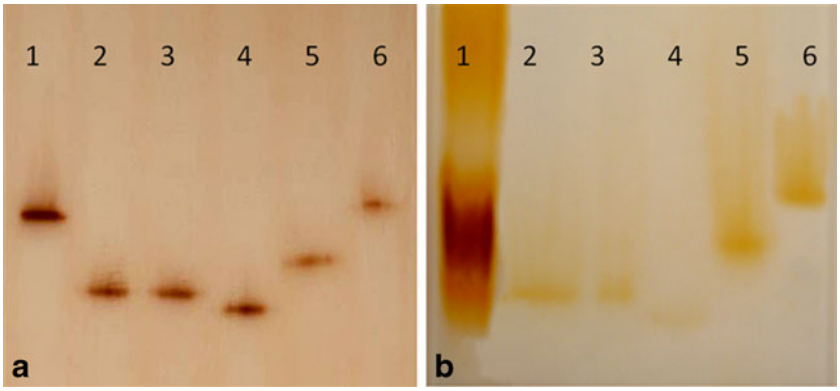

Fig. 1 **a** Amine-degrading activity showed by nondenaturing polyacrylamide gel staining from LAB cell-free extracts revealed with DAB. *Lane 1* is *T. versicolor* laccase, and *lanes 2 to 4* belong to *L. plantarum* J16, Lb 132, and Lb 291, respectively. *Lane 5* corresponds to *L. farciminis* CRL 678 and *lane 6* to *P. acidilactici* CECT 5930. **b** MCO activity by gel staining from LAB cell-free extracts and purified enzymes. *Lane 1* belongs to *T. versicolor* laccase, *lane 2* to purified protein SufI from *L. plantarum* J16, *lanes 3 and 4* belong to cell-free extracts from Lb 132 and Lb 291, respectively. *Lane 5* corresponds to *L. farciminis* CRL 678 extract and *lane 6* to the purified protein D2EK17 from *P. acidilactici* CECT 5930

using DAB, was purified by a combination of ammonium sulfate precipitation, anion exchange chromatography, and two different electrophoreses in polyacrylamide gels. Enzyme activity was recovered in the precipitate of 75 % saturation of ammonium sulfate fractionation, employed as the first step in the purification, and subsequently chromatographed on Macro-Prep Q. The BA-degrading activity eluted as a single peak around 300 mM NaCl, as revealed by in-gel with DAB assay. The fractions showing the highest levels of enzyme activity containing 11.5 mg/mL of protein were pooled and electrophoresed on a semipreparative native 8 % PAGE, followed by an SDS 8 % PAGE (strikingly, the enzyme retains its catalytic activity under SDS-denaturizing conditions, as shown in Fig. 2). The single brown band obtained after DAB gel staining was at the same position as a clear and isolated band visualized after Coomassie blue staining of a twin lane, indicating an excellent purification degree. Protein from the SDS-PAGE band was excised, digested with trypsin, and identified as a putative uncharacterized protein of *P. acidilactici* 7_4 with UniProt ID: D2EK17. This protein was cited in Prosite and Interpro Databases as a multicopper oxidase (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR001117). Matched peptides covered the 36 % of the complete sequence of the identified protein (Fig. 3).

Amine-degrading enzyme from L. plantarum ENOLAB J16

Due to a lower enzymatic activity in *L. plantarum* ENOLAB J16 than in *P. acidilactici*, a larger amount of starting material was required and an additional purification step. A cation exchange chromatography on SP-Sepharose FF was added between ammonium sulfate precipitation and Macro-Prep Q chromatography. Dialysis steps with appropriated buffers were carried out in order to connect the different purification phases properly. Excellent purification was achieved when fraction containing 0.25 mg/ml of protein was loaded in native 8 % PAGE, followed by SDS-10 % PAGE (Fig. 4). The rest of the protein purification procedure and identification was essentially identical to that employed for *P. acidilactici* CECT 5930. Results from the Mascot Search software analysis showed that peptides (Fig. 5) pertained to the cell division protein SufI. This protein has been described as a provisional multicopper oxidase with accession number UniProt ID: C6VK53. Significant peptides covered 54 % of the complete protein sequence. In the laccase and multicopper oxidase engineering database (LccED) (http://www.lcced.uni-stuttgart.de), the cell division SufI was classified as MCO belonging to SUBFamily J (Bacterial CueO).

Evaluation of MCO activity from bacterial extracts and enzymes

To support the results provided by the Mascot identification software which identified purified proteins of *L. plantarum* and *P. acidilactici* as MCOs, their activity toward the canonical substrate of this kind of enzymes (DMP) was assayed in-gel (Fig. 1b). Yellow-orange bands appeared in-gel in both cases when DMP was added. The yellow-orange stained bands resulting after DMP staining were placed at the same positions as brown bands revealed with BAs and DAB in a twin-gel.

To find out if the enzymes responsible for amine degradation in the 76 cell-free extracts of LAB also had multicopper oxidase activity, they were tested in-gel with DMP as substrate. See Table 2 for the data. Forty-seven extracts (61 %) possessed activity toward DMP after 10 min of staining. Comparison of BA-degrading activity revealed with DAB shows that all the positive strains, except two, were also positive in

Table 2 Enzymatic activities of cell free extracts from LAB on biogenic amines (histamine, tyramine, and putrescine mixture) and DMP assayed under nondenaturing polyacrylamide gels

LAB strain	Enzymatic activities		LAB strain	Enzymatic activities	
	BAs	DMP		BAs	DMP
E. faecium C1	+	−	*L. paracasei* Lb 446L	+	+
E. faecium C2	+	−	*L. paracasei* Lb 446R	−	−
L. brevis Lb 67	+	+	*L. paracasei* Lb 451	−	+
L. brevis Lb 131	+	+	*L. pentosus* Lb 445	+	+
L. brevis Lb 250	−	−	*L. pentosus* Lb 453	+	+
L. casei CECT 475^T	−	−	*L. plantarum* CECT 748^T	+	+
L. collinoides Lb 373	+	+	*L. plantarum* C24	+	+
L. collinoides Lb 404	−	−	*L. plantarum* C51	+	+
L. curvatus C9-19C	−	−	*L. plantarum* C145	+	+
L. curvatus C13-48	−	−	*L. plantarum* J16	+	+
L. delbrueckii CECT 286	+	+	*L. plantarum* J33	+	+
L. farciminis CRL 678	+	+	*L. plantarum* J39	+	+
L. fermentum CHMDW 5A	−	−	*L. plantarum* Lb 98	+	+
L. hilgardii L6	−	+	*L. plantarum* Lb 102	+	+
L. hilgardii L21	−	−	*L. plantarum* Lb 132	+	+
L. hilgardii L27	−	+	*L. plantarum* Lb 135	+	+
L. hilgardii L41	+	+	*L. plantarum* Lb 140	+	+
L. hilgardii L44	+	+	*L. plantarum* Lb 153	+	+
L. hilgardii L56	−	+	*L. plantarum* Lb 291	+	+
L. mali C 46	−	+	*L. plantarum* MRS 6	+	+
L. mali Lb 44	−	−	*L. plantarum* MRS 69A	+	+
L. mali Lb 45	+	+	*L. sakei* CECT 906^T	−	−
L. mali Lb 47	−	−	*L. vini* CECT 7072^T	−	+
L. mali Lb 52	+	+	*L. vini* Lb 154	+	+
L. mali Lb 53	−	−	*L. vini* Lb 209P	−	−
L. mali Lb 75	+	+	*P. acidilactici* CECT 5765^T	+	+
L. mali Lb 110	+	+	*P. acidilactici* CECT 5930	+	+
L. mali Lb197	−	−	*P. parvulus* P 205	−	−
L. mali Lb 206	−	−	*P. parvulus* P 486 BL	−	−
L. mali Lb 334	+	+	*P. parvulus* P 487	−	+
L. paracasei L51	−	−	*P. parvulus* R210 1A	−	−
L. paracasei L54	−	+	*P. parvulus* R210 2B	−	−
L. paracasei Lb 309	−	−	*P. parvulus* R211A	+	+
L. paracasei Lb 340	+	+	*P. parvulus* R211B	−	−
L. paracasei Lb 362	−	−	*P. pentosaceus* MRS 12	−	−
L. paracasei Lb 365	−	−	*P. pentosaceus* MRS 14	−	−
L. paracasei Lb 380	−	−	*P. pentosaceus* MRS 45	+	+
L. paracasei Lb 444	−	+	*P. pentosaceus* MRS 77	+	+

the DMP assay. Twelve strains gave a positive response for DMP but not for DAB at the conditions used, and they belonged to *L. hilgardii*, *L. paracasei*, *L. vini*, and *P. parvulus* species. A special case is that of two strains of *E. faecium* that exhibited positive DAB staining in-gel but were not positives for DMP degradation in-gel conditions. This fact suggests that different enzymes may catalyze amine and DMP degradation. In this case, the enzyme able to degrade amines may be an AO.

The results confirm that the enzymes purified and identified used histamine, tyramine, putrescine, and DMP as substrates and are responsible for the oxidation of these compounds, although the chemical nature of the reaction is as yet unknown. In addition, the results compiled in Table 2

Table 3 Degradation percentages of three amines in modified Dapkevicius' medium supplemented with 150 mg/L of amines, and adjusted to 5.5 pH, after 48 h of incubation

LAB	Strain	Degradation (%)[a,b]		
		Histamine	Tyramine	Putrescine
L. delbrueckii	CECT 286	33±0.25	6.3±0.52	18.0±0.12
L. farciminis	CRL 678	n.e.	33.7±0.24	25.2±0.28
L. paracasei	ENOLAB Lb 444	11.3±0.12	n.e.	n.e.
L. plantarum	ENOLAB J16	4.7±0.17	33±0.13	26.2±0.42
L. plantarum	ENOLAB Lb 98	7.3±0.18	41.7±0.32	13.8±0.38
L. plantarum	ENOLAB Lb 132	15.3±0.55	42.9±0.22	14.5±0.15
L. plantarum	ENOLAB Lb 291	18.6±0.15	39±0.41	26±0.21
L. plantarum	ENOLAB J33	6.4±0.11	n.e.	14.8±0.58
L. plantarum	ENOLAB J39	16.4±0.21	n.e.	5.8±0.12
L. plantarum	ENOLAB Lb 140	33.9±0.25	8.6±0.46	15.7±0.51
L. plantarum	ENOLAB C145	14.6±0.32	n.e.	6.2±0.11
P. acidilactici	CECT 5930	13.8±0.15	40±0.23	19.3±0.14
E. faecium	C1	3.6±0.25	n.e.	16.8±0.13

n.e. no effect was observed

[a]Activity is expressed as a percentage of amine concentration present in the inoculated sample in relation to the uninoculated sample

[b]Mean values (n=3)

suggest that the same kind of MCO could be present in extracts showing positive reaction for BAs and DMP. A result supporting this hypothesis is the fact that the band stained brown (with amines and DAB) and yellow orange (with DMP) was located at the same position in twin gels (Fig. 1).

BA degradation by purified enzyme SufI and commercial laccase from *T. versicolor*

The percentages of degradation by purified SufI protein were 36 % for histamine, 80 % for tyramine, and 17 % for putrescine, in the presence of ABTS after 48-h incubation. In the absence of ABTS, degradations of histamine, tyramine, and putrescine were considerably lower, but tyramine was the least susceptible to the lack of mediator, with a 30 % of degradation.

To verify that known MCO could act on BAs, the commercial laccase of *T. versicolor* was assayed for amine degradation in the same way as the SufI protein. The assay was performed incubating the laccase with amines in the presence/absence of the mediator compound ABTS, and degradation quantifications were done by HPLC. After a 24-h incubation without ABTS, tyramine was degraded at almost 100 %, up to 42.2 % histamine was degraded but only in the presence of the mediator ABTS, and only 10 % of putrescine was eliminated in the presence or absence of the mediator.

The above results suggest that the enzyme responsible for degradation of amines in several LAB species is a single MCO enzyme acting on different substrates such as histamine, tyramine, putrescine, and DMP.

Amplification of genes encoding for SufI and D2EK17 proteins

The primers Lac Lp1/Lp2 were used to test for the presence of SufI encoding gene in the strains of *L. plantarum* described in Table 2. All strains tested showed a band of 765 bp, the expected size, and the sequencing results for *L. plantarum* J16 and *L. plantarum* CECT 748^T amplified fragments confirmed the presence of the gene encoding the protein SufI (Fig. 6). A nucleotide identity of more than

Table 4 Degradation percentages of three amines in red wine supplemented with 40 mg/L of amines

LAB	Strain	Degradation (%)[a,b]		
		Histamine	Tyramine	Putrescine
L. farciminis	CRL 678	n.e.	16.2±0.24	44±0.22
L. plantarum	ENOLAB J16	13.4±0.35	22.5±0.14	26.5±0.25
L. plantarum	ENOLAB Lb 98	27.8±0.21	25±0.11	41.1±0.34
L. plantarum	ENOLAB Lb 132	14.7±0.15	28.4±0.36	35.5±0.13
L. plantarum	ENOLAB Lb 291	15.6±0.16	17.8±0.52	29.8±0.22
P. acidilactici	CECT 5930	13.5±0.35	18.8±0.21	35.7±0.11

n.e. no effect was observed.

[a]Activity is expressed as a percentage of amine concentration present in the inoculated sample in relation to the uninoculated sample after one week incubation

[b]Mean values (n=3)

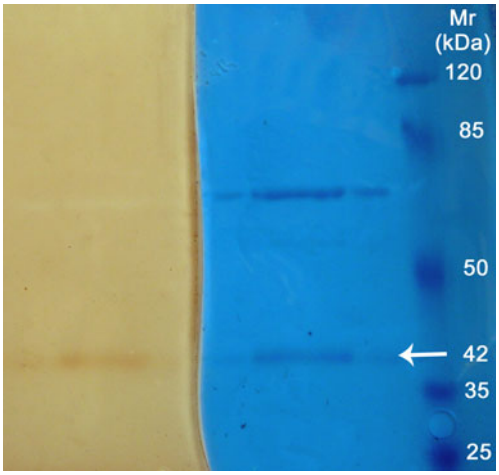

Fig. 2 8 % SDS-PAGE of purified protein from *P. acidilactici* CECT 5930. *Lane 1* purified protein stained with DAB, *lane 2* purified protein stained with Coomassie blue, *lane 3* molecular weight marker stained with Coomassie blue

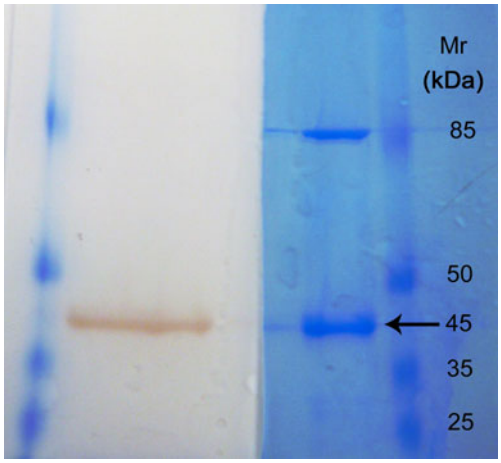

Fig. 4 10 % SDS-PAGE of purified protein from *L. plantarum* J16. *Lane 1* molecular weight marker, *lane 2* purified protein stained with DAB, *lane 3* purified protein stained with Coomassie blue, *lane 4* molecular weight marker stained with Coomassie blue

99 % with gene sequence GenBanK ID NC_012984.1 encoding SufI protein was recorded.

Amplification using the primers Lac Pa1/Pa2 and the conditions described in "Materials and methods" gave an expected fragment of 485 bp in the strains of *P. acidilactici* CECT 5765^T and CECT 5930 (Fig. 6). Sequences of both amplicons showed a sequence identity higher than 99 % with gene GenBanK ID NZ_GG730086.1 encoding for D2EK17 protein.

Thus, we can confirm the presence of the fragments from the genes encoding for MCOs in *L. plantarum* and *P. acidilactici* strains showing positive reaction for amine degradation and DMP oxidation.

Discussion

In this work, the screening of LAB able to degrade biogenic amines in wine was performed by a biochemical approach using the procedure used by Leuschner et al. 1998 to search for amine oxidase activities. The main reason for this methodology was to assure the presence of the enzymes catalyzing these reactions. The microbiological approach could give false-negative results if problems related to entry of the amine into the cell existed. The methodology used in this study has provided realistic information about the presence of enzymes acting on biogenic amines. From 76 LAB extracts screened, 40 of them (53 %) showed activity against BAs in-gel. Active extracts belonged to almost every species tested: *E. faecium*, *L. brevis*, *L. collinoides*, *L. delbrueckii*, *L. farciminis*, *L. hilgardii*, *L. mali*, *L. paracasei*, *L. pentosus*, *L. plantarum*, *L. vini*, *P. acidilactici*, *P. parvulus*, and *P. pentosaceus*. However, extracts from *L. casei*, *L. curvatus*, *L. fermentum*, and *L. sakei* gave negative results. In 1998, Leuschner et al. found lower percentages than us (42 %) of LAB able to degrade histamine or tyramine. The LAB species showing activity on histamine were *L. pentosus*, *L. plantarum*, *L. sakei*, and *P. acidilactici*, being one strain of *L. plantarum* and one of *P. acidilatici* the most active. Only one strain of *L. plantarum* was able to degrade tyramine. Differences in percentages of strains able to

Fig. 3 Mascot output corresponding to the identification of the protein of *P. acidilactici*. Peptides identified by MALDI-TOF in purified protein matching with several peptides of putative uncharacterized D2EK17 protein from *P. acidilactici* are indicated in *bold underlined letters*

MITKYL **YDENAYDYHDGGYRPLKKAPGEEHPLNVPAFLKPDR** IEGNEIYYTVTAQAGETK

ILPGKPTHTWGYNGSILGPAIQFETGK **TYHVTLK** NELDEVTTFHWHGLNIVGPYEDGGPH

APVYPHGERK**ITFTVDQPAANIWLHPHPCPETARQVWNGLAAPVIITDGHEQSLK**LPR**RW**

GVNDFPVVLQDRSYHDNQLDYK ADYDVDGTLGDYALVNGTVNPVVNVTKPIVRLRFLNGS

NRREWRLHFADYHPFTQIGSDGGLLPEAVEMDR **IMLTCAER** ADVLVNFSDYQPGQEVILQ

TDDFNLIKFKIGDIKKENMLLPSPLAEIPALSVDENTPVFK **TVMSGMDDQVRLDGKLFDMQR**

IDTRQQVDQTQIWEVSNTNDMEGGMIHPFHIHGCQFQLIDR **NGHAVNPNEHGWKDTIGVNPNETVR**

IKVKFTKLGIFMYHCHILEHEDTGMMAQIEIFDPDHPIEYHLMPMNHKM

MAKK **VYTDYFFDEPAYNTHDGGYIPLVTPKVEPQPLAIPPLLKPDRQTDTDDYYTVTAQE**

SETQFLPGKK TKTWGYNAGFLGQTIVFRNGK **QTHIDLENK** LPELTTFHWHGLNVPGPITD

GGCHAPVYPGETNHIDFKVHQPAATTWLHAHPCPSTATQVWK **GLATMVIIKDDVEDQLPL**

PRNYGVDDIPLVLQDREFHDDNQFDYRADYDPDGVQGHTALVNGTVNPYFDVTTQR VRLR

ILDGSNRR EWRLHFNDDLEFAQVASDGGILPAPVYMTK **VMMTCAER** DEIVVDFGQYQPGD

EVTLMTDDTPLCRFRIK **SFVPDDTKLPEHLVDIPDETPTPDLPVRTITMDGMDDEVALDG**

KKFDMSR IDAR **QKVGDVAIWEIRNTNSTENGMVHPFHVHGTQFR** VLAR **NDGPVYPNEHGL**

KDTVGVNPGETVR IKVKFELTGVYMYHCHIIEHEDGGMMAQIESYDPQHPQTYHLMDMDT

LRNAFAKEQGIKPEDVWMPGM

Fig. 5 Mascot output corresponding to the peptides identified in purified protein of *L. plantarum*. Matching of the peptides identified by MALDI-TOF with several peptides of *L. plantarum* SufI protein are indicated in *bold underlined letters*

degrade amines between the above study and our work could be attributed to the different approaches used in each work: microbiological versus biochemical. In both cases, various strains of *L. plantarum*, *P. acidilactici*, and *P. pentosus* showed positive responses for amine degradation. Our results show that the ability to degrade biogenic amines is strain dependent in the majority of the species, with the exception of *L. plantarum* in which all the extracts tested were active against a mixture of histamine, putrescine, and tyramine. This observation could mean that this activity is a general trait for this species and the results in Leuscher et al. (1998) support this affirmation. In some cases, the low number of extracts of different strains belonging to the same species is not enough to affirm that amine-degrading activity is strain dependent, as in the cases of *E. faecium*, *L. delbrueckii*, *L. farciminis*, *L. pentosus*, and *P. acidilactici*. Our results do not show a positive response of *L. sakei* CECT 906^T extract; however, Dapkevicius et al. (2000) reported strains of this species isolated from fish able to degrade histamine, thereby supporting the strain dependent activity in this species. However, the most active species in histamine and tyramine degradation belong to the actinobacteria group, especially *Arthrobacter*, *Micrococcus*, *Rhodococcus*, and *B. linens* strains, as Leuschner et al. (1998) and Fadda et al. (2001) have demonstrated. Nevertheless, these microorganisms are not described in the winemaking environment.

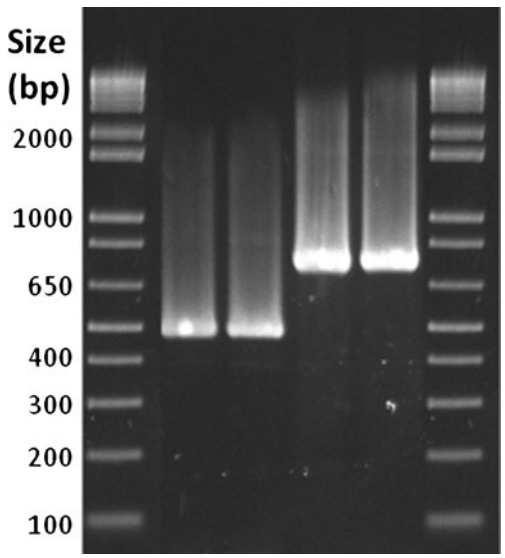

Fig. 6 Results of PCR amplification reactions obtained from *L. plantarum* and *P. acidilactici* strains. *Lanes 1 and 6* molecular weight marker 1 Kb Plus, *lanes 2 and 3 P. acidilactici* CECT 5930 and *P. acidilactici* CECT 5765^T amplification fragments obtained with Lac Pa 1/Pa 2, *lanes 4 and 5 L. plantarum* J16 and *L. plantarum* CECT 748^T amplification fragment obtained with Lac Lp1/Lp2

The degrading activity of wine strains whose extracts showed the quickest and strongest response in-gel was quantified for each individual amine. The most active wine strains degrading histamine, tyramine, and putrescine in Dapkevicius' medium belonged to *L. plantarum*, able to degrade up to 34, 43, and 26 %, respectively. Recently in 2012, Capozzi et al. reported five strains of *L. plantarum* able to degrade BAs. These results agree in part with the results published by García-Ruiz et al. (2011) in which the most active degrading strains of these three amines in culture medium were *Lactobacillus* and *Pediococcus* strains. However, Capozzi et al. (2012) did not test the activity of strains in wine and García-Ruiz et al., who did, found that none of the strains were active in wine. In our work, we have obtained percentages of degradation ranging from 13 to 30 % for histamine, 18 to 30 % for tyramine, and 26 to 41 % for putrescine, after 1 week of using *L. plantarum* strains. In some cases, the percentages of degradation were higher in wine than in the culture medium, pointing to the existence of mediator compounds in wine that collaborate in amine degradation. Dapkevicius et al. (2000) found a similar behavior in *L. sakei* strains: they degraded higher percentages of histamine in ensiled fish slurry than in synthetic medium.

