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ESTUDIO DE LOS PROCESOS DE POLIMERIZACIÓN Y DEPOLIMERIZACIÓN DEL POLI-3-HIDROXIBUTIRATO (P3HB) EN CULTIVOS DE *AZOTOBACTER VINELANDII* BAJO CONDICIONES CONTROLADAS DE TENSIÓN DE OXÍGENO DISUELTO

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ÍNDICE GENERAL

Contenido Página

Listado de Abreviaturas 8

Resumen 9

Abstract 10

1. ANTECEDENTES 11

1.1 Poli-3-hidroxibutirato (P3HB) 11

1.1.1 Aplicaciones del P3HB 11

1.1.2 Importancia del peso molecular del P3HB 13

1.2 Microorganismos productores de polihidroxialcanoatos (PHAs) 13

1.2.1 Metabolismo del P3HB y el efecto del oxígeno disuelto sobre la síntesis del biopolímero 14

1.2.1.1 Etapa de síntesis 15

1.2.1.2 Etapa de degradación 15

1.2.1.3 Efecto del oxígeno disuelto sobre la síntesis del P3HB 16

1.3 Generalidades de las enzimas involucradas en el ciclo del P3HB 16

PHA sintasa 17

β-cetotiolasa 19

Acetoacetil-CoA reductasa 20

PHA depolimerasa 21

1.4 Influencia de la síntesis y degradación sobre el peso molecular del P3HB 21

2. HIPÓTESIS 25

3. OBJETIVOS 25

3.1 Objetivo general 25

3.2 Objetivos específicos 25

4. ESTRATEGIA EXPERIMENTAL 26

5. MATERIALES Y MÉTODOS 28

5.1 Mantenimiento de la cepa, medio de cultivo y preparación del inóculo 28

5.2 Cultivos lote en biorreactor 28
5.3 Métodos analíticos 29
5.3.1 Determinación de biomasa por peso seco 29
5.3.2 Determinación de proteína 29
5.3.3 Determinación de sacarosa por el método de β-fructofuranosidasa-DNS 30
5.3.4 Cuantificación del contenido de P3HB en la biomasa 32
5.3.5 Extracción del P3HB 32
5.3.6 Determinación del peso molecular del P3HB 33
5.3.7 Obtención del extracto libre de células para análisis enzimáticos 35
5.3.8 Análisis enzimáticos 35
β-cetotiolasa 35
Acetoacetil-CoA reductasa 36
P3HB sintasa 37
P3HB depolimerasa 37
Recuperación de gránulos nativos 38
6. RESULTADOS Y DISCUSIÓN 40
6.1 Cinéticas de cultivos de *A. vinelandii* cepa OP en biorreactor bajo condiciones limitantes y no limitantes de oxígeno disuelto 40
6.2 Análisis del peso molecular del P3HB sintetizado bajo condiciones de limitación y no limitación de oxígeno 43
6.3 Análisis de las enzimas que participan en el ciclo del P3HB 46
6.4 Caracterización cinética y análisis del peso molecular del P3HB producido por la cepa mutante *phbZ*⁻ de *A. vinelandii* 50
6.5 Análisis de las enzimas que participan en el ciclo del P3HB en cultivos de la cepa mutante phbZ- de A. vinelandii 54
7. CONCLUSIONES 58
8. APORTACIONES Y PERSPECTIVAS 59
9. REFERENCIAS 60
10. ANEXOS 66
ANEXO A. Productos Generados 66
ANEXO B. Establecimiento de la metodología de extracción del P3HB y análisis del peso molecular por GPC 113

ÍNDICE DE FIGURAS

Figura **Página**

Figura 1. Estructura química del monómero constituyente del P3HB. 11
Figura 2. Ruta de biosíntesis y degradación del P3HB. 14
Figura 3. Modelo del mecanismo catalítico de las PHAs sintasas. 18
Figura 4. Modelo donde se representan las 4 subunidades que conforman a la enzima β-cetotilasa. 19
Figura 5. Modelo donde se representan las 4 subunidades que conforman a la enzima reductasa. 20
Figura 6. Estrategia experimental propuesta. 26
Figura 7. Curva estándar de proteína empleada para la cuantificación de la concentración de proteína. 30
Figura 8. Curva estándar de sacarosa empleada para la cuantificación de la concentración de la fuente de carbono. 31
Figura 9. Curva de calibración utilizada para la cuantificación del peso molecular de las muestras de P3HB empleando el software *Empower*. 34
Figura 10. Cinéticas de (a) crecimiento celular, (b) concentración de P3HB, (c) acumulación de P3HB y concentración de sacarosa (d) en cultivos de la cepa OP de *A. vinelandii* en condiciones de limitación (1 % de TOD) y no limitación (15 % de TOD) de oxígeno disuelto. 41
Figura 11. Evolución del peso molecular promedio del P3HB producido por la cepa OP de *A. vinelandii* bajo condiciones de limitación (1 % de TOD y no limitación (15 % de TOD) de oxígeno disuelto. 44
Figura 12. Distribución del peso molecular del P3HB acumulado en condiciones de TOD constante de 1y 15 % de saturación a diferentes fases de cultivo. Fase exponencial de crecimiento (a), fase estacionaria temprana (b) y fase estacionaria tardía (c). 45
Figura 13. Actividades de las enzimas β-cetotiolasa (a), acetoacetil-CoA reductasa (a), P3HB sintasa (b) y P3HB depolimerasa (c) en cultivos de *A. vinelandii* cepa OP realizados en condiciones controladas de TOD a 1 %. 47
Figura 14. Distribución de pesos moleculares de los gránulos de P3HB antes y después del ensayo de degradación *in vitro* obtenidos de cultivos a 1 % de TOD a 12 y 20 h de cultivo. 49
Figura 15. Cinéticas de (a) crecimiento celular, (b) concentración de P3HB, (c) concentración de sacarosa y (d) acumulación de P3HB en cultivos de la cepas OP y la mutante *phbZ*$^-$ de *A. vinelandii* en condiciones de limitación (1 % de TOD) de oxígeno. 51
Figura 16. Peso molecular promedio (a) y distribución de pesos moleculares (b-c) del P3HB producido por las cepas OP y *phbZ*$^-$ de *A. vinelandii* en condiciones de limitación de oxígeno. 53
Figura 17. Peso molecular promedio (a) y actividad de las enzimas β-cetotiolasa (b) y sintasa (c) del P3HB producido por las cepas OP y *phbZ*$^-$ de *A. vinelandii* en condiciones de limitación de oxígeno. 55
Figura 18. Esquema del ciclo del P3HB donde se presenta la actividad P3HB sintasa (barras azules) y P3HB depolimerasa (barras rojas) en la fase de crecimiento y estacionaria en los cultivos de las cepas OP y phbZ- de A. vinelandii bajo condiciones de limitación de oxígeno disuelto. 56
Figura B1. Distribución característica de pesos moleculares de muestras de P3HB sometidas a diferentes tratamientos de ruptura celular. 115