The fact that brown bands appeared at the same position in-gels when they were revealed with each individual amine supports the idea that a single enzyme is responsible for the degradation of the three amines.

Until now, BA-degrading activities have been attributed exclusively to amine oxidases. Various authors have found bacteria able to degrade amines in several fermented foods (Dapkevicius et al. 2000; Fadda et al. 2001; García-Ruiz et al. 2011; Leuschner et al. 1998; Martuscelli et al. 2000; Tapingkae et al. 2010; Voigt and Eitenmiller 1978); and have even used them to reduce the amine concentration in cheeses, fermented sausages, fish, fish sauce, and fish slurry (Dapkevicius et al. 2000; Gardini et al. 2002; Leuschner and Hammes 1998a, b; Mah and Hwang 2009; Yongsawatdigul et al. 2007). All of them assumed that that the ability to degrade biogenic amines was due to the action of the AO; so therefore, the ability to degrade more than one amine would be due to the presence of several of these enzymes. However, none of them identified the enzymes responsible.

Several AOs have been identified in *Arthrobacter crystallopoietes*, *C. boidinii*, *Klebsiella aerogenes*, *M. rubens*, *S. lutea*, and *Rhodococcus erythropolis* (Cooper 1997; DeSa 1972; Haywood and Large 1981; Ishizuka et al. 1993; Murooka et al. 1979; Okamura et al. 1976; Ota et al. 2008; Sekiguchi et al. 2004; Van Hellemond et al. 2008; Yagodina et al. 2002; Yamada et al. 1965a, c), but never in LAB. Our results demonstrate that the ability to degrade BAs in at least two strains of *L. plantarum* and *P. acidilactici* is linked to the presence of a single enzyme. The purified enzyme from *L. plantarum* J16 showed two conserved domains present in protein SufI of *L. plantarum* and other MCOs compiled in the laccase and multicopper oxidase engineering database (LccED database). *P. acidilactici* protein D2EK17 is not included in this database, but it has the domain PRK10965 that is common to other MCOs; hence, it has been described as a putative MCO. We have proven the MCO activity of both enzymes, demonstrating their ability to oxidize DMP. In addition, we suggest that other strains belonging to these and other species have the same type of enzymes. The brown and yellow-orange bands appearing at the same position in twin gels when revealed with amines and DMP, respectively, supports the dual function of the enzyme. In addition, five of the species showing activity against BAs and DMP (*L. brevis*, *L. delbrueckii*, *L. paracasei*, *L. plantarum*, and *P. pentosaceus*) have enzymes classified in the same Subfamily J (Bacterial CueO) as the protein identified in *L. plantarum* J16 as can be seen in LccED database.

MCOs are encoded in the genomes of Eukarya, Bacteria, and Archaea. Their characteristic catalytic center contains four catalytic copper atoms conventionally classified into three types according to the copper's coordination and spectroscopic properties (Messerschmidt and Huber 1990). Type 1 (T1) copper (blue copper) is a mononuclear center involved in substrate oxidation, while T2 copper and binuclear T3 copper form a trinuclear cluster, the oxygen binding and reduction site.

With these four redox-active copper sites, the multicopper oxidases catalyze the four-electron ($4e^-$) reduction of dioxygen to $2H_2O$, an activity that they only share with terminal heme-containing oxidases. Most MCOs exhibit broad specificity toward various aromatic substrates such as diphenols, methoxy-substituted monophenols, and amines (Quintanar et al. 2007; Solomon et al. 1996). MCOs also can participate in cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds among other reactions (Sharma et al. 2007). The most studied MCOs are those belonging to lignin-degrading fungi, and these enzymes are mainly involved in wood decay. More recent are the descriptions of bacterial MCOs. Sharma et al. (2007) reviewed this kind of enzymes describing some of their characteristics, but the discovery of other novel bacterial MCO is in progress (Reiss et al. 2011).

Activity of MCO toward amines has been studied in very few works. In 2003, Arias et al. found a very low activity toward tyramine of a purified MCO from *Streptomyces cyaneus* CECT 3335. In 2008 and 2009, Kudanga et al., working on the functionalization of wood surfaces to bind fungicides, reported the ability of laccases to mediate the covalent binding of aromatic amines (including tyramine) to a lignin model molecule. They reported that tyramine was not a substrate for *Trametes hirsute* or *Bacillus* SF spore cotA laccases. In contrast, in this work we have demonstrated, for the first time, the ability of the *T. versicolor* laccase to degrade not only tyramine but also histamine and putrescine, although these latter two less efficiently.

As stated in the "Introduction," the only approach reporting a successful BA degradation in wine used a *P. citrinum* extracellular fraction (Cueva et al. 2012). The extract was not characterized by the authors, and they did not provide additional data about the nature of the enzyme responsible for BA degradation. As already stated, Rundberget et al. (2004) reported that some strains of the species *P. citrinum* are mycotoxin producers. The practical application to remove BA in wine with the Cuevas et al. procedure is not immediate. More research should be done to check for the presence of mycotoxins in the extract to ensure the safety of this procedure and to find out what enzyme is responsible for BA degradation. In our case, the *L. plantarum* strains able to remove BAs in wine have been isolated from the winemaking process and have been characterized as safe. In addition, the possibility of using purified MCO enzymes instead of microbial cells provides a new biotechnological alternative. Thus, our work offers the possibility of new safe procedures for reducing BAs in wine with biological approaches. However, more work on the enzymes is needed (biochemical and technological

characterization) before they can be used industrially. An exciting new research area awaits basic and applied results.

As far as we know, this is the first demonstration that LAB degrade BAs in wine and this is the first report in which the enzymes responsible for this reduction have been identified. They are MCOs and are able to degrade histamine, tyramine, and putrescine, in addition to their canonical substrate DMP. We have strong evidence that this kind of enzymes are present in various species of LAB and the most of them are active against the three amines, showing that these bacteria are a new source of MCOs. In addition, we have proven that laccase of *T. versicolor* is able to reduce tyramine, histamine, and putrescine.

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Anexo 2

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Ability of *Kocuria varians* LTH 1540 To Degrade Putrescine: Identification and Characterization of a Novel Amine Oxidase

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Ability of *Kocuria varians* LTH 1540 To Degrade Putrescine: Identification and Characterization of a Novel Amine Oxidase

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Supporting Information

ABSTRACT: This work describes the identification and characterization of an amine oxidase from *Kocuria varians* LTH 1540 (syn. *Micrococcus varians*) primarily acting on putrescine. Data from MALDI-TOF MS/MS and the identification of Δ^1-pyrroline as degradation product from putrescine indicate that the enzyme is a flavin-dependent putrescine oxidase (PuO). Properties of partially purified enzyme have been determined. The enzyme oxidizes diamines, putrescine and cadaverine, and, to a lesser extent, polyamines, such as spermidine, but not monoamines. The kinetic constants (K_m and V_{max}) for the two major substrates were 94 ± 10 μM and 2.3 ± 0.1 μmol/min·mg for putrescine and 75 ± 5 μM and 0.15 ± 0.02 μmol/min·mg for cadaverine. Optimal temperature and pH were 45 °C and 8.5, respectively. Enzyme was stable until 50 °C. *K. varians* PuO is sensitive to human flavin-dependent amine oxidase inhibitors and carboxyl-modifying compounds. The new enzyme has been isolated from a bacterial starter used in the manufacture of fermented meat. One of the problems of fermented foods or beverages is the presence of toxic biogenic amines produced by bacteria. The importance of this works lies in the description of a new enzyme able to degrade two of the most abundant biogenic amines (putrescine and cadaverine), the use of which could be envisaged to diminish biogenic amines content in foods in the future.

KEYWORDS: *amine oxidase, biogenic amines, putrescine, cadaverine, Kocuria varians*

INTRODUCTION

Biogenic amines (BA) commonly occur at high concentrations in fermented products with a high protein content. During the fermentation process the protein breakdown products, peptides and amino acids, represent precursors for amine formation by spoilage and fermentative microorganisms.[1,2] Putrescine is a low molecular weight organic base, belonging to the group of aliphatic amines. This diamine is synthesized in small quantities by living cells and appears to be a growth factor necessary for cell division.[3] Putrescine is the most abundant biogenic amine in wine[4,5] because it has two precursors from which it can be formed: ornithine, via ornithine decarboxylase enzyme, or arginine, via agmatine deiminase.[6] Although putrescine, contrary to vasoactive amines such as tyramine, histamine, tryptamine, and phenylethylamine, does not have adverse health effects by itself, it can promote the harmful activity of these amines by inhibiting the detoxifying enzymes diamine oxidase and hydroxymethyl transferase.[7] In addition, the presence of ethanol in wine enhances the toxicological effect of these vasoactive amines due to its inhibitory action on detoxifying enzymes present in the human gut.[8]

Putrescine can be present in grapes at different concentrations depending on grape variety, type of soil, fertilizer used, and geographic location.[9] Putrescine and cadaverine are normally associated with poor sanitary conditions of grapes.[10] Putrescine in grapes remains in the wine concomitantly. Putrescine and cadaverine, together with other amines, may even increase during fermentation causing further serious health problems in consumers and economic losses for producers. Moreover, putrescine and cadaverine can be detrimental to the aroma of wine, conferring putrefaction and a rotten meat smell.[11]

Many microorganisms can convert BA, via an oxidation step, into products that they can use as a carbon and/or energy source or as a nitrogen source.[12] Flavoprotein oxidases present in some microorganisms catalyze the oxidation of a wide range of compounds, BA among them, through the concomitant reduction of oxygen to hydrogen peroxide.[13] Putrescine oxidase (PuO) is a flavin-containing amine oxidoreductase that was previously described in *Micrococcus rubens*[14] (now reclassified as *Kocuria rosea*[15]) and in *Micrococcus luteus*.[16] Although *M. rubens* and *M. luteus* cells have not been used to eliminate putrescine in foods, cells of the related species *M. varians* have been employed for tyramine degradation during sausage ripening[17] or to control BA production in low-acid salami.[18] In the first case the tyramine degradation was caused by the strain expressing an amine-degrading activity.[19] However, the responsible amine-oxidizing enzyme was neither isolated nor identified. Among the referred bacteria, only the enzyme PuO from *M. rubens* has been biochemically characterized.[20]

To diminish or to eliminate BA in foods, bacteria harboring BA degrading activity can be added to the food during a processing stage or can be used directly as a starter in fermented foods. It is for this reason that the starter cultures capable of degrading BA have gained more interest.[21] Various species of microorganisms have been used to remove BA

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(mainly histamine and tyramine) from different foods and foodstuffs: dry and raw sausages, cheese, fish slurry, Thai fish sauce, Myeolchi-jeot salted and fermented anchovy.[22−27] Some examples of these species are *Staphylococcus xylosus* (sausages), *Brevibacterium linens* (cheese), *Lactobacillus sakei* (fish slurry), and *Virgibacillus* sp. (Thai fish sauce). In contrast to García-Ruíz et al.,[28] who failed to show the ability of wine lactic acid bacteria (LAB) to reduce BA in wine, we have recently found that wine-associated strains of *L. plantarum*, as well as other LAB from sausages (*L. farciminis*) and beer (*P. acidilactici*) are competent to degrade histamine, tyramine, and putrescine in wine.[29]

In this work we show that cells of *K. varians* LTH 1540 are able to degrade putrescine in wine, providing the first report on putrescine degradation in wine by a species of *Micrococcus* genus. We also describe the purification, identification, and biochemical characterization of a novel amine oxidase enzyme responsible for putrescine degradation.

MATERIALS AND METHODS

Chemicals. Commercial standard proteins, reagents, and amines (putrescine, cadaverine, histamine, tyramine, methylamine, propylamine, ethanolamine, triethylamine, spermidine, and spermine) were obtained from Sigma-Aldrich (Steinheim, Germany).

HPLC analytical grade organic solvent methanol was supplied by Panreac. Reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate solution (AQC solution) was purchased from Waters (Milford, MA, USA). Horseradish peroxidase was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

Organism and Culture Conditions. The strain *K. varians* LTH 1540, formerly isolated from artisanal fermented sausage and deposited in Dr. Hammes' culture collection (Universität Hohenheim, Germany), was kindly provided by Professor Dr. Herbert Schmidt. Cells were cultured overnight at 30 °C in nutrient broth medium supplemented with putrescine at 10 mg/L under stirred aerobic conditions.

Assay for Putrescine Degradation by *K. varians* Cells. The ability of *K. varians* cells to degrade putrescine in buffer system, synthetic media, and wine was tested. Putrescine degradation assays in 50 mM sodium phosphate buffer, pH 7.4, were performed using cells from a 6 unit OD_{600} culture, collected by centrifugation, washed twice in that buffer, and resuspended in the same buffer supplemented with 100 or 1000 mg/L of putrescine. Reaction mixtures were incubated at 28 °C with shaking, aliquots were taken at different time periods, and their putrescine content was measured by LC-FLD according to a previously reported method[30] and described below. The ability of *K. varians* cells to degrade putrescine was also tested in modified Dapkevicius' medium.[23] To this end, 100 μL of an overnight culture grown on nutrient broth medium was added to 10 mL of the medium supplemented with 150 mg/L of putrescine (pH 5.5). After static incubation for 48 h at 28 °C, residual putrescine was measured by LC-FLD.

For the analysis of putrescine degradation in wine, cells from 20 mL of nutrient broth medium culture (OD_{600} around 1 unit) were collected by centrifugation, washed twice with 50 mM sodium phosphate buffer, pH 7.4, and finally resuspended in 2 mL of red wine containing 40 mg/L putrescine (pH 3.5). Cell suspension was incubated at 28 °C under shaking conditions for 1 week, and then putrescine concentration was determined by LC-FLD as described below. In addition, viable cell counts on nutrient agar plates at 0 and 7 days were performed to estimate survival of *K. varians* LTH 1540 in wine.

BA from synthetic medium and wine samples were extracted with Oasis MCX 1 cm^3 extraction cartridges (Waters) as described in Peña-Gallego et al.[31] An Agilent 1200SL LC-FLD system equipped with an in-line degasser, autosampler, column heater, and fluorescence detector was employed. Chromatographic separation was carried out on an LC-FLD Luna C18 silica Phenomenex column (250 × 4.6 mm) with a guard precolumn (20 × 4.6 mm) of the same type. A solution of 140 mM sodium acetate and 17 mM triethanolamine (TEA, pH 5.05) was used as mobile phase A, and methanol was used as mobile phase B. A volume of 10 μL of samples was mixed with 25 μL of a solution of 0.2 M sodium borate (pH 8.8) and 5 mM EDTA. The derivatization reaction was performed by adding 15 μL of AQC solution according to the optimized procedure described by Cohen and De Antonis.[32] A volume of 5 μL of the derivatized sample was injected for each analysis. Gradient conditions used for separation were described by Hernández-Orte et al.[30] Column temperature was kept at 65 °C, flow rate was 0.8 mL/min, and total elution time was 70 min. The excitation and emission wavelengths of the fluorescence detector were set at 250 and 395 nm, respectively. Quantification was performed by comparing integrated peak areas of the sample with the control.

Purification and Identification of Putrescine Oxidase (PuO) Enzyme. Resting cells from a 2 L culture grown overnight were harvested by centrifugation at 10000 rpm at 4 °C for 20 min (Beckman coulter Avanti J-E, JA 10 rotor) and washed with 50 mM sodium phosphate buffer, pH 7.4. Cells were then resuspended in 10 mL of the same buffer and disrupted in a Mikro-Dismembrator-S (Sartorius) with 1 g of 106 μm glass beads. Cell suspensions were subjected to 10 cycles of breakage at 3000 rpm for 40 s each, alternating with cooling periods of 5 min on ice. Cell homogenate was centrifuged at 10000 rpm for 15 min at 4 °C, and the supernatant was saved as whole-cell extract.

To isolate the putrescine-oxidizing enzyme, the whole-cell extract was further ultracentrifuged at 47000 rpm for 1 h in an SW55Ti rotor (Beckman L-70 Ultracentrifuge), and the resulting supernatant was subjected to fractionation by ammonium sulfate precipitation.[29] The fraction showing enzyme activity, as revealed by an in-gel assay (see later), was retained. After dialysis against equilibration buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% Tween 20), redissolved proteins were fractionated by anion-exchange chromatography on a Macro-Prep Q (Bio-Rad) column (0.9 × 6 cm) and eluted with a 0.1−0.6 M NaCl gradient as described previously.[29] Fractions containing the highest putrescine-degrading activity, as determined by the liquid PuO activity assay described below, were pooled and used for further purification by preparative gel electrophoresis. First, an 8% polyacrylamide nondenaturing gel electrophoresis was performed as described by Callejón et al.[29] After revealing in-gel PuO activity, the resulting brown bands were cut off, fragmented by passing them through a hole at the bottom of a microcentrifuge tube, and the resulting small gel fragments were resuspended in SDS-PAGE loading solution. The suspension, without boiling, was directly loaded on an 8% polyacrylamide gel containing SDS, and after running, PuO activity was also revealed by the in-gel assay (see later). Protein purity was checked by Coomassie blue staining of a parallel lane loaded with identical sample. The brown band corresponding to PuO enzyme was cut out and sent to the Experimental Research Support Centre (SCSIE, Universitat de València) for protein identification.

Tryptic digest of the protein from the excised gel fragment was analyzed by MALDI-TOF-TOF MS/MS on an Autoflex speed instrument (Bruker). Samples were prepared using 4-cyano-4-hydroxycinnamic acid (CHCA) as matrix on Prespotted Anchorchip targets (Bruker Daltonics). Calibration was performed by an external mode using a peptide calibration standard kit (Bruker Daltonics). Spectra were processed using Flex Analysis 3.3 software (Bruker Daltonics). In MS analysis, peak lists were generated using the signals in the m/z 800−4000 region, with a signal-to-noise threshold >3. The SNAP algorithm included in the software was used to select the monoisotopic peaks from the observed isotopic distributions. After removal of m/z values corresponding to usually observed matrix cluster ions, an internal statistical calibration was applied. The final list of the resulting peaks was used to identify the proteins by peptide mass fingerprint, once keratin and trypsin peptides were removed. For MS/MS analysis, the most significant peptides were analyzed. The Mascot 2.2 program (Matrix Science Ltd., London, UK) was used to search the NCBInr_20100430 database, limiting the search to actinobacterial

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proteins. Searches were done with tryptic specificity allowing one or two missed cleavages and a tolerance on the mass measurement of 100 ppm in MS mode and 0.5 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met as variable modification.

Putrescine Oxidase Assays. The amine oxidase activity assay on putrescine in a nondenaturing PAGE was carried out essentially as previously described.[29] Whole-cell extracts or fractions from the different purification steps were mixed with an equal volume of 2× loading buffer (100 mM Tris-HCl, pH 6.8, bromophenol blue 0.04%, and glycerol 10%), loaded on a stacking 4% polyacrylamide gel and resolved on a nondenaturing 8% polyacrylamide gel, using Tris−glycine as electrophoretic buffer (25 mM Tris, 192 mM glycine). These native gels were run at 30 mA for an hour. After electrophoresis, gels were soaked in sodium phosphate buffer (50 mM, pH 7.4) containing 1 mM putrescine and incubated at room temperature with gentle agitation for 15 min. Then, the solution was replaced by the same buffer containing horseradish peroxidase (HRP; 1000 U/L) and diaminobenzidine (DAB, 0.25 mM) as chromogenic substrate. The appearance of a brown band, due to the formation of a colored precipitated product from DAB oxidation, revealed putrescine-oxidizing activity. This brown color takes usually 10 min to appear. Control assays were carried out without putrescine.

As the enzyme PuO maintains activity after SDS treatment, the identity of the band corresponding to the PuO enzyme could be checked in SDS-PAGE. To do that, samples were dissolved in SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue), but not boiled, and then loaded onto standard SDS-8% polyacrylamide gels. Following electrophoresis, gels were washed in 50 mM sodium phosphate buffer, pH 7.4, and PuO activity was revealed by the same procedure described above for native gels.

To assess which fractions of the chromatographic purification process contained PuO activity, the liquid PuO assay described by Federico et al.,[33] but modified and adapted to microplates, was also employed. This kind of assay was likewise used for the biochemical characterization of the enzyme. Briefly, 2 μL from the chromatographic fractions was mixed with 198 μL of a peroxidase-coupled reaction solution containing 2 mM putrescine, 0.5 mM 4-amino-antipyrine (AAP), 5 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS), and 1000 U/L of HRP in 50 mM sodium phosphate buffer, pH 7.4. Relative PuO activity was determined by registering absorption at 515 nm (ε_{515} = 26000 M^{-1} cm^{-1})[33] by a microplate reader, FLUOstar Optima (BMG Labtech), over a time period of 30 min.

Mass Spectrometry Analysis. Putrescine degradation assays by cells or by the partially purified enzyme, to detect Δ^1-pyrroline as PuO reaction product that forms spontaneously from 4-aminobutanal as described by DeSa and Richard,[34] were performed in 50 mM tetraborate buffer, pH 8.0. Cells at DO_{600} of 6 were incubated in the buffer cited containing 1 g/L putrescine for 6 h. After that, sample was centrifuged at 13500 rpm for 10 min, cells were discarded, and supernatant was directly injected into the mass spectrometry equipment for analysis. In the same way, partially purified enzyme was incubated for 1 h and then injected. Samples from the mixtures were taken at time 0 and processed for mass spectrometry examination as controls. Analyses were performed using the Turbo V source in an electrospray positive ion mode at an ionspray voltage of −4500 V on an AB SCIEX TripleTOF 5600 system (Applied Biosystems, Life Technologies, Canada). Curtain gas (CUR) was set at 25 psi, nebulizer gas (gas 1) at 35 psi, drying gas (gas 2) at 35 psi, and temperature at 650 °C, scan TOF MS. Data acquisition was performed using the software Analyst and was qualitatively evaluated using PeakView software (Applied Biosystems, Life Technologies, Canada).

Biochemical Characterization of the Enzyme PuO. Fractions from anion-exchange chromatography containing partially purified enzyme were used for biochemical characterization of *K. varians* PuO. Enzymatic activity was measured by liquid assay in microplates, as described previously.

The relative molecular mass of the catalytic polypeptide was estimated by comparison with molecular weight markers after a SDS-10% PAGE and subsequent in-gel assay to identify the protein with PuO activity. Molecular size determination of the native protein was performed by size exclusion chromatography and also by sucrose gradient ultracentrifugation. Chromatography was developed in a column (0.9 × 60 cm) of Sephacryl S-200 HR (GE Healthcare) equilibrated with 50 mM sodium phosphate buffer, pH 7.4, and operated at room temperature with a flow rate of 15 mL/h. Fractions of 0.5 mL were collected and analyzed for PuO activity by liquid assay. The column was calibrated using as standard molecular mass markers catalase (240 kDa), alcohol dehydrogenase (120 kDa), ovalbumin (47 kDa), and cytochrome *c* (12.5 kDa). For ultracentifugation experiments, linear sucrose gradients (7−18%) in 50 mM sodium phosphate buffer, pH 7.5, were used. Partially purified PuO was layered on the top of an 11 mL gradient and centrifuged in a Beckman SW41Ti rotor at 40000 rpm for 20 h. After centrifugation, tubes were bottom-punctured, and fractions of 0.45 mL were recovered and subjected to PuO activity liquid assay. In a parallel gradient, a mixture of the same molecular mass markers used in size exclusion chromatography was also ultracentrifuged and employed for gradient calibration.

To determine the optimal pH value of the enzyme, the following buffers (and their corresponding pH ranges) were employed at a concentration of 50 mM: acetic acid−sodium acetate buffer (for a pH range of 3.7−5.5), citric acid−Na_2HPO_4 (for pH values between 4.5 and 7.4), NaH_2PO_4−Na_2HPO_4 (pH 6.5−8.0), Tris-HCl (pH 7.2−9.0), and glycine−NaOH (pH 8.0−9.5). All samples were incubated at 28 °C. Immediately after starting the reaction by addition of 0.007 U of enzyme, absorbance at 515 nm was registered each minute for a total time of 10 min, and the initial slope of plots of A_{515} versus time, representing relative PuO activity, was calculated.

Temperature dependence was tested by an end point procedure in which the reaction mixtures were incubated at different temperatures between 4 and 75 °C. Tubes, containing 198 μL of 50 mM sodium phosphate buffer, pH 7.4, plus putrescine, and the components for the peroxidase-coupled reaction, were pre-incubated at the corresponding temperature for 5 min before the start of the reaction by adding 0.007 U of enzyme. Relative PuO activity was determined as absorbance at 515 nm. The maximum absorbance registered was considered as 100% relative activity.

For thermal stability tests, aliquots of enzyme solution were pre-incubated at different temperatures at 28, 30, 45, 55, 65, 75, 85, and 100 °C for 10 min and then were placed at 28 °C for 15 min and assayed by the PuO liquid assay. The activity obtained at 28 °C was considered as 100% relative activity.

To determine substrate specificity, different monoamines (histamine, tyramine, methylamine, propylamine, and ethanolamine), diamines (putrescine and cadaverine), and polyamines (spermine and spermidine) were used at concentrations of 2 and 50 mM, and enzyme activity was tested by liquid assay at 28 °C. In all cases reaction was initiated by the addition of 0.007 U of PuO enzyme. Absorption at 515 nm was regularly measured over 30 min in a microplate reader, and then amine oxidase activity was determined from the initial slope of plots of ΔA_{515} versus time. PuO activity on putrescine was considered as 100% relative activity. Enzyme activity on different amines was confirmed by in-gel activity assay, as previously described.

Kinetic parameters K_m and V_{max} were determined for putrescine and cadaverine by liquid assay, with various amine concentrations from 0.1 to 5 mM. Reactions were initiated by the addition of 0.007 U of PuO enzyme. Rate constants were deduced from the slope and intercept of double-reciprocal plots of initial velocity versus diamine concentration.

Bipyridyl, phenanthroline, *N*-(3-(dimethylamino)propyl)-*N*′-ethyl-carbodiimide (EDC), deprenyl, cyclopropilamine, rasagiline, pargyline, and semicarbazide were assessed as putative inhibitors of *K. varians* PuO. Inhibitor compounds were added to the reaction mixture to final concentrations of 0.1, 0.5, 1.0, and 5.0 mM. Enzyme (0.007 U) was pre-incubated at 28 °C for 5 min in the presence of each inhibitor before initiation of the reaction by adding putrescine. PuO activity measured in the absence of any inhibitor was considered as 100%

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relative activity (control). As negative control a reaction mixture containing heat-denatured enzyme was used.

Given the potential use of the enzyme in wine, the effect of ethanol on enzyme activity was tested by adding it to the reaction mixture to a concentration between 0 and 15% (v/v). The PuO liquid assay with 0.007 U of enzyme was employed in these experiments. The volume of buffer solution was adjusted accordingly to maintain the final reaction volume. Enzyme activity in the absence of ethanol was taken as 100% relative activity (control).

To investigate the ability of PuO to degrade putrescine in wine, 10 μL of enzyme solution containing 2.8 mg/mL of partially purified enzyme was mixed with 10 volumes of red wine supplemented with 40 mg/L putrescine (pH adjusted to 3.5). Following incubation at 37 °C for 24 h, putrescine content was quantified by LC-FLD, as described above. Two controls were performed: one containing an identical non-incubated (time zero) reaction mixture and a second sample without enzyme but incubated in the same conditions as the test samples. The percentage of degradation in the test samples was calculated by comparison with those controls.

Statistical Analyses. All data are presented as the mean ± SE. Group means were compared using one-way ANOVA followed by Duncan's multiple-range tests to identify differences among groups when appropriate. Two-group comparisons of control and inhibitor-exposed groups were carried out using a two-tailed Student's *t* test. All analyses were carried out using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Percentage data were normalized by arcsine transformation prior to the analysis.

RESULTS AND DISCUSSION

Ability of *K. varians* Cells To Degrade Putrescine. Previous experiments using different strains of this species isolated from fermented foods provided indications of their potential to degrade BA. We therefore started out by investigating the ability of *K. varians*, a microorganism initially obtained from dry sausages, to eliminate putrescine in different media: a defined buffered solution, Dapkevicius' medium, and wine. In the buffer solution, when the initial concentration was 100 mg/L, all putrescine was degraded by cells in <24 h. Under similar conditions, when the concentration of putrescine was 1000 mg/L, more than half of the amine was eliminated after only 4 h of incubation.

In Dapkevicius' medium, putrescine was completely removed after 48 h when the initial concentration was 150 mg/L. Furthermore, *K. varians* cells were able to degrade up to 30%, from an initial concentration of 40 mg/L in wine, after 7 days of incubation (Figure 1). At this time no viable *K. varians* cells were recovered from wine.

Purification and Identification of Putrescine Oxidase from *K. varians*. Twelve milliliters of cell-free extract with a protein concentration of 10.4 mg/mL (Table 1) was obtained after bead beating of *K. varians* LTH 1540 cells. Putrescine oxidase activity (5.6 U/mL) was recovered in the ammonium sulfate 70% saturation fraction (Table 1). PuO liquid assay of collected fractions from the Macro-Prep Q chromatography showed a single enzyme activity peak at fractions eluted around 300 mM NaCl. Table 1 summarizes the yield and purification of the *K. varians* putrescine oxidase in the first three steps of the procedure described under Materials and Methods. We performed a native gel electrophoresis as an additional purification step, which led us in the end to identify the protein with PuO activity. To do this, the Macro-Prep Q fraction showing the highest activity was loaded on a semipreparative 8% native polyacrylamide gel electrophoresis. PuO activity was revealed as a brown band by the in-gel assay. The native electrophoresis has not been included in the purification table (Table 1) because the in-gel activity assay, employed after this purification step, is neither quantitative nor comparable with the liquid assay used in the previous steps. Nonetheless, native electrophoresis generated the highest degree of purification of our protocol, given that the protein band corresponding to the enzyme accounts for >82% of total protein after this step (based on a densitometry analysis with ImageJ software of the gel after SDS-PAGE, see Figure 2, lane 5). Taking into account the protein amount (estimated also by densitometry) remaining after native-PAGE and supposing, as an upper limit, the absence of activity loss, a 4400-fold purification overall may be roughly estimated by our purification procedure. In any case, native electrophoresis was an essential step for the identification of *K. varians* PuO. When the PuO brown band from the native gel was cut out and subjected to an 8% PAGE in the presence of SDS, a single band was visualized after Coomassie blue staining, which moreover matched exactly with the brown band revealed by in-gel PuO assay on a parallel lane (Figure 2). Subjecting the brown band to a second SDS-10% PAGE, again, only one Coomassie blue stained band appeared and also coincident with a brown band resulting from the PuO in-gel assay (results not shown). Putrescine oxidase activity was recovered following exposure to, and subsequent removal of SDS, indicating that denaturation of the enzyme did not result in an irreversible loss of activity. This property of the enzyme together with the excellent resolution of proteins by SDS-PAGE enabled us to purify and identify the catalytically active polypeptide of the enzyme oxidizing putrescine. Figure 2 shows the protein analysis by SDS-PAGE corresponding to the different steps of PuO purification described under Materials and Methods. The single band with PuO activity, which appears as a homogeneous polypeptide, migrated to a position corresponding to a molecular mass of 43 kDa. Digestion of the excised brown band by trypsin gave eight internal peptides that were resolved

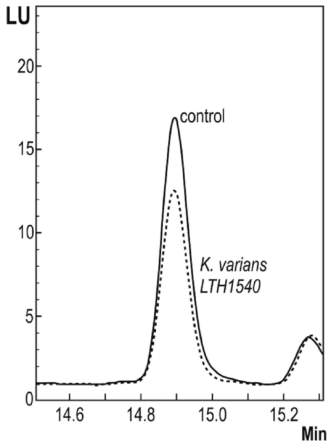

Figure 1. Degradation of putrescine in wine after incubation in the presence of *K. varians* LTH 1540 cells (equivalent to 10 OD_{600} units, approximately 7.6 × 10^{10} CFU/mL) (dotted line). Wine was supplemented with 40 mg/L of putrescine prior to inoculation of cells. Remaining putrescine in wine was quantified by LC-FLD. The graph shows the retention time on the *x*-axis versus fluorescence units (LU) recorded by the detector (*y*-axis). As negative controls, an identical mixture not incubated obtained at time zero (control 1) and wine supplemented with putrescine without the addition of cells incubated equally (control 2) were used. Because both controls gave the same peak area, only control 1 is shown. Only the chromatographic profile region corresponding to the putrescine peak is shown.

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Table 1. Purification of *Kocuria varians* LTH 1540 Putrescine Oxidase[a]

step	volume (mL)	protein concentration (mg/mL)	enzyme activity[b] (units/mL)	specific activity (units/mg)	purification (-fold)
crude extract	12	10.4	1.22	0.12	1
$(NH_4)_2SO_4$ precipitation	2.4	7.4	5.60	0.76	6.4
Macro-Prep Q chromatography	1.0	2.8	6.70	2.40	20

[a]The table shows results of typical experiments starting from 2 L of a culture of cells grown overnight. [b]Enzyme activity is expressed in enzyme activity units (U), where 1 unit is defined as that amount of enzyme which oxides 1 μmol of substrate per minute, in the reaction mixture for the microplate PuO liquid assay as described under Materials and Methods.

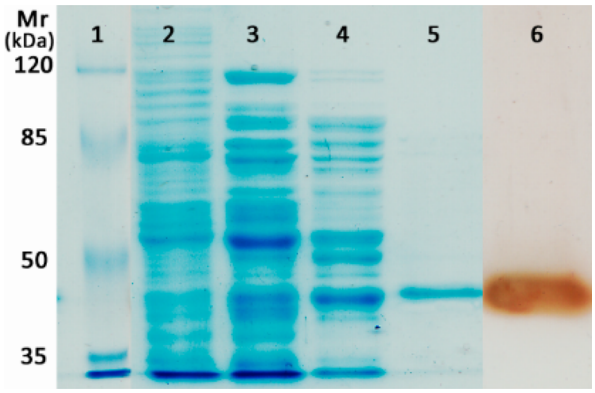

Figure 2. Electrophoretic analysis of proteins at different steps of the PuO purification in SDS-polyacrylamide gels. Lanes: 1, prestained molecular weight markers (Fermentas); 2, crude extract (~42 μg); 3, 70% saturation of ammonium sulfate precipitate (~42 μg); 4, Macro-Prep Q chromatography eluted fraction (34 μg); 5 and 6, proteins of the brown band resulting from native PAGE in which 250 μg of Macro-Prep Q partially purified protein was loaded. Lanes 1−5 were stained with Coomasie blue dye, and lane 6 was cut off and subjected to the in-gel PuO activity assay. Molecular masses of marker proteins are indicated on the left (kDa).

by mass spectrometry. Six of these peptides matched the putrescine oxidase enzyme of *K. rhizophila* (gil184199732) with a score of 321.4, covering 11.5% of protein sequence; 2 peptides matched the putrescine oxidase of *K. rosea* (gil730425) (syn. *M. rubens*) at a score of 229.3, which covers 8.2% of sequence; and 3 peptides were common to the two cited proteins (Supporting Information, Supplementary Figure 1). From this score value the putrescine degrading protein from *K. varians* LTH 1540 was unequivocally identified as a putrescine oxidase, because its score was >81, which is considered to be statistically significant for identification.[35] This protein is an ortholog of putrescine oxidase of *K. rhizophila* and belongs to the EC 1.4.3.10 class.

Supporting identification of the enzyme as PuO by mass spectrometry is the fact that Δ^1-pyrroline was detected by Mass qTOF as the product of putrescine degradation. Δ^1-Pyrroline is the product of the spontaneous condensation of 4-amino-butanal, which is the final product of putrescine oxidation by flavin-dependent PuO enzymes. A peak of m/z 70.06, corresponding to Δ^1-pyrroline, was obtained after incubation of putrescine with cells or partially purified enzyme in tetraborate buffer (Supporting Information, Supplementary Figure 2). The intensity of the Δ^1-pyrroline peak generated by the enzyme was 5-fold higher than that generated by cells.

Properties of the *K. varians* PuO. Partially purified enzyme from anion-exchange chromatography was employed to estimate the molecular mass of native PuO by size exclusion column chromatography and by sucrose density gradient ultracentrifugation. Analysis by size exclusion chromatography on Sephacryl S-200 HR yielded reproducibly an apparent PuO molecular mass of ~150 kDa. However, PuO molecular mass estimated by sucrose gradient centrifugation was around 87 kDa (results not shown). Because gel filtration and sedimentation should give similar values for globular macromolecules, our results strongly suggest that the shape of native *K. varians* PuO is elongated. Consequently, the actual molecular mass of the native enzyme probably lies somewhere between 87 and 150 kDa. Most importantly, these data demonstrate that the native form is notably higher than that of the catalytic single polypeptide obtained by SDS-PAGE (Figure 2), indicating that the *K. varians* native PuO must be a protein oligomer. The *K. varians* native enzyme is likely to be a dimer of identical subunits, given the homology with PuO from the nearby species *K. rosea*, which has been described in its native form as a homodimer with a molecular mass around 88 kDa,[34] similar to our ultracentrifugation results with *K. varians* PuO. To analyze the dependence of PuO enzyme activity on pH, different buffers with overlapping buffering capacities were used. *K. varians* PuO was found to be active over a relatively broad range of pH, which includes values from 5.5 to >9.5 (Figure 3A). No significant activity was detected below pH 5.0.

The optimal temperature for PuO activity was found to be 45 °C, although the enzyme retained activity in a rather wide temperature range, from 4 to 75 °C (Figure 3B). Moreover, temperature stability experiments revealed that *K. varians* PuO is a relatively thermostable enzyme compared to other amine oxidases. As deduced from the heat inactivation experiments (Figure 3C), the enzyme retained >50% of the activity after a pre-incubation treatment at 55 °C. In contrast, it was found that the putrescine oxidase from *K. rosea* suffered rapid inactivation at 50 °C.[20] Temperatures >65 °C completely inactivated the *K. varians* enzyme (Figure 3C).

Our substrate specificity analysis showed that none of the tested monoamines (histamine, tyramine, methylamine, propylamine, and ethanolamine) was oxidized by *K. varians* PuO (data not shown). The diamine putrescine was by far the best substrate for this enzyme, although the diamine cadaverine and, to a much lesser extent, the polyamine spermidine were also oxidized by the enzyme, as can be deduced from the relative activity data obtained from each substrate (Figure 4A). These results are consistent with previous data reported for known PuO enzymes from different species: *K. rosea*,[34,36] *M. luteus*,[16] and *R. erythropolis*.[37] Similarly to these enzymes, *K. varians* PuO apparently requires substrates with a minimum of two amino groups and, moreover, given that spermine cannot act as substrate (not shown) unlike spermidine, a free 4-aminobutyl moiety also seems to be essential for catalysis. Reactivity of *K. varians* PuO toward cadaverine and spermidine was verified by an in-gel assay after native 8% PAGE (Figure 4B), replacing putrescine with one of these other amines to reveal activities. In

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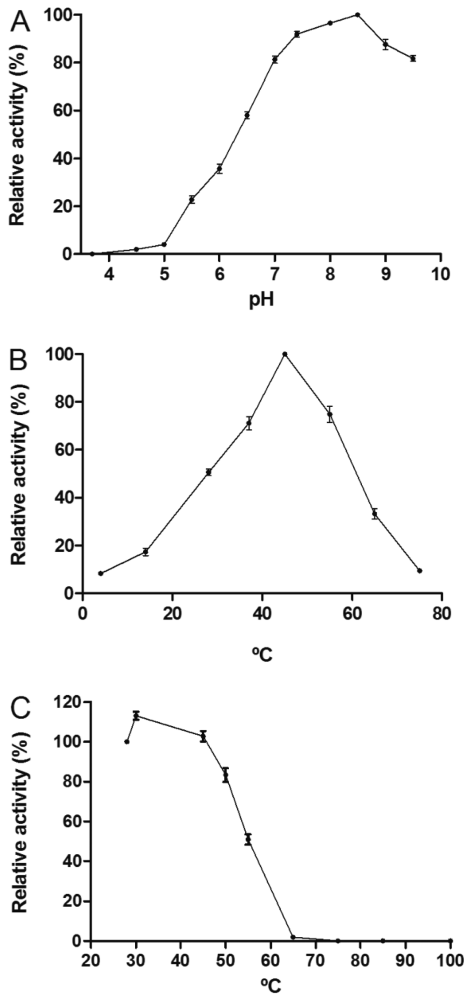

Figure 3. pH effect, temperature dependence, and thermal stability of the *K. varians* PuO. (A) pH effect. Reaction mixtures prepared with 50 mM of different buffers, at the shown pH, were assayed by the liquid procedure as described under Materials and Methods. The activity measures corresponding to the overlapping pH values were employed for normalization, and the resulting data expressed as percentage with respect to the maximum are plotted versus pH. (B) Temperature dependence. Liquid enzymatic reaction solutions were incubated at the indicated temperatures for 5 min. A_{515} was measured with a microplate reader and plotted as relative percentage to the maximum value. (C) Thermal stability. Equal aliquots of the enzyme solution were pre-incubated at the shown temperature for 10 min before being re-equilibrated to 28 °C. Putrescine was added to aliquots and degradation measured by the standard liquid assay. The residual enzymatic activity is represented as percentage relative to the value at 28 °C.

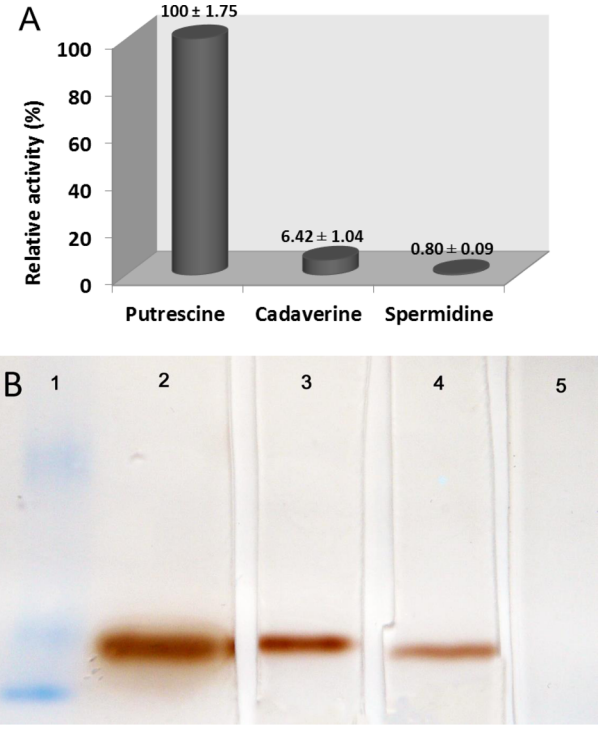

Figure 4. Substrate specificity analysis of partially purified *K. varians* LTH 1540 PuO. (A) Results from the liquid assay. Oxidation progression of the substrates, putrescine, cadaverine, and spermidine (2 mM), was determined as the absorption increase at 515 nm in a microplate reader. The initial slope of A_{515} versus time, expressing activity, was calculated and depicted as a percentage relative to that obtained with putrescine (100%). (B) Enzyme activity determined on a native−8% polyacrylamide gel and revealed by in-gel assay with 2 mM putrescine (lane 2), cadaverine (lane 3), or spermidine (lane 4). Lane 5 is a negative control without amine. Lane 1 was loaded with prestained protein markers (Fermentas) and used as reference during the electrophoresis progression.

all cases, a brown band was observed, but whereas it took only a few minutes to become visible when putrescine was the substrate, it lasted around 30 min with cadaverine and about 2 h with spermidine. No brown band was obtained in the absence of amine. These results evidence different preferences for substrate of *K. varians* LTH 1540 PuO enzyme.

We determined the steady state kinetic parameters for the diamine substrates, putrescine and cadaverine. The double-reciprocal plot of calculated initial velocities versus substrate concentrations was used to deduce K_m and V_{max}. The resulting kinetics parameters were as follows: K_m = 94 ± 10 μM and V_{max} = 2.30 ± 0.1 μmol/min·mg for putrescine; and K_m = 75 ± 5 μM and V_{max} = 0.15 ± 0.02 μmol/min·mg for cadaverine. *K. varians* PuO K_m for putrescine is in the same value range as that described for the homologue *K. rosea* PuO, slightly higher than that reported by DeSa[34] (38 μM), and a little lower than that reported by Okada et al.[36] (240 μM). It is, however, substantially higher than that for *R. erythropolis* (8.2 μM).[37] V_{max} values and results shown in Figure 4 clearly indicate that cadaverine (the five carbon analogue of putrescine) is worse than putrescine as substrate of *K. varians* PuO. However, the K_m value for cadaverine is slightly lower than that for putrescine, which suggests, as proposed for *K. rosea* PuO,[36] that hydrophobic interaction between the enzyme and the amine substrate must increase with methylene chain length.

Percentages of *K. varians* PuO enzymatic activity after the addition of different compounds, which are known inhibitors for amine oxidases from diverse origins, are shown in Table 2. In short, no inhibitor at a concentration of 100 μM showed any significant effect on enzyme activity (not shown). However, at concentration of 0.5 mM, EDC already revealed an important inhibitor effect, and at 1 mM rasagiline and pargyline also showed a decrease in putrescine oxidation. Deprenyl, cyclopropylamine, bipyridyl, phenanthroline, and semicarbazide did not affect PuO activity at the same concentration of 1 mM. When the concentration of inhibitors was 5 mM, PuO activity was diminished by deprenyl, semicarbazide, rasagiline, EDC, and pargyline, as can be seen in Table 2. EDC was described as

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Table 2. Effect of Various Inhibitors on *K. varians* PuO[a]

	concentration (mM)	inhibition (%)
EDC	0.5	47 ± 1.63**
	1	70 ± 0.53**
	5	99 ± 0.02**
rasagiline	0.5	0
	1	10 ± 1.20*
	5	52 ± 1.46**
deprenyl	0.5	0
	1	0
	5	23 ± 0.50*
pargyline	0.5	2 ± 0.62
	1	14 ± 0.24*
	5	75 ± 1.14**
cyclopropylamine	0.5	0
	1	0
	5	0
bipyridyl	0.5	0
	1	0
	5	0
phenanthroline	0.5	0
	1	0
	5	0
semicarbazide	0.5	0
	1	0
	5	30 ± 0.43**

[a]Enzyme activity was measured by the 4-APP/DCHBS/HRP liquid assay as described under Materials and Methods. Results are expressed as percent inhibition related to the control in the absence of inhibitor. The negative control of the heat-denatured enzyme showed 100% inhibition. The experiments were done in triplicate. Asterisks indicate a *P* value of (*) <0.05 or (**) <0.01.

a carboxyl group modifying agent that affects the catalytic properties of PuO from *K. rosea*, which resulted in changes in substrate specificity and a ∼95% reduction of its catalytic potential.[38] As Table 2 shows, *K. varians* PuO was also inhibited by EDC, although to a lesser extent (∼70% reduction). It remains to be determined if modification by EDC causes the same alterations of properties in *K. varians* PuO as have already been described in *K. rosea*.

Rasagiline, deprenyl, and pargyline inhibit human monoamine oxidases (MAO)[39] through the formation of adducts with the FAD cofactor, and thus, unsurprisingly, the highest concentrations of these agents also inhibit the flavin-dependent PuO from *K. varians*. In contrast, cyclopropylamine, another MAO inhibitor, acts through alkylation of the active site.[40] Because the catalytic center of human MAOs differs from bacterial PuO as has been demonstrated by Van Hellemond et al.,[37] cyclopropylamine should act in a different way not exercising an inhibitory effect on *K. varians* PuO.

The fact that two metal chelating agents, bipyridyl and phenanthroline, did not affect the enzymatic activity of PuO suggests that no metallic cofactor is involved in catalytic activity, although both compounds significantly inhibit the activity of Cu-dependent amine oxidases.[41] Furthermore, phenanthroline at 1 mM did not affect enzymatic activity of the ortholog PuO enzyme from *K. rosea*.[42]

Semicarbazide is a carbonyl-modifying agent and a representative potent inhibitor of a specific group of amine oxidases, named semicarbazide-sensitive amine oxidases (SSAO). A high sensitivity to inhibition by semicarbazide, around 0.1 at 1 mM, distinguishes SSAOs from other amine oxidases, particularly from MAO.[43] *K. varians* PuO was not affected at all by 0.5 or 1 mM semicarbazide (Table 2) but was inhibited (by 30%) at the higher tested concentration of semicarbazide (at 5 mM), in contrast with *K. rosea* enzyme, which was not inhibited with up to 10 mM.[42]

Although the cofactor requirements of the enzyme have not been specifically analyzed in this work, MALDI-TOF identification and various physical properties including structure and substrate specificity similar to those of phylogenetically close species indicate that it is a flavin-dependent putrescine oxidase. The fact that it is inhibited by deprenyl, rasagiline, pargyline, and EDC, specific inhibitors of flavin-dependent amine oxidase, lends further support to this assumption. Likewise, the detection of Δ^1-pyrroline as product of putrescine degradation by Mass qTOF (Supporting Information, Supplementary Figure 2) confirms this idea.

Putrescine degradation in wine experiments showed that partially purified enzyme was able to degrade only 5% of putrescine after 24 h of incubation. Clearly wine conditions are not optimal for PuO activity; possible limiting factors could be ethanol and the low pH. Probably, ethanol is not the most limiting factor, as the enzyme maintains 82% activity in the presence of 15% ethanol (a higher concentration than normally found in wine). The most limiting factor should be thus the low pH of the wine (3.5 units in this case), a value at which PuO is almost completely devoid of activity (see Figure 3A). Higher reduction of putrescine in wine was achieved (30% approximately) using whole cells. Possibly inside cells, the enzyme may find a more appropriate environment for performing putrescine degradation, even if they are not growing.

Although the preferred methods for controlling BA in food are those involving prevention, it is not always possible to apply this kind of measure. Nowadays, the most widespread method for controlling BA in food is refrigeration. However, some bacteria can grow and produce BA below 5 °C, which demonstrates that refrigeration alone cannot always prevent the increase of BA and, thus, emerging control measures should be considered. New emerging control measures for delaying BA formation include high hydrostatic pressure, irradiation, packaging, microbial modeling, and the use of food additives or preservatives. These methods operate primarily through the inhibition of bacterial growth or the inhibition of the decarboxylase enzyme activity responsible for BA production.[21] In the case of wine, strategies that could be adopted to avoid BA formation are mainly reduction of precursor levels (amino acids) and prevention of growth of spoilage bacteria. Reduction can be achieved by avoiding practices such as long skin maceration, aging on lees, and amino acid addition for enhancing yeast growth during alcoholic fermentation, but these practices are not possible when full-bodied wines are intended. On the other hand, the prevention of bacterial growth is difficult due to the limited action of preservatives (lysozyme and SO_2) approved for enological use.[44]

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Some authors have used BA-degrading bacteria to reduce BA content in several fermented products, either as starters for food fermentation or as additives. For instance, Leuschner and Hammes[22] used three different strains of *Brevibacterium linens* to eliminate tyramine and histamine in cheese. These authors also demonstrated the applicability of the strain *K. varians* LTH 1540 to degrade tyramine during sausage ripening.[17] Our results suggest that the use of this bacterium to eliminate putrescine in this process should be evaluated. Likewise, two different strains of *L. sakei* were applied to remove histamine in fish slurry by Dapkevicius et al.[23] Furthermore, Gardini et al.[24] achieved reduced concentrations of tyramine, spermine, and spermidine in dry sausages by inoculating a mixed starter consisting of *L. sakei* G20 and *S. xylosus* S8. Similarly, the inoculation of 10% (w/w) of *Virgibacillus* sp. SK33 in Thai fish sauce fermentation reduced histamine content by 50%.[26] More recently, Mah and Wang attained BA reduction in Myeolchi-jeot, a salted and fermented anchovy (*Engraulis japonicas*), by applying starter cultures of *S. xylosus* during ripening.[27] Finally, it is worth mentioning that multiple lactic acid bacteria strains are able to degrade amines in wine as described by Callejón et al.[29]

Purified enzyme amine oxidases have also been applied to reduce the BA content in food. With this aim, Dapkevicius et al.[23] utilized purified amine oxidase from porcine kidney to reduce histamine in ensiled fish slurry. Similarly, U.S. patent 4725540 describes the use of histaminases from *Lactobacillus* bacteria and from *Candida* yeast cells to eliminate BA from foodstuffs, beverages, and forages.[45] Therefore, cells containing amine oxidase activity or the direct use of the purified enzyme can be used to eliminate BA already generated in food. This strategy would be a potential measure of control when all other prevention practices have been ineffective.[21] In our case, the limited activity of the partially purified enzymatic fraction in wine does not support its use to decrease putrescine content. However, the fact that entire cells are able to degrade 30% of the amine opens the possibility of using them as an alternative to the enzyme. Because it has been shown that cells are unable to grow in wine, the use of high densities of nonproliferating cells is proposed. A similar strategy has been used to perform malolactic fermentation in wines in which very low pH values or high ethanol content prevented bacterial growth.[46]

In this work, we have demonstrated that *K. varians* LTH 1540 degrades diamines, mainly putrescine, in buffer, Dapkevicius' medium, and wine. We have identified the enzyme responsible for diamine degradation in this bacterium as a PuO; it has been biochemically characterized, and a strategy is proposed to degrade putrescine in wine. The applicability of *K. varians* LTH 1540 PuO in the food industry is a new challenge to be considered in the near future.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Anexo 3

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation

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Cloning and characterization of a new laccase from *Lactobacillus plantarum* J16 CECT 8944 catalyzing biogenic amines degradation

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Abstract In our search for degrading activities of biogenic amines (BAs) in lactic acid bacteria, a protein annotated as laccase enzyme was identified in *Lactobacillus plantarum* J16 (CECT 8944). In this study, the gene of this new laccase was cloned and heterologously overexpressed in *Escherichia coli*. The recombinant laccase protein was purified and characterized biochemically. The purified laccase showed characteristic spectroscopic properties of blue multicopper oxidases. The enzyme has a molecular weight of ~62.5 kDa and activity toward typical laccase substrates 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (2,6-DMP). The pH optima on ABTS and 2,6-DMP were 3.5 and 7.0, respectively. Kinetic constants K_m and V_{max} were of 0.21 mM and 0.54 U/mg for ABTS and 1.67 mM and 0.095 U/mg for 2,6-DMP, respectively. The highest oxidizing activity toward 2,6-DMP was obtained at 60 °C. However, after a preincubation step at 85 °C for 10 min, no residual activity was detected. It has been demonstrated that recombinant *L. plantarum* laccase oxidizes biogenic amines, mainly tyramine, and thus presents new biotechnological potential for the enzyme in eliminating toxic compounds present in fermented food and beverages.

Keywords Laccase · Biogenic amines · Tyramine · Histamine · Putrescine · *Lactobacillus plantarum*

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Introduction

Laccases (benzenediol/oxygen oxidoreductases; EC 1.10.3.2) belong to a diverse group of multicopper oxidases. Their catalytic centers contain four reactive copper atoms, giving them a characteristic blue color. The catalytic centers conventionally are classified into three types according to the copper's coordination and spectroscopic properties (Messerschmidt and Huber 1990). Type 1 (T1) copper (or blue copper) is a mononuclear center involved in substrate oxidation, while T2 copper (or normal copper) together with T3 copper (or coupled binuclear copper center) form a trinuclear cluster containing the oxygen binding and reduction site. Laccases couple the four-electron reduction of molecular oxygen to water with the oxidation of a broad range of substrates including phenols such as methoxyphenols, polyphenols, and non-phenolic substrates such as aromatic amines, arylamines, anilines, thiols, and some cyanide complexes of metals (Giardina et al. 2010; Thurston 1994). Due to the broad substrate spectrum of laccases and their wide spectrum of catalyzed reactions, which include cross-linking of monomers, degradation of polymers, ring cleavage, and oxyfunctionalization of aromatic compounds, they are considered to be industrially relevant enzymes with potential for diverse applications (Madhavi and Lele 2009; Giardina et al. 2010).

Laccases are widely distributed among fungi, higher plants, and also bacteria (Claus 2003; Giardina et al. 2010; Hoegger et al. 2006). In fungi, they are involved in degradation of

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lignin, pigment production, and plant pathogenesis, while in plants, they participate in the lignification process (Giardina et al. 2010; Hoegger et al. 2006; O'Malley et al. 1993). To date, only a few bacterial laccases have been studied although rapid progress in genome analysis suggests that these enzymes are also widespread in bacteria. Physiological functions of these enzymes in microorganisms have been investigated and include roles in melanin production, spore coat resistance, involvement in morphogenesis, cell division, electron transport, and detoxification of copper (Giardina et al. 2010; Sharma et al. 2007). Bacterial laccase was first reported in Givaudan et al. (1993). The enzyme was isolated from *Azospirillum lipoferum* in rice rhizosphere. However, the most well-known and representative of this kind of enzymes is CotA from *Bacillus subtilis*, an endospore coat protein with high thermostability (Hullo et al. 2001). Bioinformatic studies on laccases have indicated that they are present in various high G+C Gram-positive bacteria and α-, γ-, and ε-proteobacteria (Alexandre and Zhulin 2000; Bains et al. 2003; Sharma et al. 2007). Most bacterial laccases are intracellular or periplasmic enzymes as shown in *A. lipoferum* and *B. subtilis*. Laccase enzymes have been described in genomic sequences and studied in various other bacterial species such as *Escherichia coli* (Roberts et al. 2002), several other *Bacillus* species (Ruijssenaars and Hartmans 2004), *Thermus thermophilus* (Miyazaki 2005), and diverse streptomycetes (Arias et al. 2003; Endo et al. 2003). Laccase enzyme genes have even been detected in metagenomes of uncultured bacteria (Ye et al. 2010).

Most of the laccases described in the literature with potential uses in industrial applications were isolated from higher fungi. Although laccase enzymes are widely distributed in bacteria, there have been only a few studies about industrial applications of bacterial laccasses (Sharma et al. 2007). However, the intrinsic properties of bacterial laccases, including their broader substrate spectrum and stability under extreme conditions of temperature, pH, salt, and so on, are overcoming the disadvantages of fungal laccases, which mean they have also extraordinary possibilities in a variety of biotechnological areas (Murugan 2014; Chandra and Chowdhary 2015). Recently, we have demonstrated that wine lactic acid bacteria (LAB) were also a source of laccases, with the distinction of having the ability to degrade biogenic amines (BAs) (Callejón et al. 2014). BAs in food and beverages are present in raw material, or they are generated by microbial decarboxylation of amino acids. BAs are undesirable in foods and beverages because, if consumed at high concentrations, they can cause health problems such as headaches, respiratory distress, heart palpitation, hypertension or hypotension, and several allergenic disorders (Silla Santos 1996). Therefore, maximum legal concentration limits have been imposed for some foods; e.g., in fish, the upper limit of histamine is 100 mg/kg, whereas in alcoholic beverages, the proposed maximum legal concentration is 2.0 mg/l (Landete et al. 2004). Clearly, understanding how LAB laccase enzymes work is of great interest, as they can be used to solve the problem of high amine concentrations in food and wine. These enzymes from wine-associated LAB would significantly improve, in a simple, safe, and competitive manner, the safety and quality of the product.

In our previous report, a purified protein from the wine-associated LAB strain *Lactobacillus plantarum* J16 with ability to degrade BA was identified as a SufI protein homologue, with enzymatic activity corresponding to the multicopper oxidase family (Callejón et al. 2014). The aim of the present work was to clone and express the corresponding gene and characterize *L. plantarum* J16 laccase biochemically, including its capacity to act on BA as a substrate.

Materials and methods

Chemicals and materials

Typical laccase substrates [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (2,6-DMP)], amines (histamine, tyramine, and putrescine), the expression inducer isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin, and also the standard proteins employed for molecular mass estimation were purchased from Sigma (Madrid, Spain). Nickel-chelating nitrilotriacetic acid (Ni-NTA) agarose gel, for purification of polyhistidine-tagged proteins, was purchased from Qiagen (Hilden, Germany). Prepacked Sephadex G-25 PD10 desalting columns were from GE Healthcare Life Sciences (Barcelona, Spain). All other chemicals and reagents were of analytical grade and were obtained from commercial sources.

Strains, growth conditions, enzymes, and plasmids

L. plantarum J16, originally isolated from grape must, was obtained from the private ENOLAB collection and deposited in the Spanish Type Culture Collection as CECT 8944. It was routinely cultivated in an MRS medium at 28 °C.

E. coli DH5α [F− Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1] was used as the host for manipulation and propagation of the cloned gene. The plasmid pET-28a(+) (Novagen, Madison, WI, USA) was used as the overexpression vector to produce the target protein. *E. coli* BL21(DE3) [F^{-} *dcm ompT hsdS* ($r_B^{-}m_B^{-}$) *gal* (DE3)] (Novagen) was used as the host to express the gene of interest under the control of the T7 promoter. *E. coli* strains were grown at 37 °C in a Luria–Bertani (LB) medium, and the transformants were grown in an LB medium supplemented with 50 μg/ml kanamycin.

Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA), T4 DNA ligase was from Roche Diagnostics (Barcelona, Spain), and DNA polymerase and its 10× reaction buffer were from Invitrogen (La Jolla, CA, USA). The UltraClean® Microbial DNA Isolation Kit, for genomic DNA isolation, UltraClean™ 6 Minute Mini Plasmid Prep Kit, and UltraClean® PCR Clean-Up Kit for PCR purification were from Mo Bio (Carlsbad, CA, USA). DNA synthetic oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Laccase activity assays

Laccase activity was assessed using two different procedures: in-gel and liquid assays. In-gel assays were carried out after subjecting the protein samples to native 8 % polyacrylamide gel electrophoresis, as previously described (Callejón et al. 2014). Following electrophoresis running during approximately 1 h at a constant current of 30 mA, gels were incubated at room temperature in 0.1 M sodium acetate buffer, pH 4.0, containing 10 mM 2,6-DMP for 5 min. Then, the solution was discarded and replaced with a new one with the same buffer containing 1 mM $CuSO_4$. Laccase activity was evidenced after 5–10 min by the presence of an orange-colored band (Callejón et al. 2014). Liquid assays were routinely performed in 96-well plates at 25 °C with 2,6-DMP as a substrate. For these assays, aliquots from the protein samples were mixed with 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM 2,6-DMP. Oxidation of 2,6-DMP was determined spectrophotometrically by measuring absorbance increases at 469 nm in a microplate reader (ε_{469}=27,500 M^{-1} cm^{-1}) (Molina-Guijarro et al. 2009).

Cloning of *L. plantarum* laccase gene

Genomic DNA from *L. plantarum* was isolated with the commercial system UltraClean® Microbial DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) following the manufacturer's instructions. Laccase from the *L. plantarum* J16 homologous to the *sufI* gene (AHN67998.1) was PCR amplified with newly designed primers SufI1c 5′- GAT***GCTAGC***-ATGGCAAAAAAAGTCTATACTGA-3′ (forward) and Suf2 5′- CCG***GGATCC***TTACATACCGGGCATCCAAAC -3′ (reverse). Recognition sites for *Nhe*I and *Bam*HI endonucleases are indicated in bold italics. PCR was performed with Taq DNA polymerase native (Invitrogen) in an Eppendorf thermocycler programmed with the following thermal profile setup: initial denaturation (95 °C for 5 min), 35 cycles of denaturation (94 °C for 1 min), primer annealing (45 °C for 1 min), and extension (72 °C for 1 min). Reactions were completed with 5-min elongation time at 72 °C followed by cooling to 10 °C. The PCR product was purified with the UltraClean® PCR Clean-Up Kit for DNA purification following the manufacturer's instructions. The purified DNA fragment was then digested with *Nhe*I and *Bam*HI endonucleases and ligated to the linearized pET-28a(+) vector (which carries the T7 bacteriophage promoter and terminator) with T4 DNA ligase (Fermentas) using a molar ratio of 1:1 over the vector (100 ng of the linear DNA vector) following the manufacturer's instructions to generate sticky-end ligation. The resulting recombinant plasmid, called pET-28a-*sufI*, was transformed into *E. coli* DH5α by electroporation and plated onto LB agar plates. Single clones grown on plate were screened for insert by direct colony PCR using SufI1c/Suf2 primers and for the entire construction using T7 promoter and the terminator commercial primers. Cells bearing laccase gene were transferred into a 50-ml flask containing 10 ml LB medium (supplemented with 50 μg/ml kanamycin) and incubated at 37 °C overnight. Consecutively, pET-28a-*sufI* plasmid was extracted with the UltraClean™ 6 Minute Mini Plasmid Prep Kit (Mo Bio, Carlsbad, CA, USA) and transformed by electroporation into *E. coli* strain BL21(DE3). Recombinant cells were selected after PCR analysis as described above. Gene cloning and plasmid construction were checked by sequencing (Servei Central de Suport a la Investigació Experimental, Universitat de València, Spain).

Expression and purification of *L. plantarum* laccase protein

Transformant *E. coli* BL21(DE3) cells carrying pET-28a-*sufI* were grown in a 50-ml Terrific–Broth (TB) medium supplemented with kanamycin at 37 °C and 200 rpm overnight. This overnight preculture was used to inoculate 1 l TB medium, which was incubated at 37 °C with stirring at 200 rpm. After approximately 2 h, at the start of exponential growth, laccase expression was induced by adding 1 mM IPTG. In order to optimize the expression of active recombinant laccase in *E. coli*, several parameters affecting growth and induction were evaluated: temperature, presence and level of $CuCl_2$, and shaking speed.

After induction, cells were harvested by centrifugation at 4 °C for 10 min at 10,000 rpm (JA10 rotor, Beckman Coulter Avanti J-E centrifuge), washed in 50 mM sodium phosphate buffer (pH 7.4) containing 0.3 mM $CuCl_2$, centrifuged again, and subsequently frozen and stored at −80 °C until use. Cells were thawed and resuspended in lysis buffer (50 mM sodium phosphate (pH 7.4), 0.3 mM $CuCl_2$, 10 μg/ml RNase, 5 μg/ml DNase, and 1 mg/ml lysozyme) and were maintained in ice for 30 min. Then, cells were disrupted mechanically with a similar volume of 106 μm glass beads in a Mikro-Dismembrator (10 cycles, 40 s; Sartorius). Cell debris was removed by centrifugation (13,500 rpm, 15 min, 4 °C). The resulting soluble extract was dialyzed overnight against equilibration buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.05 % Tween 20), and after centrifugation

under the same conditions as before, the resulting supernatant was collected and saved as crude extract.

Purification was performed in one step by immobilized metal ion affinity chromatography (IMAC) using the NTA–agarose matrix (Qiagen). The sample was loaded onto a 2 ml Ni^{2+}-NTA–agarose column, previously equilibrated with equilibration buffer. Non-retained proteins were washed out with five column volumes of equilibration buffer containing 20 mM imidazole, and subsequently, the column was eluted with the same buffer but containing 250 mM imidazole. Fractions displaying laccase activity, as detected using a 2,6-DMP oxidation liquid assay, were pooled and dialyzed overnight against 50 mM sodium phosphate, pH 7.4, containing 1 mM $CuCl_2$ and 0.05 % Tween 20. The protein content of these fractions and the crude extract were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to check the purity achieved in the purification procedure. Laccase activity was further verified by in-gel activity assay, throughout the process of enzyme extraction and purification. Protein concentration was determined by bicinchoninic acid (BCA) assay (Smith et al. 1985), using bovine serum albumin as standard. In some experiments, Sephadex G-25 PD10 desalting columns were used as an alternative in order to change the buffer composition quickly. Purified laccase enzyme samples were stored at −80 °C until use.

Biochemical characterization of *L. plantarum* laccase

UV–visible absorption spectra (300–800 nm) of pure recombinant *L. plantarum* laccase in 50 mM potassium phosphate buffer, pH 6.5, were acquired using a Beckman Coulter DU® 800 UV/Vis spectrophotometer.

The pH dependence of laccase activity was determined for ABTS and 2,6-DMP both at a concentration of 5 mM, by liquid assay. Several buffers were used to cover the pH range from 3 to 8: 50 mM sodium acetate (for pH values from 3 to 4), 50 mM sodium succinate (for pH values between 4 and 6), and 50 mM sodium phosphate (for pH values from 6 to 8). Oxidation of ABTS and 2,6-DMP was determined by an absorbance increase in microplate wells at 420 nm (ε_{420}=36,000 M^{-1} cm^{-1}) and 469 nm (ε_{469}=27,500 M^{-1} cm^{-1}), respectively.

The effect of temperature on laccase activity in the range 14 to 99.5 °C was determined by measuring 2,6-DMP oxidation in the liquid assay. Before enzyme addition, the reaction mixture was set at the appropriate temperature. Enzyme thermostability was measured at 45, 60, 70, and 85 °C by incubating the enzyme solution in 50 mM sodium phosphate buffer, pH 6.5, for 10 min. Then, the samples were cooled and residual activity was determined under standard liquid 2,6-DMP assay conditions.

Kinetic parameters of the purified recombinant laccase were determined by liquid assay in sodium phosphate buffer, pH 7.4, at 37 °C and using different concentrations of ABTS (0.1–50 mM) and 2,6-DMP (0.1–50 mM). Absorbance increases were recorded for 10 min, and the initial rates were deduced from the slope. Enzymatic assays were performed in triplicate. Data were fitted to the Michaelis–Menten equation to obtain V_{max} and K_m values for each substrate. One enzyme activity unit (U) was defined as the amount of enzyme that oxidizes 1 μmol of substrate/min in the liquid assay.

The effect of metal-chelating agents, such as bipyridyl, phenanthroline, and EDTA, in addition to other compounds that could affect enzyme activity, such as *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), pargyline, clorgiline, and semicarbazide, was checked by incubating the enzyme solution for 5 min at 28 °C with 100 μM of these compounds. After preincubation, the reaction was initiated by the addition of the substrate 2,6-DMP and the residual activity was determined. Enzyme without any agent was taken as control.

Oxidation of amines by *L. plantarum* laccase

The amine-oxidizing capacity of the recombinant *L. plantarum* laccase was studied by incubation of the enzyme with various amines, under different conditions, and then by quantification of remaining amines in the reaction mixtures (Callejón et al. 2014). Optimal working conditions of the enzyme activity, including enzyme concentration, the presence and concentration of ABTS (0–10 mM) as mediator, the presence of $CuCl_2$ (100 μM), temperature (4–45 °C), pH (2.5–10.5), and agitation, were determined using tyramine as a substrate. Assays were carried out using 1.5-ml tubes with 100 μl of the reaction volume in 50 mM sodium phosphate buffer, pH 6.5. The pH effect was determined using sodium citrate–phosphate buffer (pH ranging from 2.5 to 8.0), sodium borate buffer (pH 8.0 to 9.0), and sodium carbonate buffer (pH 9.0 to 10.5). Histamine and putrescine were also tested as a potential substrate for the *L. plantarum* laccase. In all cases, an amine concentration of 150 mg/l was used. Reaction mixtures were incubated at 28 °C for 24 h, after which residual amine concentration was determined by liquid chromatography with fluorescence detection (LC-FLD). As negative controls, reaction mixtures without enzyme or with heat-inactivated enzyme, incubated under identical conditions as those previously described, were used.

Amines were quantified by reverse-phase LC-FLD after precolumn derivatization with *o*-phthaldialdehyde (OPA). To this end, the samples were centrifuged at 13,500 rpm for 5 min, filtered through 0.22-μm nylon membranes (Fisher), and then incubated with the derivatizing reagent, which consists of 10 mg OPA in 0.9 ml methanol, supplemented with 0.1 ml of 0.1 M boric acid (pH 10.5) and 10 μl of 2-

mercaptoethanol (MCE) (Sigma). Derivatization reactions were carried out directly in the chromatograph injector. Separation of OPA-amine derivatives was performed on a Kinetex™ PFP column (100×4.6 mm) with a KrudKatcher ultra in-line filter, using an Agilent 1200SL LC-FLD system equipped with an in-line degasser, an autosampler, a column heater, and a fluorescence detector. A mixture of 50 mM sodium tetraborate buffer (pH 8.5), acetonitrile, and methanol (10:45:45) was used as mobile phase A and methanol as mobile phase B. The gradient conditions were as follows: from 10 to 85 % of solvent A in 11.4 min, followed by 100 % of methanol for 3 min, and finally, 3 min from 0 to 10 % of solvent A to return to initial conditions. Compounds were identified by comparing retention times of known standards of tyramine, histamine, and putrescine (Sigma). Fluorometric detection was done using excitation and emission wavelengths at 340 and 430, respectively. Amine concentrations were calculated using the peak areas relative to the area of internal standard.

Statistical analyses

All data are presented as the mean±SE. Group means were compared using one-way ANOVA followed by Duncan's multiple range tests to identify differences among groups when appropriate. All analyses were carried out using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Percentage data were normalized by arcsine transformation prior to the analysis.

Results

Isolation and sequence analysis of the laccase gene from the *L. plantarum* J16 homologous to the *sufI* gene

The *L. plantarum* J16 protein associated to multicopper oxidase (MCO) activity was purified and identified as a homologue of SufI, a previously known protein involved in cell division (Callejón et al. 2014). The new pair of primers SufI1c/SufI2 was designed from the *L. plantarum* JDM *sufI* gene published as a part of its complete genome sequence (Zhang et al. 2009), in order to isolate, clone, and characterize the homologous *L. plantarum* J16 gene. After amplification of the complete gene (~1500 bp), a comparison of the *L. plantarum* J16 nucleotide sequence with that of the *sufI* gene (GenBank ID CP001617.1, from regions 333,125 to 334,630) from *L. plantarum* JDM1 showed only two differences at positions 831 (C→T) and 1074 (T→G) but these substitutions do not change the amino acid sequence of the protein.

All members of the MCO family contain four copper-binding motifs, easily recognized at primary amino acid sequences, accounting for a total of ten histidine residues and a cysteine residue. The copper ligands are four highly conserved motifs, HXHG, HXH, HXXHXH, and HCHXXXHXXXXM/L/F, arranged successively within the amino acid sequence (Reiss et al. 2013). DNA sequence analysis of the *L. plantarum* J16 *sufI* homologue with MEGA 5 software showed the four strictly conserved copper ligand motifs within the encoded protein (Fig. 1). Likewise, multiple sequence alignment, performed with ClustalW software (http://www.ebi.ac.uk/clustalw/), showed that laccase protein from *L. plantarum* J16 has 42 % amino acid sequence identity with laccase from the common mushroom *Trametes versicolor* (UniProt ID: Q96UK8). When compared to other well-known bacterial laccases, the SufI homologue from *L. plantarum* J16 showed 30 % identity with *Bacillus* sp. CotA and 47 % with *E. coli* CueO (UniProt ID: H8WCE3 and P36649, respectively).

Cloning and optimization of laccase expression

The amplified laccase gene (*sufI* homologue) from *L. plantarum* J16 strain was cloned into the pET28a(+) expression vector, and the resulting plasmid was transformed into the *E. coli* BL21(DE3) cells for protein expression. When expression was performed under standard conditions at 37 °C, the resulting expressed protein was mainly found in inclusion bodies and showed no laccase enzyme activity. However, we detected very low laccase activity by the in-gel assay using 2, 6-DMP as a substrate on the overexpressed protein when the induction was carried out in a medium containing $CuCl_2$ at 37 °C and 200 rpm (data not shown). This result indicates that the *L. plantarum* J16 laccase activity was affected by the presence of Cu^{2+} in the culture broth. Nevertheless, neither overnight incubation of the expressed protein with up to 10 mM $CuCl_2$ nor dialysis against 50 mM potassium phosphate, pH 7.5, containing 1 mM $CuCl_2$ increased enzyme activity (results not shown). Furthermore, when induction was carried out at a lower temperature and under microaerobic conditions, held by the absence of agitation, there was a drastic increase in soluble laccase activity recovery. Thus, in short, the best expression level of active laccase was achieved by the following procedure: recombinant *E. coli* BL21(DE3) cells, carrying the pET-28a-*sufI* plasmid, were grown at 37 °C with stirring at 200 rpm to reach the exponential phase of growth. Then, IPTG was added (1 mM, final concentration) to induce protein expression while the medium was simultaneously supplemented with 1 mM $CuCl_2$. The culture was then incubated at 20 °C with a shaking regimen of 120 rpm for 2 h, followed by 16 h (overnight) without stirring. This change in aeration, from shaking (aerobic) to static incubation (microaerobic culture), was essential to recover a high yield of laccase activity in a soluble form suitable for characterization.

```
        10         20         30         40         50         60
MAKKVYTDYF FDEPAYNTHD GGYIPLVTPK VEPQPLAIPP LLKPDRQTDT DDYYTVTAQE
        70         80         90        100        110        120
SETQFLPGKK TKTWGYNAGF LGQTIVFRNG KQTHIDLENK LPELTTFHWH GLNVPGPITD
       130        140        150        160        170        180
GGCHAPVYPG ETNHIDFKVH QPAATTWLHA HPCPSTATQV WKGLATMVII KDDVEDQLPL
       190        200        210        220        230        240
PRNYGVDDIP LVLQDREFHD DNQFDYRADY DPDGVQGHTA LVNGTVNPYF DVTTQRVRLR
       250        260        270        280        290        300
ILDGSNRREW RLHFNDDLEF AQVASDGGIL PAPVYMTKVM MTCAERDEIV VDFGQYQPGD
       310        320        330        340        350        360
EVTLMTDDTP LCRFRIKSFV PDDTKLPEHL VDIPDETPTP DLPVRTITMD GMDDEVALDG
       370        380        390        400        410        420
KKFDMSRIDA RQKVGDVAIW EIRNTNSTEN GMVHPFHVHG TQFRVLARND GPVYPNEHGL
       430        440        450        460        470        480
KDTVGVNPGE TVRIKVKFEL TGVYMYHCHI IEHEDGGMMA QIESYDPQHP QTYHLMDMDT
       490        500
LRNAFAKEQG IKPEDVWMPG M
```

Fig. 1 Deduced amino acid sequence of *L. plantarum* J16 laccase homologue of SufI. *Encased inside boxes* are the motifs that form the four copper ligands and are highly conserved in MCOs (conserved sequence of these motifs are HXHG, HXH, HXXHXH, and HCHXXXHXXXXM/L/F; Reiss et al. 2013)

Purification of recombinant *L. plantarum* laccase

After setting appropriate conditions of *E. coli* cultures for a good expression level of active recombinant laccase, cells from a preparative culture obtained under those conditions were harvested, lysed mechanically, and centrifuged and the resulting supernatant was saved as crude extract. In the extract, the amount of recombinant laccase protein represented more than 12 % of the total protein (estimated by ImageJ software densitometry of SDS-PAGE, see Fig. 2, lane 1). Next, the recombinant *L. plantarum* laccase, containing the artificially added polyhistidine (6× His) tag, was purified in one step by affinity chromatography on Ni^{2+}-NTA–agarose. Column fractions were assayed for laccase activity and analyzed by SDS-PAGE (Fig. 2). Fractions with the highest enzyme activity showed an intense blue color and, after pooling, desalting, and aliquoting, were frozen and stored at −80 °C until use for enzyme characterization. Approximately 10 mg of the purified protein was routinely obtained from 1 l of cell culture. The specific activity of purified recombinant laccase with ABTS as a substrate was around 0.55 U/mg. SDS-PAGE analysis of the expressed enzyme showed a band with an apparent molecular weight of 65 kDa (Fig. 2). Given that the recombinant protein has a 2.5-kDa N-terminal extension, corresponding to the polyhistidine tag plus a short linker, the proper molecular weight of the polypeptide chain of *L. plantarum* J16 laccase may be around 62.5 kDa, which is fairly similar to that inferred theoretically from the cloned sequence (59.3 kDa).

Otherwise, it is worth mentioning that, after chromatographic purification of the recombinant laccase, it was necessary to remove the imidazole used for elution from the column Ni^{2+}-NTA–agarose, since, over time, it caused inactivation and precipitation of the enzyme. We were able to maintain protein solubility and enzyme activity by dialyzing the active chromatographic fractions against a sodium phosphate buffer, pH 7.4, containing 1 mM $CuCl_2$ (alternatively, buffer exchange was carried out with PD10 desalting columns, with similar results). We found that the presence of Cu in dialysis buffer improved enzyme activity recovery, probably because

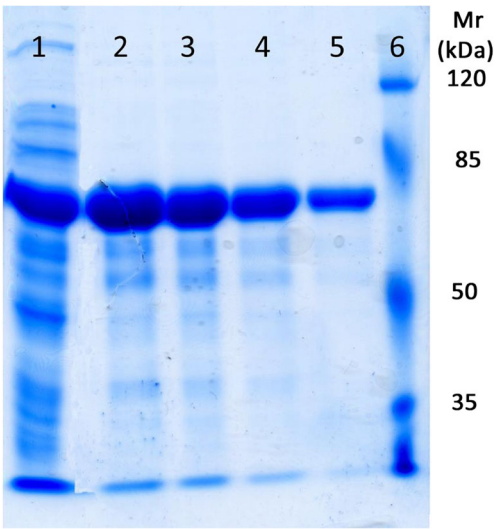

Fig. 2 SDS-PAGE analysis during purification procedure of recombinant *L. plantarum* laccase. *Lane 1* crude cell extract; *lanes 2–5* fractions 1 to 4, respectively, obtained after elution of bound proteins to Ni-NTA–agarose with 250 mM imidazole; *lane 6* molecular size markers (Fermentas)

it avoids unloading and loss of Cu from the protein (Durão et al. 2008).

Identification and characterization of the *L. plantarum* J16 SufI homologue as laccase

The purified protein exhibited a strong blue color common to laccase enzymes. The UV–visible absorption spectrum of the purified recombinant protein showed a maximum peak at 590 nm (corresponding to the T1 or blue copper center) as can be seen in Fig. 3. The 280/600 nm absorbance ratio of the purified enzyme was determined to be 11.2.

The pH effect was studied in the range from 3.0 to 8.0, employing different buffer compounds. The pH optimum of *L. plantarum* laccase was dependent on the substrate; for 2,6-DMP, the highest oxidation activity was found at pH 6.5, while for ABTS, it was found at pH 3.5. The relative activities of the enzyme at the analyzed pH range for each substrate are shown in Fig. 4.

The optimal temperature for laccase activity, on 2,6-DMP as a substrate, was found to be 60 °C, but the enzyme showed activities above 50 % over a rather wide temperature range, from 37 to 100 °C (Fig. 5a).

Thermal stability experiments showed that recombinant *L. plantarum* laccase was activated by 10 min of preincubation at 45–70 °C, but higher temperatures promoted inactivation (Fig. 5b). No activity was detected after 10 min of preincubation at 85 °C or higher.

Kinetic parameters, K_m and V_{max}, of recombinant *L. plantarum* J16 laccase were determined by the liquid assay for ABTS and 2,6-DMP, using various concentrations, and at room temperature. Dependence of the rate on substrate concentration followed Michaelis–Menten kinetics. The K_m and V_{max} values were 0.21 mM and 0.54 U/mg for ABTS and 1.67 mM and 0.095 U/mg for 2,6-DMP, respectively.

L. plantarum laccase retained 100 % activity with 2,6-DMP as a substrate in the presence of clorgiline or pargyline, two of the specific amine oxidase inhibitors tested. Only semicarbazide, a carbonyl-modifying agent which inhibits a specific subgroup of copper-dependent amine oxidases, produced a 25 % decrease in enzyme activity. On the other hand, although with a 100 μM concentration EDTA did not

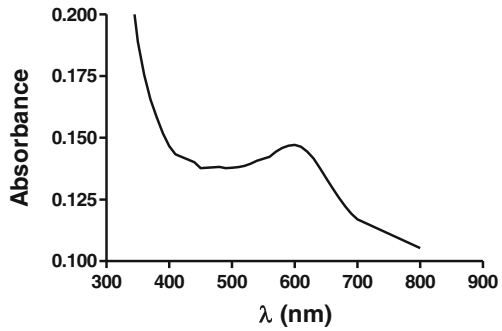

Fig. 3 UV–visible spectrum of purified recombinant laccase (3.5 mg/ml) in 50 mM sodium phosphate buffer, pH 6.5. Only the range from 300 to 800 nm is shown

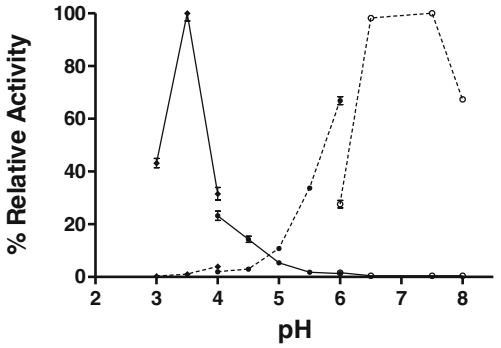

Fig. 4 pH effect on activity of the recombinant *L. plantarum* enzyme toward canonical laccase substrates: ABTS (*solid line*) and 2,6-DMP (*dashed line*). *Filled diamond* indicates sodium acetate buffer (pH range 3–4), *filled circle* sodium succinate buffer (pH range 4–6), and *open circle* sodium phosphate buffer (pH range 6–8). All buffer compounds were used at a concentration of 50 mM. The values are means±standard deviations of triplicate assays

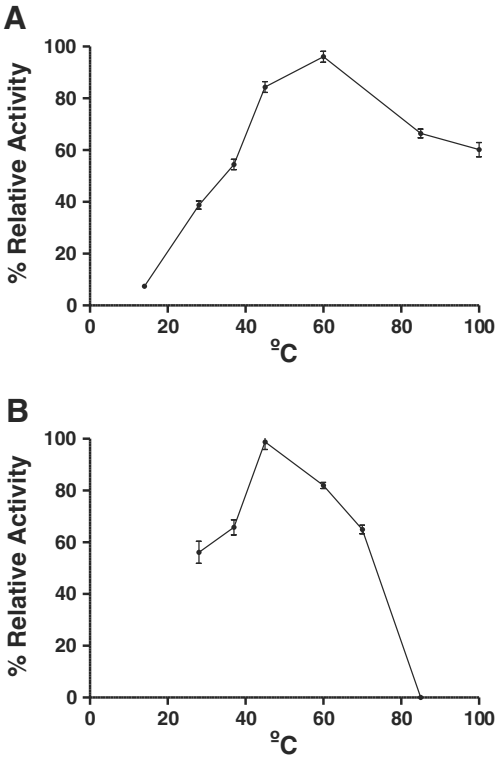

Fig. 5 Temperature dependence and thermal stability of recombinant laccase. **a** Temperature effect was determined by incubation of the reaction mixtures at the indicated temperatures and using the standard liquid assay with 2,6-DMP. Enzyme activity is plotted as a percentage relative to maximum value (% relative activity). **b** Thermal stability was analyzed by preincubation of small portions of the enzyme solution, at the indicated temperatures, for 10 min before being employed for the standard liquid assay. Residual enzymatic activity, relative to the maximum value, is graphed against the pretreatment temperature. The values are means±standard deviations for triplicate assays

diminish the catalytic activity, the other two metal-chelating agents tested, bipyridyl and phenanthroline, considerably inhibited the activity of the enzyme by 90 and 95 %, respectively. The carboxyl modifier 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was the most effective inhibitor, producing nearly complete inhibition (~99.5 %) as can be seen in Fig. 6.

Amine-oxidizing activity of *L. plantarum* laccase

Given that *L. plantarum* J16 laccase was previously identified as the enzyme responsible for degrading BA (Callejón et al. 2014), we studied in more detail its oxidizing activity on BA most frequently described in foods: tyramine, histamine, and putrescine. For these analyses, amines were mixed with the recombinant enzyme and, after incubation, the remaining amine concentration in the reaction solution was quantified by reverse-phase LC-FLD. First, we assessed the dependence of the amine degradation with the recombinant enzyme concentration, employing tyramine as a substrate. As shown in Fig. 7, in the tested range, the percentage of tyramine degradation increased with the concentration of added enzyme. Figure 7 also shows that an addition of copper to the reaction solution, as $CuCl_2$ at 100 μM, increased tyramine degradation by 6 % while higher addition did not improve degradation (not shown).

Next, we tested whether ABTS, acting as a mediator, could increase the amine-oxidizing capacity of *L. plantarum* laccase. As shown in Fig. 8, the addition of ABTS to the reaction mixture significantly increased tyramine oxidation. Notably, the inclusion of only 1 mM ABTS doubled tyramine degradation, indicating that ABTS acts as an effective mediator for amine oxidation by *L. plantarum* laccase. Higher concentrations of ABTS achieved higher tyramine degradation percentages to nearly 100 % with 5 mM (Fig. 8). Taking into account these results, the reaction conditions used in subsequent studies with amines were as follows: 1 mM ABTS, 100 μM $CuCl_2$, 19 μg of recombinant laccase protein, and static incubation time of 24 h. In contrast to canonical laccase substrates, the optimum temperature for tyramine degradation was found to be considerably lower, 28 °C instead of 60 °C. *L. plantarum* laccase was able to degrade 70 % of tyramine at 28 °C, and furthermore, even at 4 °C, a reduction of 44 % was obtained. Enzyme activity on tyramine degradation was strongly dependent on pH. It can be seen from Fig. 9 that two peaks of activity were obtained, one at pH 4 and the second at pH 9.5, in which the enzyme degrades tyramine up to 85 and 40 %, respectively. Curiously, neither of these two peaks coincide with those obtained with more conventional substrates of laccases, ABTS and 2,6-DMP (see Fig. 4).

Laccase from *L. plantarum* J16 also has the ability to oxidize, although to a lesser extent, two other biogenic amines, histamine and putrescine. *L. plantarum* degraded 36 % of histamine and 17 % of putrescine in phosphate buffer (pH

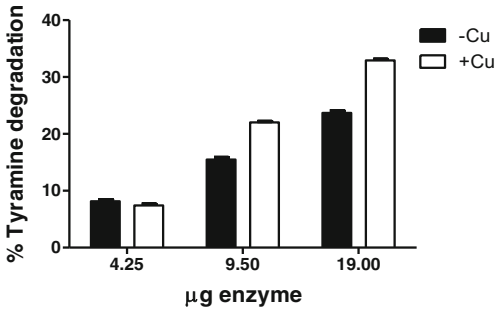

Fig. 7 Dependence of tyramine oxidation on the concentration of recombinant *L. plantarum* J16 laccase and effect of Cu addition (100 μM). The % tyramine degradation data are relative to a control without enzyme and are means±standard deviations of triplicate assays. Values represented on the *x-axis* are amounts of recombinant laccase in reaction mixtures

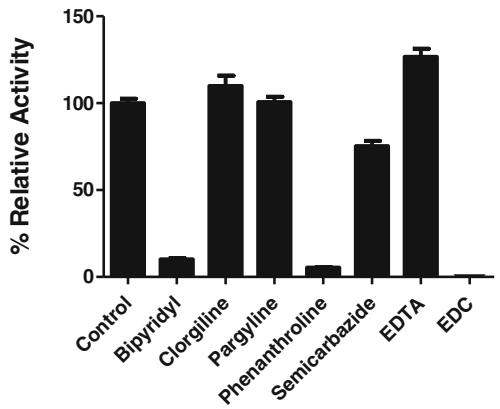

Fig. 6 Effect of potential inhibitors on *L. plantarum* J16 laccase. Liquid assays using 2,6-DMP as a substrate were carried out in the presence of 100 μM of the indicated compounds. *Control* means an enzyme assay in the absence of inhibitors. Values are means±standard deviations of triplicate assays

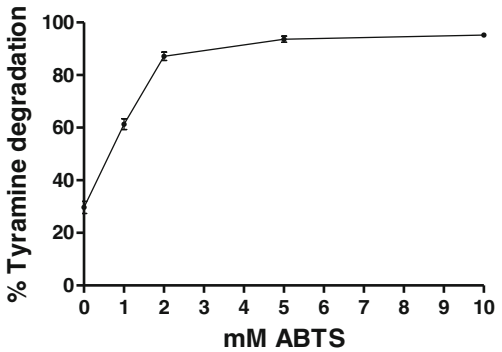

Fig. 8 Effect of the addition of the mediator ABTS on oxidation of tyramine by *L. plantarum* J16 recombinant laccase. Tyramine degradation was determined as the decrease of concentration in the reaction solution by reverse-phase LC-FLD. The values are means± standard deviations for triplicate assays

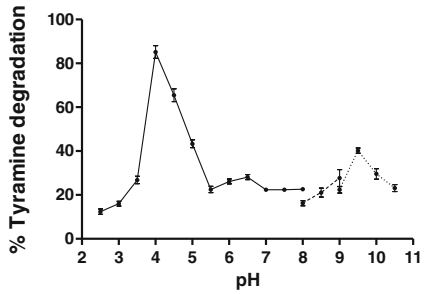

Fig. 9 Effect of pH on the tyramine-oxidizing activity of recombinant laccase. Data of % tyramine degradation are means±standard deviations for triplicate assays. *Solid line* represents the values obtained with sodium citrate–phosphate buffer (pH 4 to 8.0), *dashed line* shows the values with sodium borate buffer (pH 8.0–9.0), and *dotted line* the values with sodium carbonate buffer (pH 9.0–10.5)

6.5) under the reaction conditions described above. Oxidation of histamine and putrescine was more dependent on the presence of ABTS as a mediator than oxidation of tyramine. In fact, in the absence of ABTS, histamine and putrescine degradation was reduced to lower values, 10 and 5 %, respectively.

Discussion

Here, we describe cloning, expression, and characterization of a new bacterial laccase from the LAB *L. plantarum* J16 whose main feature is the ability to degrade BA. The identified laccase gene from *L. plantarum* is homologous to *E. coli* gene *sufI*, encoding for a protein localized at the septal ring in the dividing cell. Gene *sufI* ["suppression of ftsI(Ts)"] was originally identified in *E. coli* cells as a multicopy suppressor of an ftsI(Ts) mutation, which generates a phenotype characterized by the inability of cells to divide (Tarry et al. 2009). The sequence of *E. coli* SufI shows that it is a member of the multicopper oxidase superfamily but lacks enzymatic activity since it does not bind metal cofactors, and it may serve as a scaffolding protein in the septal ring (Tarry et al. 2009). In this work, however, we show that the SufI homologue from *L. plantarum* J16 is a genuine laccase. The four archetypical copper-binding motifs of MCOs were deduced from the protein sequence (Reiss et al. 2013), and the pattern M4 corresponding to the classification and analysis system for laccases and related multicopper oxidases (Sirim et al. 2011) was found. Sequence alignment analyses have indicated significant amino acid sequence identity with laccases from diverse bacterial and fungal species. In addition to sequence data, the purified recombinant *L. plantarum* J16 laccase exhibits the strong blue color, characterizing all "blue" copper oxidases, due to its absorbance peak at 600 nm, corresponding to the presence of type 1 copper center, and also the 280/600 nm absorbance ratio was in the typical range for bacterial laccases (Xu et al. 2007). Particularly, the oxidizing enzymatic activity of canonical substrates of laccase, like ABTS and 2,6-DMP, confirms its authenticity as a multicopper oxidase, subtype laccase.

The heterologous expression of an active form of recombinant laccase was enhanced by exogenous copper addition, by decreasing the post-induction temperature, and by setting microaerobic conditions. When expression was carried out in the absence of copper at 37 °C, subsequent $CuCl_2$ addition to the solution of overexpressed protein did not result in an increase of enzymatic activity. Insufficient copper incorporation or misfolding of the polypeptide or both probably leads to a catalytically inactive form of the enzyme. Previously, it has been reported that under usual expression conditions of recombinant proteins, intracellular copper accumulation may be inadequate to produce the appropriate state for folding and formation of catalytically active laccase (Mohammadian et al. 2010). Similar results were reported for other bacterial laccases. For example, CotA laccase apoprotein from *Bacillus licheniformis* did not recover oxidizing activity toward ABTS after treatment with copper (Koschorreck et al. 2008). The active holoenzyme CotA from *B. subtilis* was also not completely restored following incubation of recombinant apo-CotA with cooper ion (Durão et al. 2008). Nevertheless, in vitro inefficacy of copper loading and activity reconstitution of the inactive form is not a general trait for bacterial laccases since it has been successfully achieved with CueO recombinant apoprotein (apo-CueO) of *E. coli*. Supplementation of apo-CueO with an excess copper ion led to the acquisition of high laccase activity (Li et al. 2007).

Under optimized expression conditions, presumably, the presence of copper during the induction of expression leads to the accumulation of intracellular Cu ions, which seems to be indispensable for the correct folding of the holoenzyme (Durão et al. 2008; Koschorreck et al. 2008; Mohammadian et al. 2010). Moreover, it has also been shown that copper incorporation into recombinant *B. subtilis* CotA laccase is not only strongly dependent on Cu supplementation of the culture media but also, and equally important, on oxygen availability. Under microaerobic conditions, cells increase intracellular copper ion accumulation and therefore enhance the proper folding of the laccase (Durão et al. 2008; Mohammadian et al. 2010). Moreover, it is known that decreasing temperature during induction also elevates the expression and specific activity of laccase enzymes (Koschorreck et al. 2008; Mohammadian et al. 2010). Consistently, in our expression experiments, a low post-induction temperature (20 °C) produced higher levels of soluble enzyme. With temperature above 30 °C, most of the overexpressed recombinant protein was found in inclusion bodies, a circumstance which has also been described for CotA laccase from *Bacillus* sp. (Mohammadian et al. 2010). In any case, a comparison of the specific activity obtained for *L. plantarum* J16 laccase with other bacterial laccases suggests that part of the expressed recombinant laccase may be

catalytically inactive, probably due to insufficient copper incorporation (Koschorreck et al. 2009).

Analysis by SDS-PAGE allows us to estimate that *L. plantarum* J16 laccase has a molecular weight around 62.5 kDa. This value is in the range reported for other bacterial laccases, most of which fall between 50 and 100 kDa (Sharma et al. 2007).

The pH optimum of recombinant *L. plantarum* laccase was strongly dependent on the type of substrate, which is a known general characteristic of laccases from bacteria and fungi. Optimum pH values for the recombinant enzyme oxidation of ABTS and 2,6-DMP were analogous to those described for other bacterial and also some fungal laccases (Kiiskinen et al. 2002; Martins et al. 2002).

The temperature optimum of recombinant *L. plantarum* J16 laccase, around 60 °C, is in the range that has been reported for bacterial laccases, such as CotA from several species of *Bacillus* (55–75 °C) (Durão et al. 2008; Koschorreck et al. 2008; Reiss et al. 2011) and CueO from *E. coli* (55 °C) (Roberts et al. 2002). Moreover, our thermal stability analysis shows that thermostability of recombinant laccase from *L. plantarum* is comparable to CueO (Martins et al. 2002). It is, however, less thermostable than CotA from *B. subtilis* (Durão et al. 2008) that retains 50 % of relative activity after more than 100 min of incubation at 80 °C or *Bacillus tequilensis* laccase that retains 80 % of activity at 70 °C after 24 h of incubation (Sondhi et al. 2014). Furthermore, a remarkable enzyme activity of the recombinant laccase from *L. plantarum* increased, almost doubling, after preincubation at 45–60 °C when compared to control samples preincubated at 28 °C. Although the molecular cause of this apparent enzyme thermal activation is unknown, a similar phenomenon has been already reported for a number of fungal and bacterial laccases (Mohammadian et al. 2010). As a tentative proposal, temperature could induce small conformation changes in the protein conducive to a reorganization in the copper centers and, consequently, to the recovery of some inactive laccase molecules.

Our kinetic analysis of the recombinant *L. plantarum* J16 laccase for ABTS and 2,6-DMP provided K_m values (0.21 and 1.67 mM, respectively) slightly higher than those reported for CotA from diverse *Bacillus* species and other bacterial laccases (Koschorreck et al. 2008; Reiss et al. 2011). Our results indicate a higher V_{max} value for ABTS than for 2,6-DMP in line with those other bacterial laccases (Gunne and Urlacher 2012; Wu et al. 2010). These kinetic data provide a relative substrate preference value (in terms of V_{max}/K_m ratio) of approximately 46-fold higher for ABTS than for 2,6-DMP. Certainly, the physiological role of *L. plantarum* laccase, as their natural substrates, is not known. Therefore, those differences, in biochemical properties, with laccases from other organisms must be related to adaptation of its main biological function.

As expected, and similar to different laccases from diverse organisms (Endo et al. 2003), *L. plantarum* J16 laccase was inhibited by the metal-chelating agents bipyridyl and phenanthroline, although not by EDTA. Possibly, the first two chelating agents are more efficient than EDTA in removing bound copper ions from the enzyme, as a result of their higher affinity for this metal. The absence of inhibition by EDTA has been previously reported for several other laccases, like that of *Streptomyces coelicolor* (Machczynski et al. 2004), and an activating effect has even been described for *Haloferax volcanii* and *Trametes hirsuta* laccases (Haibo et al. 2009; Uthandi et al. 2010). Interestingly, we have found a strong inhibition of *L. plantarum* laccase by EDC, a modification agent for carboxyl groups, suggesting the involvement of a carboxyl group in the catalytic process or substrate binding, as has been already proposed for some fungal laccases (Salony et al. 2008).

Laccases have awakened our interest as enzymes able to degrade BA in foods. Recently, we have demonstrated oxidation of BA by the commercial laccase from the fungus *T. versicolor* and we have identified the laccase SufI from LAB *L. plantarum* J16 as the enzyme responsible for eliminating BA in wine (Callejón et al. 2014). In the present work, we have investigated the activity of recombinant *L. plantarum* laccase on the most frequent and dangerous BA in foods: tyramine, histamine, and putrescine. We found that recombinant laccase catalyzes oxidation of these three amines, with tyramine as far the best amine substrate. This greater oxidizing activity toward tyramine is probably due to the phenolic structure of this substrate (Reiss et al. 2013). Furthermore, it is known that laccase-mediated catalysis can be extended to other substrates through mediators which, once converted to highly reactive cation radicals by laccase, oxidize the additional compounds that laccase alone cannot, or only with much difficulty (Shraddha et al. 2011). We have demonstrated that ABTS serves as a redox mediator for tyramine oxidation in the reaction catalyzed by recombinant laccase. The inclusion of ABTS in the reaction solution, in a dose-dependent manner, considerably enhances tyramine degradation.

Since *L. plantarum* J16 has the ability to oxidize amines and therefore potential interest for BA elimination in foods, the effect of known inhibitors for amine oxidases was studied and compared to that of common inhibitors of laccases. *L. plantarum* J16 laccase was not affected by flavin amine oxidase inhibitors such as clorgiline and pargyline, but it was inhibited by semicarbazide, a carbonyl modifier, which is a representative inhibitor of a subtype of copper amine oxidases. Inhibition by semicarbazide has been reported for the laccase CueO (Roberts et al. 2002).

Given the concerns over the presence of BA in foods and beverages, the recognition of *L. plantarum* laccase as an enzyme able to degrade them opens up new prospects for

industrial application. In addition, its activity and substrate range could be improved for potential industrial application by means of directed evolution, which is much easier to apply to bacteria than to fungal laccases.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Anexo 4

Recombinant laccase from Pediococcus acidilactici *CECT 5930 with ability to degrade tyramine*

Recombinant laccase from *Pediococcus acidilactici* CECT 5930 with ability to degrade tyramine

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Research Article

Recombinant laccase from *Pediococcus acidilactici* CECT 5930 with ability to degrade tyramine

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Keywords: Laccase, Biogenic amines, Tyramine, *Pediococcus acidilactici*.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); **BA,** biogenic amines; **DMP,** 2,6-dimethoxyphenol; **HBT**, (N-hydroxybenzotriazole); **LAB,** lactic acid bacteria; **LC-FLD,** liquid chromatography with fluorescence detector; **MCO,** multicopper oxidases; **EDC,** N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide.

Abstract

Biogenic amines degradation by bacterial laccases is little known, so we have cloned and heterologously expressed, in *E. coli,* a new laccase from *Pediococcus acidilactici* CECT 5930 (Lpa5930), a lactic acid bacterium commonly found in foods able to degrade tyramine. The recombinant enzyme has been characterized by physical and biochemical assays. Here we report the optimization of expression and purification procedures of this laccase. DNA encoding sequence of laccase from *P. acidilactici* was amplified by PCR and cloned into the expression plasmid pET28a for induction by isopropyl-β-D-thiogalactoipyranoside. Protein expression was performed in *E. coli* BL21(DE3) harboring pGro7 plasmid expressing a chaperone folding assistant induced by arabinose. Purification was performed by column metal-chelating chromatography on Ni-NTA-agarose. The laccase enzyme obtained has an apparent molecular mass of ~60 kDa, an optimum temperature activity toward 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) of 28 °C, and was quickly inactivated at temperatures higher than 70°C. The apparent K_m value for ABTS was 1.7 mM and the V_{max} obtained was 24.05 U/mg. In addition to ABTS, recombinant Lpa5930 laccase degraded the biogenic amine tyramine at pH 9.5 and pH 4.0 with or without ABTS as a mediator. Tyramine degradation by laccases could solve the problems generated in food due to the presence of this toxic compound.

1 Introduction

Pediococcus acidilactici is a Gram-positive bacterium that can survive and grow in a wide range of pH, temperatures, and osmotic pressures, allowing them to inhabit the entire digestive tract of humans and different animals [1]. *P. acidilactici* is labeled as a *Generally Regarded As Safe* (GRAS) bacteria by the Food and Drug Administration (FDA). It can be found in fermented vegetables, fermented dairy products, and meat as a starter kit to add flavor and texture [2-4]. Its importance has increased since some strains of this species have been proposed as probiotic bacteria [5, 6]. *P. acidilactici* abilities include preventing the colonization and growth of pathogens, regulating and optimizing the function of the natural microorganisms. In addition, *P. acidilactici* strains also increase the nutritional value and digestibility of feed nutrients. There are available preparations with *P. acidilactici* (CNCM) MA 18/5M as additive Bactocell PA that has already been authorized by the European Food Safety Authority (EFSA) to be used as a feed additive for shrimp, pigs and chickens. Some strains of this species also can produce bacteriocines, so Cintas et al. [8] described a heat-stable bacteriocine active against gram-positive bacteria of the genera *Listeria*, *Staphylococcus* and *Enterococcus*. Later Nieto-Lozano et al. [9] described another one that was able to inhibit the growth of *Listeria monocytogenes* and *Clostridium perfringens* in raw meat. Moreover, *P. acidilactici* has been used as microbial cell factories for bioconversion of lignocellulosic feedstocks. Boguta et al. [10] and Ventorino et al. [11] described diverse lactic acid bacteria (LAB) strains, *P. acidilactici* among them, capable of utilizing pentose sugars, as xylose and arabinose, and highly resistant towards common inhibitors from pretreated lignocellulosic biomass, such as furan derivatives, phenolic compounds and, weak acids. Recently we reported multicopper oxidases from LAB with a special new capacity toward biogenic amines (BA) [12]. BA are organic compounds of low molecular weight present naturally in animals, plants and microorganisms that possess biological activity [13]. These compounds are produced by decarboxylation of amino acids in foods containing free amino acids and which exhibit conditions that allow microbial and

biochemical activity [13-15]. Thus, any fermented or raw product exposed to microbiological contamination may contain BA [16]. Foods with high risk of forming BA are ripened cheeses, fermented beverages (including beer and wine), fermented vegetables (such as sauerkraut), fermented fish or meat foods and fish or also fishery products under process of deterioration [16, 17]. Consumption of foods containing high concentrations of BA are a concern for the consumer because they are related to health disorders [18]. Tyramine is associated with different symptoms such as difficulty breathing, hives, sweating, heat, burning mouth, palpitations, headache, nausea, diarrhea and hypertension [13, 14, 17, 19]. Some authors have reported relationships between poisoning outbreaks and tyramine concentrations of foods [13, 20].

Laccases (benzenediol:oxygen oxidoreductase, p-diphenol oxidase EC 1.10.3.2) are blue multicopper oxidases (MCO), abundantly present in many plants, fungal and bacterial species [21-23]. Laccases catalyze the monoelectronic oxidation of substrates at the expense of molecular oxygen and, generally they had a broad range of substrates including phenols, such as methoxyphenols, polyphenols, and nonphenolic substrates, including aromatic amines, arylamines, anilines, thiols, and some cyanide complexes of metal.

Laccases can expand their substrate range through the use of redox mediators, which are low molecular weight molecules that are able to shuttle electrons between laccases and target molecules that otherwise could not be oxidized [24]. In a laccase mediator system, laccase oxidizes the mediator and the oxidized mediator in turn oxidizes the final substrate [25]. Common artificial mediators are TEMPO (2, 2, 6, 6-Tetramethylpiperidine-1-yl) oxyl; HBT (N-hydroxybenzotriazole), violuric acid, and ABTS 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [24]. Several naturally occurring compounds that mediate laccase activity have been identified as well [26, 27].

Laccase enzymes are being increasingly evaluated for a variety of biotechnological applications due to their broad substrate range [22]. In addition, these enzymes are used in green

chemistry [28], for paper and pulp processing, in textile and petrochemical industries [25], in polymer synthesis and also in wine and beverage production. They have also used for bioremediation of contaminated soils and for detoxification of industrial effluents [29]. Laccases are also used as catalysts for the manufacture of anticancer drugs and even as ingredients in cosmetics, and they have also been applied to nanobiotechnology as biosensors or bioreporters [29, 30]. Laccases are catalogued as eco-friendly enzymes since they work with air and produce water as the only by-product [28]. Therefore, it seems an important innovation the study of laccases with novel capacities (e.g. BA degradation) to address new expectations about their potential use with biotechnological purposes.

To date, laccases have mostly been isolated and characterized from plants and fungi, and only fungal laccases are being currently used in biotechnological applications. Bacterial laccases, in contrast, have been less studied and applied to solve practical problems. So far only one report has described the use of a bacterial laccase in bioremediation of industrial wastes [31]. Moreover, the detailed properties of bacterial laccases and their biotechnological applications are very little known. Only a few studies have been reported on the catalysis mechanism and stability at high pH and temperature of prokaryote laccases [32-34]. However, in principle, bacterial laccases could solve some of the limitations of fungal laccases in industrial and biotechnological processes. Thus, large-scale production of laccases from fungi is restricted due to their slow growth rates. In addition, activities of fungal laccases are unfavorable in submerged aquatic environments and low desired pH ranges, limiting their applications. In contrast, bacterial laccases have a wide range of bioremediation activities because through biostimulation processes, they can stimulate the indigenous organisms, providing a favorable environment or nutrients needed for increasing the microbial activity [31]. By nature, bacterial laccases retain catalytic activity in a wider pH range [35, 36]. In contrast to fungal enzymes, laccases from Bacilli shown activity in an extensive interval of pH, from neutral to extreme alkaline values [32, 37]. Furthermore, bacterial laccases generally exhibit higher thermal

stability and often they are more tolerant towards organic solvents, high salt concentrations, and common laccase inhibitors compared to fungal laccases [25]. Likewise, bacterial laccases can be expressed with more feasibility than the fungal laccases in standard prokaryotic expression systems like *E. coli*, although sometimes their heterologous expression results in enzymes that do not contain a full complement of copper ions. Therefore, activity yields of bacterial laccases in heterologous expressions are unsatisfactory and constitute a concern [25] .

Bacterial laccase was first reported by Givaudan et al. [38] from *Azospirillum lipoferum* isolated from rice rhizosphere. However, the most well-known and representative of this kind of enzymes is CotA from *Bacillus subtilis*, an endospore coat protein with high thermostability [39]. Bioinformatic studies on laccases have indicated that they are present in various high G + C gram-positive bacteria and also in α-, γ-, and ε-proteobacteria [40-42]. Laccase enzymes have been described in genomic sequences and analyzed in various other bacterial species such as *Escherichia coli* [43], several *Bacillus* species [32], *Thermus thermophilus* [44], diverse streptomycetes [45, 46] and one lactic acid bacterium strain *L. plantarum* J16 [47].

The aim of the present work was to clone and express the *P. acidilactici* CECT 5930 gene encoding the protein D2EK17, a putative MCO, and to characterize the expressed recombinant protein biochemically and physically including its capacity to react on BA.

2 Materials and methods

2.1 Materials

Usual laccase substrates [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,6-dimethoxyphenol (2,6-DMP)], amines histamine, tyramine, and putrescine, expression inducers isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose, antibiotics kanamycin, and chloramphenicol, and also standard proteins used for molecular weight determination were

obtained from Sigma (Madrid, Spain). Nickel-chelating nitrilotriacetic acid (Ni-NTA) agarose was from Qiagen (Hilden, Germany). All other chemicals and reagents were of analytical grade.

2.2 Strains, enzymes and plasmids

P. acidilactici CECT 5930 was obtained from Spanish Type Culture Collection (CECT) and routinely cultivated in MRS medium at 28 °C.

Escherichia coli DH5α [F− Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1] was used as host for manipulation, amplification and propagation of the D2EK17 encoding gene. The employed overexpression vector was the plasmid pET-28a(+) (Novagen, Madison, WI, USA). *E. coli* BL21(DE3) [F- *dcm ompT hsdS* (rB^-mB^-) *gal* (DE3)] (Novagen), harboring also the pGro7 plasmid, was used as the host to express the gene of interest under the control of the T7 promoter. The plasmid pGro7, designed to enable efficient expression of multiple chaperones (groES- groEL) that work cooperatively in the protein folding process [48], was used for the coexpression of the protein D2EK17. *E. coli* cells were grown at 37°C in Luria–Bertani (LB) medium, while transformants, when appropriate, were grown in LB medium supplemented with kanamycin (50 µg/ml) or with kanamycin plus chloramphenicol (20 µg/ml).

Genomic DNA was purified by using the Ultra Clean® Microbial DNA Isolation Kit, plasmid DNA with the Ultra Clean™ 6 minute mini plasmid prep Kit and for purification of PCR products the Ultra Clean® PCR Clean-Up Kit was employed, all these kits from MoBio (Carlsbad, CA). DNA synthetic oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Restriction enzymes were obtained from New England Biolabs (Beverly, MA), T4 DNA ligase was from Roche Diagnostics (Barcelona, Spain) and DNA polymerase and the corresponding 10X reaction buffer were from Invitrogen (La Jolla, CA).

2.3 Cloning of gene encoding the D2EK17 protein and construction of the expression plasmid

To produce the recombinant enzyme tagged with a polyhistidine sequence at the N-terminus, the pET28a vector containing the nucleotide sequence encoding for the 6xHis

tag was employed. The polyHis tag plus linkers added 20 amino acids to the recombinant protein at N-terminus.

The *P. acidilactici* CECT 5930 gene corresponding to the D2EK17 protein as a putative MCO was PCR amplified from purified genomic DNA with newly designed primers LacaPat1 5′- GAT***GCTAGC***ATGATTACAAAGTATCTATATGA-3′ (forward) and LacaPat2 5′-CCG***GGATCC***CTACATTTTATGGTCCATTGG-3′ (reverse). Recognition sites for *NheI* and *BamHI* endonucleases are indicated in bold italics. PCR was carried out using Taq DNA polymerase native (Invitrogen), in an Eppendorf thermocycler. The thermal profile setup was: initial denaturation (95°C for 5 min), 35 cycles of denaturation (94°C for 1 min), primer annealing (54°C for 1 min), and extension (72°C for 1 min). Finally, reactions were completed with 5-min elongation time at 72°C followed by cooling to 10°C. The PCR products were purified with the UltraClean® PCR Clean-Up Kit following the manufacturer's instructions. The resulting purified fragment, containing the entire coding sequence plus linkers, and also the pET-28a plasmid vector were digested with *NheI* and *BamHI*. Following of the treatment of the linearized plasmid and after dephosphorylation with alkaline phosphatase, a ligation reaction was carried out with 100 ng of both D2EK17 and pET-28a vector and using T4 DNA ligase (2 U/ml) under standard experimental conditions, to obtain the plasmid pET-28a-Lpa5930. This recombinant plasmid was amplified in *E. coli* DH5α after transformation by electroporation. The pET28a-Lpa 5930 plasmid was then extracted, purified, and introduced, by heat shock transformation, into *E. coli* BL21(DE3) cells which already contained the pGro7 (TaKaRa) plasmid expressing GroES/GroEL chaperones. In parallel, transformation of *E. coli* BL21(DE3) without pGro7 was also performed. *E. coli* BL21(DE3) harboring the two plasmids, pET28a-Lpa 5930/pGro7, were grown at 37°C in LB medium containing kanamycin and chloramphenicol. Cells harboring only the pET28a-Lpa5930 plasmid were grown in medium with kanamycin.

2.4 Optimization of bacterial expression of recombinant D2EK17 protein

In order to find appropriated conditions for an efficient production of the *P. acidilactici* CECT 5930 recombinant laccase, several parameters affecting culture growth and expression were tested, including post-induction temperature, regimen of stirring, presence of copper cation, and mediation of molecular chaperones expressed from a second co-existing plasmid.

E. coli transformed cells were grown in LB medium, supplemented with the corresponding antibiotics, under shaking conditions (250 rpm) at 37°C for about 16 h. Fifty ml of this preculture was employed to inoculate one liter of pre-warmed Terrific-Broth medium, containing the appropriated antibiotics (kanamycin and chloramphenicol) plus 2 mg/ml of arabinose, when needed to induce pGr07 plasmid. The culture was incubated at 28°C with orbital shaking at 250 rpm and, when the OD_{600} was around 0.6, IPTG was added to a final concentration of 1 mM to induce the expression of the recombinant laccase protein. Incubation of the culture was maintained under different conditions and time periods as described below. Two different final concentrations of copper (0.3 and 1 mM) in the growth medium were evaluated by adding $CuCl_2$ at different stages of the cell culture. Two different post-induction temperatures, 37 °C and 20°C, were checked [47]. After addition of IPTG cultures were incubated at 37°C (250 rpm) for 4 h before harvesting or at 20°C and shaking at 120 rpm for 4 h followed by an additional static incubation overnight (20°C) [47, 49]. In all cases cells were finally harvested by centrifugation at 4°C at 13.500 rpm for 15 min (Beckman coulter Avanti J-E, JA10 rotor) and the resulting pellets were frozen and stored at -80°C.

2.5 Recombinant laccase purification

Cell biomass from one liter culture was thawed and resuspended in 10 ml of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0), containing 1 mg/ml lysozyme, 5 µg/ml DNase, 10 µg/ml RNase and, 1mM phenylmethylsulfonyl fluoride (PMSF). Cell suspension was incubated on ice for 30 min, and afterwards cells were disrupted

mechanically with a similar volume of glass beads (106 µm) in a Mikro-Dismembrator (Sartorius) by setting 10 cycles of 40s. Cell debris was removed by centrifugation at 13,500 rpm for 15 min at 4 °C (Multifuge 1 S-R, Heraeus). The supernatant was collected and loaded onto a Ni^{2+}-NTA–agarose column (0.8 x 2.5 cm), previously equilibrated with equilibration buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). Non-retained proteins were washed out with five column volumes of equilibration buffer. The retained protein was eluted with elution buffer (equilibration buffer containing 250 mM imidazole). Fractions of 0.5 ml were collected, tested for relative protein concentration by monitoring A_{280}, and examined for laccase activity, using ABTS as substrate, in a liquid assay (described below). Fractions displaying activity were pooled and dialyzed overnight against 50 mM sodium phosphate, pH 7.4, containing 1 mM $CuCl_2$ and 0.05 % Tween 20. The content and complexity of proteins present in the chromatographic fractions and in the crude extract were analyzed by sodium dodecyl sulfate‑polyacrylamide gel electrophoresis (SDS-PAGE) to check the degree of purity achieved in the purification procedure. Enzyme activity throughout the process of enzyme extraction and purification was also evaluated by the in-gel assay described by Callejón, Sendra, Ferrer and Pardo [12]. Protein concentrations of all samples were measured by bicinchoninic acid (BCA) assay with bovine serum albumin as protein standard [50].

2.6 Laccase activity assays

Laccase activity was assessed by both in-gel and liquid assays [47]. Briefly, in-gel assays were performed after protein separation by native 8% polyacrylamide gel electrophoresis [12]. Gels were subsequently incubated at room temperature in 0.1 M sodium acetate buffer, pH 4.0, containing 10 mM 2,6-DMP for 5 min. Then, the gel was incubated with 1 mM $CuSO_4$ in the same buffer where the laccase activity was visualized by the presence of an orange-colored band. Liquid assays were carried out by mixing aliquots from the different fractions with 100 µL of buffer (50 mM of acetate buffer, pH 4.0, with 5 mM ABTS as a substrate), and incubation

257 at room temperature. Oxidation of ABTS was determined by absorbance increase in microplate
258 wells at 420 nm (ε_{420}=36,000 M^{-1} cm^{-1}) in a 96-well microplate reader.

259 **2.7 Biochemical characterization of P. acidilactici D2EK17 enzyme**

260 Pure recombinant *P. acidilactici* D2EK17 protein, dissolved in 50 mM potassium phosphate
261 buffer, pH 6.5, was employed to acquire UV–visible absorption spectra (300–800 nm) by using
262 a Beckman Coulter DU® 800 UV/Vis spectrophotometer.

263 The relative molecular weight of the enzyme polypeptide was determined by comparison with
264 molecular weight markers in a SDS-8% PAGE using the LMW-SDS Marker Kit (GE Healthcare
265 Life Sciences).

266 To determine the pH dependence of the recombinant laccase towards ABTS (5 mM) as a
267 substrate, the standard liquid assay was employed by using 50 mM citrate-phosphate buffer to
268 cover a range from 2.5 to 8.0. Oxidation of ABTS was measured by the absorbance increase at
269 420 nm (ε_{420}= 36,000 M^{-1} cm^{-1}) in microplate wells.

270 The effect of temperature on D2EK17 laccase activity in the range 4 to 65°C was studied by
271 measuring ABTS oxidation in the standard liquid assay. The reaction mixtures were set at the
272 appropriate temperature for 10 min previously to the enzyme addition. For thermal stability
273 tests, aliquots of the enzyme solution, in 50 mM acetate buffer, pH 4.0, were pre-incubated at
274 different temperatures at 28, 35, 40, 45, 60, 75, and 85°C for 10 min and then, after cooling of
275 the samples, the residual activity was determined under standard liquid ABTS assay conditions.

276 To determine kinetic parameters V_{max} and K_m of the recombinant laccase for ABTS it was
277 incubated with increasing concentrations of ABTS (0.1–50 mM) in acetate buffer, pH 4.0, at
278 28°C. Reactions in triplicate were initiated by the addition of the enzyme and the absorbance
279 increases at 420 nm were recorded for 10 min. The initial rates were deduced from the slope
280 of plots of A_{420} versus time. Data were fitted to the Michaelis–Menten equation to determine

the V_{max} and K_m values. One enzyme activity unit (U) was defined as the amount of enzyme that oxidizes 1 µmol of substrate/min in the standard liquid assay.

The effect of metal-chelating agents, such as bipyridyl, phenanthroline, SDS and EDTA, in addition to other compounds that potentially could affect the oxidizing activity of the enzyme, such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and semicarbazide, was assessed by incubating the enzyme solution for 5 min at 28°C in the presence of 100 µM of these compounds. After this preincubation, the reaction was initiated by the addition of the substrate 2,6-DMP and the residual activity was determined by the liquid assay. Enzyme activity in the absence of any agent was considered as 100 % relative activity (control).

2.8 Oxidation of amines by *P. acidilactici* D2EK17 laccase

The amine-oxidizing ability of the recombinant *P. acidilactici* laccase, under different conditions, was analyzed by quantification of remaining amine in the reaction mixtures after incubation of the enzyme with different amines [47]. Different enzyme activity assay conditions, including enzyme concentration, the presence and concentration of ABTS (0-10 mM) and HBT (5 and 10 mM) as mediators, the presence of $CuCl_2$ (100 µM), temperature (range 4-45°C), pH (2.5-10.5) and agitation regimen were assessed using tyramine as a substrate. Incubations were carried out with 100 µL of reaction volume in 50 mM acetate buffer, pH 4.0, except for pH dependence assays that was evaluated by using 50 mM citrate-phosphate (pH range from 2.5 to 8.0) and 50 mM carbonate-NaOH (range from 9.0 to 10.5). Histamine and putrescine were also tested as potential substrates for the *P. acidilactici* laccase. The initial concentration of the all amines was 150 mg/l. Reaction mixtures were incubated at 28 °C for 24 hours, after which remaining amine concentration was determined by LC-FLD (see below). As negative controls and under identical conditions, reaction mixtures without enzyme or with heat-inactivated enzyme were used.

Amines were quantified by reverse-phase LC-FLD as previously described by Callejón, Sendra, Ferrer and Pardo [47]. Identification of compounds was performed by comparing retention times of known standards of tyramine, histamine, and putrescine (Sigma). Fluorometric detection was done with the excitation and emission wavelengths at 340 and 430, respectively. The amine concentrations were determined using the peak areas relative to the area of internal standard. The percentage of amine degradation was calculated respect to a negative control (incubation in the absence of enzyme), and used to deduce the relative activity as a percentage of the highest degradation value obtained in each experiment.

2.9 Statistical analyses

All data are represented as the mean ± SE. Group means were compared using one-way ANOVA followed by Duncan's multiple range tests to identify differences among groups when appropriate. All analyses were carried out using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA). Percentage data were normalized by arcsine transformation prior to the analysis.

3 Results

3.1 Isolation and sequence analysis of the D2EK17 gene from *P. acidilactici* CECT 5930

Previously we purified a protein associated to an oxidizing activity of canonical MCO substrates and different amines from *P. acidilactici* CECT 5930 which was identified as a homologue of D2EK17 protein from *P. acidilactici* 7_4 [12]. Currently, in the UniProtKB database, D2EK17 is described as a putative multicopper oxidase. As described in Material and Methods section, two new primers, LacaPat1/LacaPat2, were designed from the D2EK17 *P. acidilactici* 7_4 gene, published as a part of its complete genome sequence (NZ_ACXB00000000), and used to amplify, clone, and characterize the D2EK17 homologous gene from *P. acidilactici* CECT 5930. DNA sequence analysis of the complete gene (1434 bp), with Mega 5 software, showed four short sequences encoding for the four strictly conserved copper ligand motifs which are characteristic of the MCO family (Figure 1).

In addition, multiple sequence alignment, performed with ClustalOmega software (http://www.ebi.ac.uk/Tools/msa/clustalo/), indicated that the MCO D2EK17-homologous protein from *P. acidilactici* CECT 5930 showed a 21.68 % amino acid sequence identity with the laccase from the common mushroom *Trametes versicolor* (UniProt ID: Q96UK8). Likewise, the comparison with other known prokaryotic laccases showed 30.61% identity with *Bacillus* sp. CotA, and 38.26% with *E. coli* CueO (UniProt ID: I3RYX9 and P36649, respectively). Furthermore it has an identity of 58.28% with *L. plantarum* JDM1 SufI cell division protein (UniProt ID: A0A023M894), the homologous of the *L. plantarum* J16 CECT 8944 SufI protein recently identified as laccase by us [47].

3.2 Cloning and improvement of the recombinant laccase expression

P. acidilactici CECT 5930 D2EK17 laccase gene was cloned into the pET28a expression plasmid and the resulting construction (pET28a-Lpa 5930) transformed into *E. coli* BL21(DE3) cells that harbor, or not, a second plasmid (pGro7) for the co-expression of a molecular chaperone system. When the *E. coli* BL21(DE3) cells did not contain the chaperone folding assistant plasmid, the post-induction expressed protein did not exhibit enzyme activity on the standard laccase substrate 2,6-DMP. Only a low enzyme activity was detected when the expression was carried out on cells that also contain the pGro7 vector, and under standard conditions at 37°C. The addition of up to 1 mM $CuCl_2$ to the already expressed protein or dialysis against a buffer containing 0.3 mM $CuCl_2$, did not result in an additional increase of enzyme activity (results not shown). On the contrary, a high 2,6-DMP oxidizing activity was obtained, in an in-gel assay, when the induction was made in a medium containing $CuCl_2$, at 37°C and agitation at 200 rpm (data not shown), indicating the requirement of Cu^{2+} in the culture medium for the proper expression of the recombinant MCO in active state. Previous studies have indicated that under microaeration conditions, as those obtained in static cultures, and temperatures lower than those commonly employed in the bacterial production of recombinant proteins, enhances the expression level and the specific activity of recombinant laccases are considerably increased

[47, 51, 52]. Thus, we have also found an important increase in the recovering of soluble and active recombinant *P. acidilactici* CECT 5930 enzyme. After testing additional different conditions, the most appropriate we found, to attain the best results on the protein expression and enzyme activity, were fairly similar to those previously described for the production of recombinant *L. plantarum* J16 CECT 8944 [47]. In brief, *E.coli* BL21(DE3) cells containing both plasmids, the pET-28a-Lpa5930 and pGro7 plasmid, are grown at 37°C in LB medium in the presence of 1 mM $CuCl_2$ and stirring at 200 rpm up to reach the exponential phase of growth. After adding inductor (IPTG 1 mM) the culture is incubated at 20°C with stirring at 120 rpm for four hours and then the stirring is turned off and the incubation is extended overnight at the same temperature. Seemingly the microaerobic state, resulting in the static incubation, enhances the intracellular accumulation of copper cation, facilitating the proper copper loading and folding of the protein, and leading to recover a high yield of soluble active recombinant laccase [47, 50, 51].

3.3 Purification of the recombinant *P. acidilactici* CECT 5930 laccase

For the preparative purification of the recombinant enzyme in a soluble and active form, a crude cell extract prepared by bead-beating lysis of cells from a liter culture subjected to the conditions described above was obtained. The densitometry analysis (with the ImageJ software) of the Coomasie-blue stained gel, after a SDS-PAGE, indicated that the amount of post-induction overexpressed recombinant laccase protein represented around 9 % of total protein (Figure 2). Recombinant *P. acidilactici* laccase that contains a polyhistidine N-terminal extension was purified from the crude extract by metal-chelating chromatography on Ni^{2+}-NTA agarose. Laccase activity in the different column fractions was revealed by in-gel assay with 2,6-DMP as a substrate, and their protein content was analyzed by SDS-PAGE (Figure 2). Those chromatographic fractions with the highest levels of enzyme activity, and that also showed an intense blue color, were pooled together and after dialysis for desalting, frozen and stored at -80°C. About 14 mg of pure recombinant protein was recovered from one liter of cell culture.

The specific activity of purified recombinant laccase on ABTS, determined by the standard liquid assay, was around 24.05 U/mg.

3.4 Properties of the recombinant *P. acidilactici* laccase

Electrophoretic analysis of the purified recombinant enzyme on SDS-polyacrylamide gel (Figure 2) revealed an apparent molecular weight of around 60 kDa. As the recombinant protein contains an artificial 2,5 kDa N-terminal extension, including the (6xHis) tag plus a short amino acid linker stretch, the molecular weight of the catalytic polypeptide chain of *P. acidilactici* laccase is very near to that inferred from the cloned gene sequence (54.36 kDa).

As it is characteristic of the laccase enzymes, the purified recombinant protein showed an intense blue color, and exhibited a maximum peak at 590 nm in the UV–visible absorption spectrum (Figure 3), which corresponds to the T1 copper center of the MCOs. The final absorbance 280/600 ratio of the purified enzyme was 1.9, indicating a good purification level [53].

The pH dependence analysis of the *P. acidilactici* laccase activity towards ABTS as a substrate revealed a sharp peak with a maximum around pH 4.0 (Figure 4). No significant ABTS-oxidizing activity was detected above pH 5.5. The optimal temperature for recombinant enzyme, on the canonical laccase substrate ABTS, was 28°C, although the enzyme retained a high level of activity in a wide temperature range, from 4 to 65°C (Figure 5A). Heat inactivation experiments revealed that *P. acidilactici* laccase is a relatively thermostable enzyme since the recombinant enzyme retained more than 80% of the activity after a pre-treatment at 60°C for 10 min (Figure 5B). Temperatures higher than 85°C, however, irreversibly inactivated the recombinant enzyme (Figure 5B).

Kinetic analysis of *P. acidilactici* laccase with ABTS as a substrate showed a hyperbolic dependence of the reaction rate on substrate concentration, and provided the K_m and V_{max} values of 1.7 mM and 24,05 U/mg, respectively.

The effect of different potential inhibitors on *P. acidilactici* laccase activity is shown in Figure 6. EDTA and SDS at 100 µM diminished the activity by 18% and 33%, respectively, although the other two tested metal-chelating agents, bipyridyl and phenanthroline, did not significantly inhibit the enzyme. The carboxyl modifier 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) produced only a weak inhibition around 18%. Finally, semicarbazide, a carbonyl-modifying reagent that inhibits a subgroup of copper-dependent amine oxidases, decreased a 63.3% of the ABTS-oxidizing activity of the recombinant enzyme.

3.5 Biogenic amine-oxidizing activity of *P. acidilactici* laccase

We previously detected in *P. acidilactici* CECT 5930 an enzyme with the capacity to degrade BA, which was identified as MCO, and in this work, it has been cloned and expressed. Therefore, we have deepened analyzing the recombinant enzyme ability to oxidize BA. Our specificity analysis have focused on those BA found most frequently in foods, tyramine, histamine and putrescine. Amine degradation by the recombinant laccase was monitored by reverse-phase LC-FLD measuring of the remaining amine in the post-incubation reaction mixture [47]. Primarily, we ascertained that the level of tyramine degradation was dependent on the recombinant enzyme concentration. Then, the inclusion of 100 µM $CuCl_2$ to the reaction medium produced an enhancement in the tyramine oxidation, similarly to our results with the *L. plantarum* laccase [47], although higher concentrations of $CuCl_2$ did not result in further increases in the amine degradation (not shown).

As it can be seen in Figure 4, the oxidizing activity of *P. acidilactici* laccase on the tyramine was maintained in a rather wide pH range, from 3.0 to near 11.0, although two clear maxima were observed, at pH 4.0 and pH 9.5, in which the relative activity of the enzyme was 82% and 100%, corresponding to a 35.6 and 43% of tyramine degradation respectively. It shows that the pH effect on the enzyme activity acting on tyramine is very different from ABTS, indicating that it depends on the type of substrate, which is a typical feature of laccases. Regarding the temperature dependence for tyramine degradation, it was found to be similar that for the

canonical laccase substrate, ABTS (Figure 5A), with an optimum temperature at 28°C. Under the assay conditions employed, the recombinant *P. acidilactici* laccase retained up to 70% of the tyramine-degrading activity, regarding a 100% of relative activity at 28°C. (Figure 5A).

As shown in Figure 7, the addition of ABTS to the incubation mixture increased the tyramine degradation by the recombinant *P. acidilactici* laccase, indicating that ABTS is acting as a mediator. The percentage of the amine degradation augmented with the ABTS concentration up to achieve a 75.2% degradation (100% relative activity) as can be seen in Figure 7.

The oxidizing potential of other BAs, histamine and putrescine, by the recombinant enzyme was initially tested using the assay conditions found to be optimal for tyramine, and described above (100 µL sodium acetate buffer 50 mM, pH 4.0, 150 mg/L BA, in the presence of 1 mM ABTS and 100 µM $CuCl_2$, 14 µg enzyme, and incubation for 24 h at 28°C). However, results showed that recombinant *P. acidilactici* laccase was unable to degrade histamine and putrescine. We neither detected oxidizing activity on these other BAs at pH 9.5 or after the addition of up 5 mM ABTS. It neither was observed degradation of histamine and putrescine when another mediator, HBT, at several concentrations, was tested. HBT besides did not affect the capacity of recombinant laccase to degrade tyramine.

4 Discussion

Amino acid sequence of the *P. acidilactici* CECT 5930 laccase clearly shows that the motifs for Cu ligands type 1 site and trinuclear cluster are present, and located in the same relative positions as found in other bacterial laccases. Furthermore, according to the Laccase Engineering Database (https://lcced.biocatnet.de/), *P. acidilactici* laccase belongs to the SUBfamily J with homologues of CueO from *E. coli*. Along with these data, its intense blue color, that characterize all “blue” copper oxidases, the 280/600 nm absorbance ratio in the typical range for bacterial laccases [53], and its oxidizing catalytic activity of representative substrates of laccase, like ABTS and 2,6-DMP, ratifies that it is a genuine multicopper oxidase, subtype laccase.

An important problem to solve in this work was to set up the proper conditions to get a full functional recombinant *P. acidilactici* laccase. One of the difficulties was related to Cu loading of the recombinant protein. Another one was about to the achievement the proper folding as laccases require four copper ions for catalysis, and a sufficient supply of copper ions in the cytoplasm is crucial for obtaining active enzyme. However, *E. coli* cells prevent intracellular accumulation of toxic copper with inducible efflux systems [54]. Copper depletion upon heterologous expression of bacterial laccases in *E. coli* has been observed frequently [35, 49, 55, 56], which causes suboptimal activity yields and thereby limits the applicability of such bacterial laccases [25]. Several strategies can be pursued to increase copper loading. Cells grown under anoxic conditions do not induce Cu efflux systems to the same extent. Thus, for the production of recombinant CotA laccase from *Bacillus subtilis*, cultivation conditions were successfully adapted to increase intracellular copper levels by simply introducing a static cultivation step after induction, which resulted in fully copper loaded enzyme [51]. The same procedure was applied for the efficient expression of *Bacillus pumilus* CotA in *E. coli* [34]. Although this resulted in fully copper-loaded enzyme, microaerobic expression inevitably led to reduced growth rates that reduced the performance of this condition for large scale production of laccases.

The addition of copper salts to the expression medium can increase the specific copper loading [49]. Further, depleted copper centers can be reconstituted after expression and purification by incubation or dialysis at high copper concentrations [44, 57]. However, reconstitution of copper centers in copper depleted bacterial laccases after expression of protein does not always result in a fully active enzyme as described Durão et al. and Callejón et al. [47, 51]. Apparently, formation of natural copper cluster geometry requires copper incorporation during the folding process. So, a low copper concentration level in the cell during the polypeptide chain folding could cause perturbations at the copper binding sites which result in an enzyme with reduced activity even although copper centers were reconstituted [25].

485 Another example, about the difficulties achieving high yield of recombinant laccase active, is
486 that referred by Gunne et al. (2013), on recombinant CotA from *B. licheniformis:* neither
487 microaerobic expression conditions nor copper centers reconstitution after expression and
488 purification resulted in a fully copper loaded enzyme [58]. Two further strategies were applied
489 for increasing the copper content of CotA by these authors: a knock-down of the host copper
490 detoxification system, and a helping coexpression of a chaperone. From the results, they
491 concluded that not only intracellular copper ion concentration, but also presence of an
492 appropriate chaperone protein influences copper ion insertion into CotA laccase.
493 In this work, we managed to increase the expression of recombinant *P. acidilactici* laccase in
494 *E.coli* in a catalytically active form, diminishing temperature during induction, by the inclusion
495 of copper to the expression medium, and maintaining a low aerobic level by removing stirring,
496 similarly as already reported for *L. plantarum* J16 CECT 8944 laccase [47], but in the present
497 case the coexpression of chaperones it was needed to prevent misfolding. Under standard
498 conditions for the expression of heterologous enzymes in *E. coli* (with higher temperatures,
499 aerobiosis and agitation), we were unable to produce active laccase, and the addition of
500 copper salts to the obtained recombinant protein solutions did not result in the recovery of
501 catalytic activity. Several previous reports have indicated that the intracellular copper
502 concentration reached under the common procedure, for the overexpression of proteins, must
503 be insufficient for the proper constitution of the catalytic copper centers and the correct
504 protein folding [49, 51, 52]. Nonetheless, in some cases the supplementation of Cu^{+2} to an
505 inactive recombinant laccase led to the successful retrieval of some enzymatic activity. For
506 example, Li et al. [59] reported that the incubation of the apoprotein apo-CueO, corresponding
507 to the *E. coli* CueO laccase, with copper ion resulted in recovering of a high level of enzyme
508 activity. It is assumed that using those optimized expression conditions, on one hand the
509 exogenously added copper and the microaeobic medium contribute to an appropriate
510 intracellular accumulation of copper which promote formation of native, catalytically active

laccase enzyme, and on the other hand, the decrease of the post-induction culture temperature enhances the level of the recombinant protein expression [49, 51, 52].

Our biochemically characterization of the *P. acidilactici* laccase has shown that the enzyme has a molecular weight around 60 kDa, which is in the range found for several other bacterial laccases, most of which fall between 50 and 100 kDa [42]. Likewise, the optimum pH of recombinant laccase when the ABTS is the substrate, a value of 4.0, was similar to that reported for other bacterial and fungal laccases [60, 61]. Besides, as many other laccases from bacteria and fungi, also the activity profile against pH of *P. acidilactici* laccase is dependent on the nature of substrate, showing a maximum around 3.5-4.0 with ABTS, and two maxima at 4.0 and 9.5 with the amine tyramine. Our data indicate an optimum temperature for recombinant *P. acidilactici* enzyme around 28°C, which is lower than those of other several bacterial laccases, such as CotA of *Bacillus* species (55-75°C) [34, 49, 51] or CueO from *E. coli* (55°C) [43]. Nevertheless, the thermostability of the *P. acidilactici* laccase is similar to *E. coli* CueO [43] and *L. plantarum* J16 laccase [47], maintaining at least 80% of activity after 10 min incubation at 65°C, although lower than CotA from *Bacillus subtilis* [51]and *Bacillus tequilensis* laccase [62], which retained more than 50% of activity after a long incubation (>100 min) at temperatures above 70°C. Regarding the kinetic analysis of the recombinant *P. acidilactici* laccase, our results provided a K_m value of 1.7 mM for ABTS, which is somewhat higher than those described for species of *Bacillus* and other bacterial laccases [34, 47, 49, 63], although fairly similar to that of *L. plantarum* J16 [47].

Strikingly, the *P. acidilactici* laccase was not inhibited by the metal-chelating agents bipyridyl and phenathroline (at 100 µM), but it was by EDTA and SDS, as expected given prior results with diverse laccases from different organisms [45]. Likewise, we previously observed a strong inhibition of the *L. plantarum* J16 laccase by EDC [34, 47, 49, 63], however in this work the recombinant *P. acidilactici* laccase was only marginally inhibited by this carboxyl-group modifying agent. Therefore, the apparent involvement of a carboxyl group in the catalysis, as

has been already proposed for some fungal laccases [64] seems to be less important in the *P. acidilactici* laccase. However, the inhibitory effect of semicarbazide, a carbonyl-modifying agent that inhibits a subgroup of copper-dependent amine oxidases, on the recombinant *P. acidilactici* enzyme was higher than on *L. plantarum J16* laccase [34, 47, 49, 63]. Inhibition by semicarbazide has been also described for the laccase CueO [43].

The ascertaining that laccase enzymes, including the commercial laccase from the fungus *Trametes versicolor* [12], are able to degrade biogenic amines in foods underscores a new interest on laccases for their potential applications in food biotechnology. However, the studies about the BA-oxidizing capability of prokaryotic and eukaryotic laccases are still scarce. Recently we have identified, cloned and characterized the SufI protein of *L. plantarum* J16, a LAB isolated from wine but that also is frequently found in diverse foods, as laccase bearing the ability to degrade the BA tyramine, and also, although in a lesser extent, histamine and putrescine [47]. In the present work, we have found that recombinant *P. acidilactici* laccase is also able to degrade tyramine but, contrary to *L. plantarum* enzyme, apparently not histamine nor putrescine. Possibly the suitability of tyramine being oxidized by the recombinant laccase is in the phenolic nature of this substrate [34]. Besides, we have found out that the activity of recombinant *P. acidilactici* laccase toward tyramine can be significantly enhanced by presence of ABTS acting as redox mediator. Overall the *P. acidilactici* laccase differs from the *L. plantarum* J16 laccase in terms of its physical and biochemical properties, such as response to inhibitory substances, optimal temperature, thermostability and kinetic parameters; however both enzymes exhibit a very similar oxidizing capability towards tyramine.

In conclusion, we have isolated, purified, and characterized a new bacterial laccase from the LAB *P. acidilactici* CECT 5930. Evidence for this was obtained from substrate specificity and from kinetic, physical, biochemical, and spectral characteristics. Optimization of the induction conditions and expression resulted in laccase activity of 24 U/mg. Laccase from *P. acidilactici* is able to degrade, in addition to ABTS and 2,6-DMP, wich are the canonical laccase enzymes

substrates, tyramine, but not histamine nor putrescine. This enzyme has an optimum temperature of 28°C for both ABTS and tyramine. Optimum pH value for ABTS is 4, whereas for tyramine the enzyme has two optimal values: 4 and 9.5. The substrate ABTS acts as redox mediator of the reaction of laccase on tyramine, increasing its degradation. It is a relatively thermostable enzyme, that is not inhibited by chelating agents, although it is partially inhibited by carbodiimide, EDTA, SDS and semicarbazide, in increasing order.

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Conflict of interest

The authors declare that they have no competing interests.

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781 **Figure legends**

782 **Figure 1.** Amino acid sequence of *P. acidilactici* CECT 5930 protein homologue of D2EK17.
783 Shaded and underlined are the motifs corresponding to the four copper ligands which are

784 strictly conserved in MCOs (conserved sequence of these motifs are, successively: HXHG, HXH,
785 HXXHXH and HCHXXXHXXXXM/L/F; [65])

786 **Figure 2.** Coomasie–blue stained gel after SDS-8% PAGE of different fractions during the
787 purification procedure of recombinant *P. acidilactici* CECT 5930 laccase. Lane 1, whole-cell
788 extract from non-induced cells; lane 2, whole-cell extract from post-induction cells; lane 3,
789 protein markers, with the molecular weights indicated on the right (kDa); lanes 4-6, successive
790 fractions of the elution from the metal-chelating chromatography on Ni^{2+}-NTA-agarose; lane 7,
791 crude extract corresponding to applied sample on the chromatography column. Arrow marks
792 recombinant protein.

793 **Figure 3.** UV-Visible spectrum of purified recombinant *P. acidilactici* CECT 5930 laccase. Protein
794 was dissolved in 50 mM sodium phosphate, pH 6.5 buffer at a concentration of 14 mg/ml. Only
795 the range from 300 to 800 nm is shown.

796 **Figure 4.** pH effect on activity of the recombinant *P. acidilactici* enzyme toward ABTS (*solid*
797 *line*); and tyramine (*dashed line*) as substrates. Citrate-phosphate buffer (pH range 2.5-8.0) and
798 carbonate-NaOH buffer (pH range 9.0-10.5) were used at concentration of 50 mM. Enzyme
799 activity is plotted as percentage relative to the maximum value for each substrate (% relative
800 activity). With tyramine as a substrate, the 100% of relative activity means a 43% of tyramine
801 degradation in the reaction mixture, determined by reverse-phase LC-FLD, under the assay
802 conditions described in Material and Methods section. The values are means ± standard
803 deviations of triplicate assays.

804

805 **Figure 5.** (A) Temperature dependence of recombinant *P. acidilactici* enzyme determined
806 by incubation of the reaction mixtures, at the indicated temperatures, and using the
807 standard liquid assay with ABTS (*solid line*) and tyramine (*dashed line*). Enzyme activity is
808 plotted as a percentage relative to the maximum value (% relative activity). When
809 tyramine was the substrate the 100% of relative activity represents a 70% of tyramine

degradation in the assay conditions described in Material and Methods section. (B) Thermal stability analyzed by preincubation of the enzyme solution at the showed temperatures for 10 min before being reequilibrated to 28°C and employed for the standard liquid assay with ABTS as a substrate. Residual enzymatic activity, relative to the maximum value (28°C), is graphed against the pre-treatment temperature. All values are means ± standard deviations for triplicate assays.

Figure 6. Effect of potential inhibitors of *P. acidilactici* laccase. Liquid assays using ABTS as a substrate were carried out in the presence of 100 µM of each of the indicated compounds. Control was an enzyme assay in the absence of inhibitor and is taken as 100% of activity. Results express the remaining activity as percentage relative to the control. Values are means ± standard deviations of triplicate assays.

Figure 7. Function of the ABTS as redox mediator on tyramine oxidation by recombinant *P. acidilactici* laccase. Tyramine concentration was quantified by reverse-phase LC-FLD after incubation with recombinant laccase and different concentrations of ABTS for 24 at 28°C. The percentages of tyramine degradation were calculated regarding a control without enzyme. Activity is expressed as percentage relative to the maximum, where a 100 % means a 75.2% of tyramine degradation under these assay conditions. The values are means ± standard deviations for triplicate assays.

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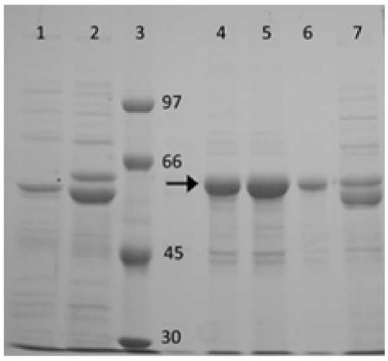
        10         20         30         40         50
MITKYLYDEN AYDYHDGGYR PLKKAPGEEH PLNVPAFLKP DRIEGNEIYY
        60         70         80         90        100
TVTAQAGETK ILPGKPTHTW GYNGSILGPA IQFETGKTYH VTLKNELDEV
       110        120        130        140        150
TTFHWHGLNI VGPYEDGGPH APVYPHGERK ITFTVDQPAA NIWLHPHPCP
       160        170        180        190        200
ETARQVWNGL AAPVIITDGH EQSLKLPRRW GVNDFPVVLQ DRSYHDNQLD
       210        220        230        240        250
YKADYDVDGT LGDYALVNGT VNPVVNVTKP IVRLRFLNGS NRREWRLHFA
       260        270        280        290        300
DYHPFTQIGS DGGLLPEAVE MDRIMLTCAE RADVLVNFSD YQPGQEVILQ
       310        320        330        340        350
TDDFNLIKFK IGDIKKENML LPSPLAEIPA LSVDENTPVF KTVMSGMDDQ
       360        370        380        390        400
VRLDGKLFDM QRIDTRQQVD QTQIWEVSNT NDMEGGMIHP FHIHGCQFQL
       410        420        430        440        450
IDRNGHAVNP NEHGWKDTIG VNPNETVRIK VKFTKLGIFM YHCHILEHED
       460        470
TGMMAQIEIF DPDHPIEYHL MPMNHKM
```

Amino acid sequence of P. acidilactici CECT 5930 protein homologue of D2EK17. Shaded and underlined are the motifs corresponding to the four copper ligands which are strictly conserved in MCOs (conserved sequence of these motifs are, successively: HXHG, HXH, HXXHXH and HCHXXXHXXXXM/L/F; [65])

Figure 1

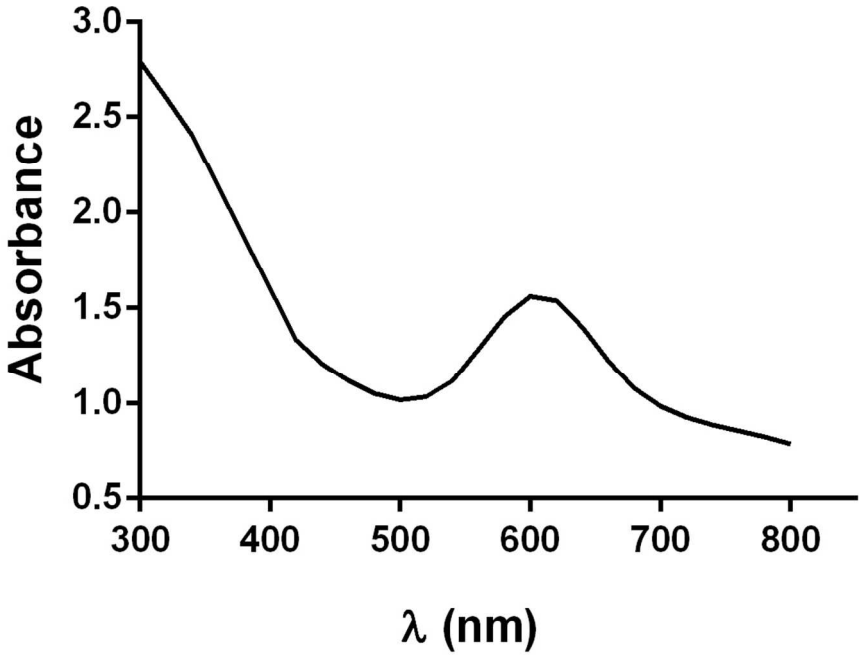
160x102mm (300 x 300 DPI)



Coomasie–blue stained gel after SDS-8% PAGE of different fractions during the purification procedure of recombinant P. acidilactici CECT 5930 laccase. Lane 1, whole-cell extract from non-induced cells; lane 2, whole-cell extract from post-induction cells; lane 3, protein markers, with the molecular weights indicated on the right (kDa); lanes 4-6, successive fractions of the elution from the metal-chelating chromatography on Ni2+-NTA-agarose; lane 7, crude extract corresponding to applied sample on the chromatography column. Arrow marks recombinant protein.

Figure 2

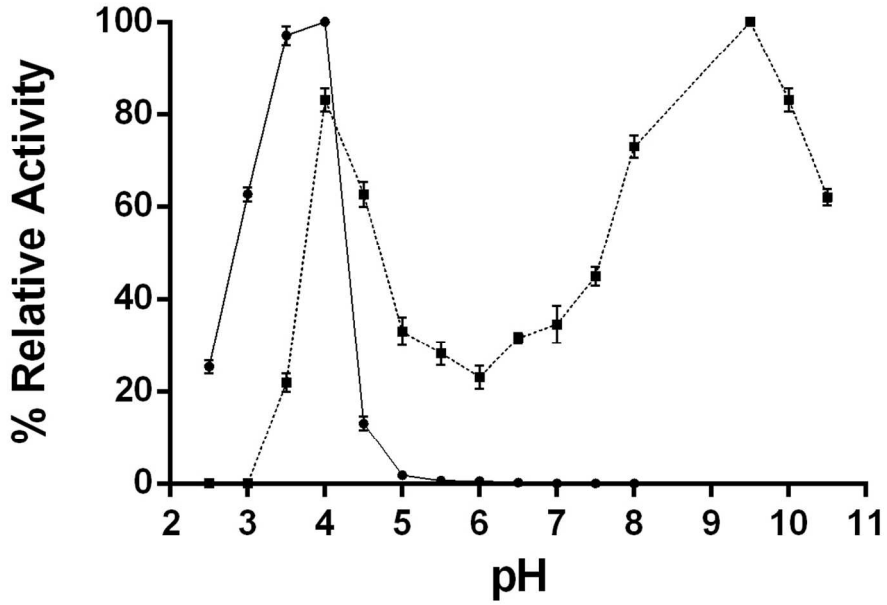
189x276mm (300 x 300 DPI)



Coomasie–blue stained gel after SDS-8% PAGE of different fractions during the purification procedure of recombinant P. acidilactici CECT 5930 laccase. Lane 1, whole-cell extract from non-induced cells; lane 2, whole-cell extract from post-induction cells; lane 3, protein markers, with the molecular weights indicated on the right (kDa); lanes 4-6, successive fractions of the elution from the metal-chelating chromatography on Ni2+-NTA-agarose; lane 7, crude extract corresponding to applied sample on the chromatography column. Arrow marks recombinant protein.

Figure 3

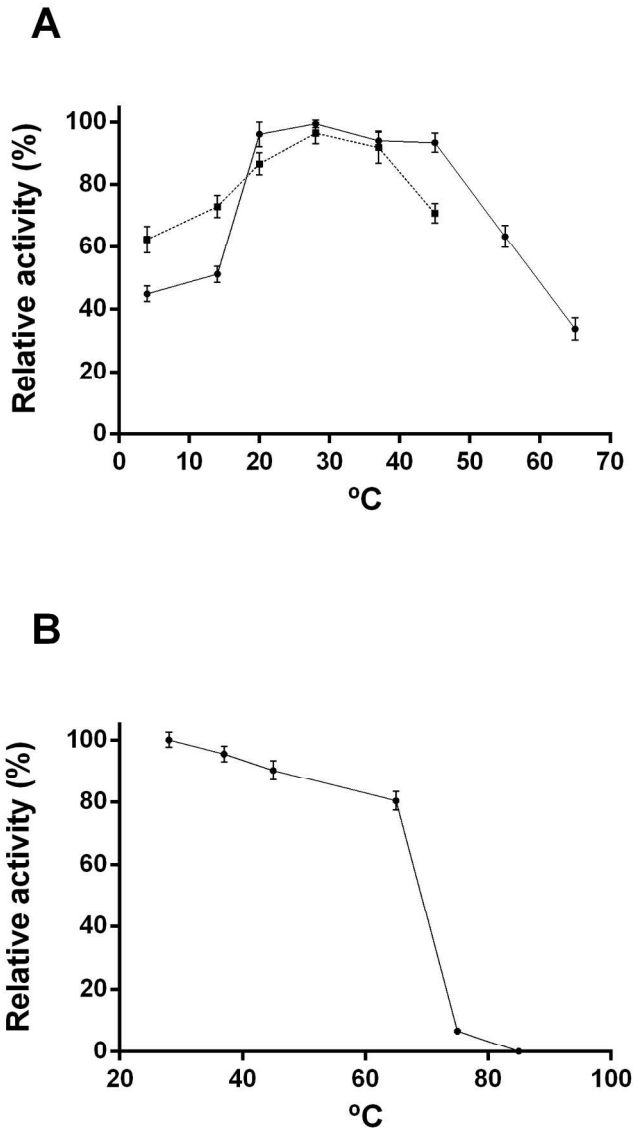
106x77mm (300 x 300 DPI)



pH effect on activity of the recombinant P. acidilactici enzyme toward ABTS (solid line); and tyramine (dashed line) as substrates. Citrate-phosphate buffer (pH range 2.5-8.0) and carbonate-NaOH buffer (pH range 9.0-10.5) were used at concentration of 50 mM. Enzyme activity is plotted as percentage relative to the maximum value for each substrate (% relative activity). With tyramine as a substrate, the 100% of relative activity means a 43% of tyramine degradation in the reaction mixture, determined by reverse-phase LC-FLD, under the assay conditions described in Material and Methods section. The values are means ± standard deviations of triplicate assays.

Figure 4

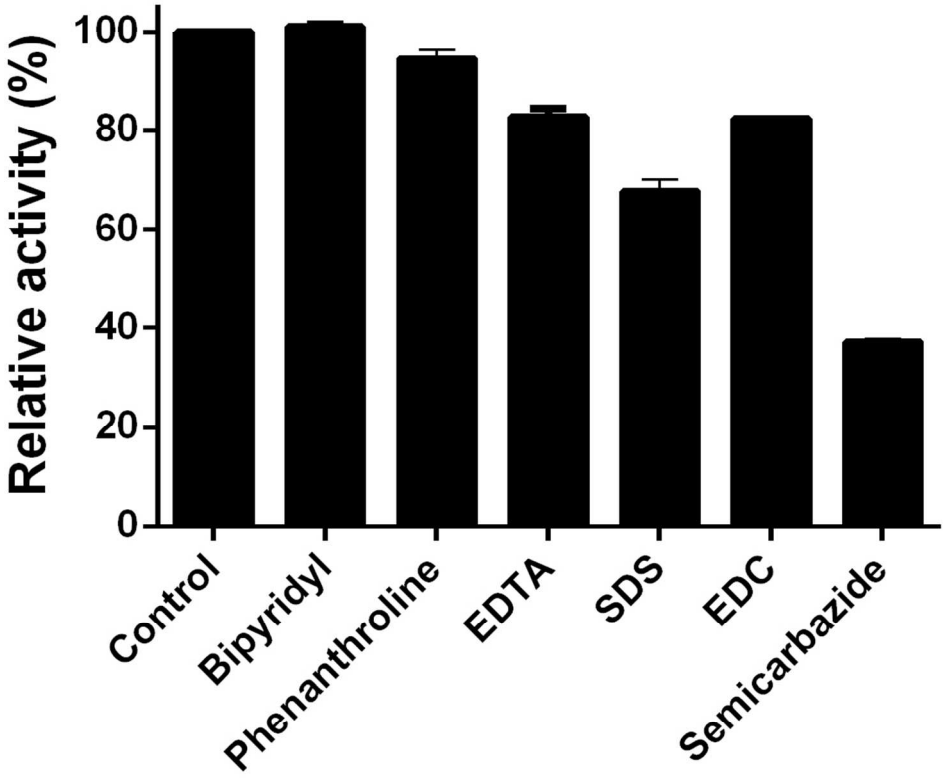
109x74mm (300 x 300 DPI)



(A) Temperature dependence of recombinant P. acidilactici enzyme determined by incubation of the reaction mixtures, at the indicated temperatures, and using the standard liquid assay with ABTS (solid line) and tyramine (dashed line). Enzyme activity is plotted as a percentage relative to the maximum value (% relative activity). When tyramine was the substrate the 100% of relative activity represents a 70% of tyramine degradation in the assay conditions described in Material and Methods section. (B) Thermal stability analyzed by preincubation of the enzyme solution at the showed temperatures for 10 min before being reequilibrated to 28°C and employed for the standard liquid assay with ABTS as a substrate. Residual enzymatic activity, relative to the maximum value (28°C), is graphed against the pre-treatment temperature. All values are means ± standard deviations for triplicate assays.

Figure 5

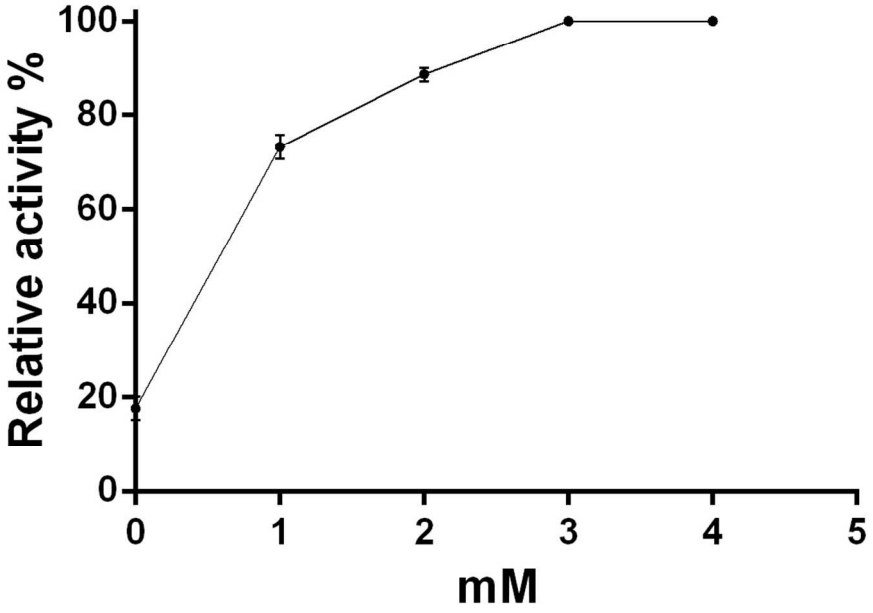
127x202mm (300 x 300 DPI)



Effect of potential inhibitors of P. acidilactici laccase. Liquid assays using ABTS as a substrate were carried out in the presence of 100 µM of each of the indicated compounds. Control was an enzyme assay in the absence of inhibitor and is taken as 100% of activity. Results express the remaining activity as percentage relative to the control. Values are means ± standard deviations of triplicate assays.

Figure 6

97x86mm (300 x 300 DPI)



Function of the ABTS as redox mediator on tyramine oxidation by recombinant P. acidilactici laccase. Tyramine concentration was quantified by reverse-phase LC-FLD after incubation with recombinant laccase and different concentrations of ABTS for 24 at 28°C. The percentages of tyramine degradation were calculated regarding a control without enzyme. Activity is expressed as percentage relative to the maximum, where a 100 % means a 75.2% of tyramine degradation under these assay conditions. The values are means ± standard deviations for triplicate assays.

Figure 7

105x74mm (300 x 300 DPI)