ÍNDICE DE TABLAS

No. tabla	Título	Página
Tabla 1.	Aplicaciones biomédicas de P3HB con diferentes pesos moleculares.	12
Tabla 2.	Clasificación de las PHAs sintasas reportadas hasta ahora.	17
Tabla 3.	Composición del buffer de lisis utilizado para la ruptura celular.	35
Tabla 4.	Concentración de los reactivos en la mezcla de reacción utilizada para medir la actividad de la enzima β-cetotiolasa.	36
Tabla 5.	Concentración de los componentes de reacción para medir la actividad de la enzima acetoacetil-coA reductasa.	36
Tabla 6.	Concentración de los componentes de reacción para medir la actividad de la enzima P3HB sintasa.	37
Tabla 7.	Concentración de los componentes de reacción para medir la actividad de la enzima P3HB depolimerasa.	38
Tabla 8.	Parámetros cinéticos y de cultivo obtenidos en biorreactores bajo condiciones de limitación y no limitación de oxígeno en cultivos de *A. vinelandii* cepa OP.	42
Tabla 9.	Porcentaje de diversas fracciones de moléculas de P3HB presentes en las muestras de 12 y 20 h de cultivo a 1 y 15 % de TOD.	46
Tabla 10.	Porcentaje de moléculas de P3HB con diferentes pesos moleculares que presentan los gránulos de polímero sometidos a degradación *in vitro* y sin degradación.	50
Tabla 11.	Parámetros cinéticos y de cultivo obtenidos de los cultivos de las cepas OP y *phbZ*¯ de *A. vinelandii* en condiciones de limitación de oxígeno.	52
Tabla B1.	Porcentajes de la distribución de pesos moleculares y peso molecular promedio de las muestras de P3HB sometidas a diferentes tratamientos de ruptura celular.	115

Listado de Abreviaturas

Abreviatura	*Significado*	*Unidades*
TOD	Tensión de oxígeno disuelto	%
Mw	Peso molecular promedio en peso	kDa
VTO	Velocidad de transferencia de oxígeno	mmol L^{-1} h^{-1}
P3HB	Poli-3-hidroxibutirato	---
HPLC	High Performance Liquid Chromatography	---
DNS	ácido 3,5 dinitrosalicílico	---
GPC	Gel Permeation Chromatography	---
PHAs	Polihidroxialcanoatos	---
NADPH	Nicotinamida adenina dinucleótido fosfato reducido	---
$NADP^+$	Nicotinamida adenina dinucleótido fosfato oxidado	---
NADH	Nicotinamida adenina dinucleótido reducido	---
NAD^+	Nicotinamida adenina dinucleótido oxidado	---
CoA	Coenzima A	---
CAT	Ciclo de los ácidos tricarboxílicos	---

Resumen

El poli-3-hidroxibutirato (P3HB) es un biopolímero de reserva de carbono acumulado en forma de inclusiones intracelulares por una gran variedad de microorganismos, entre los que se encuentra *Azotobacter vinelandii*. El P3HB es un termoplástico biodegradable y biocompatible que presenta propiedades similares a la de algunos polímeros petroquímicos. Debido a estas propiedades, el P3HB se ha propuesto como sustituto de estos polímeros y como material de soporte en la ingeniería de tejidos. El peso molecular del P3HB determina sus propiedades termomecánicas, así como su degradabilidad. En *A. vinelandii* están presentes enzimas que participan en las etapas de biosíntesis y degradación del biopolímero, lo que sugiere que el peso molecular del P3HB es función del balance de los procesos de polimerización y depolimerización. Es por eso que el objetivo de este trabajo fue entender de qué forma se regula el peso molecular del P3HB en cultivos de *A. vinelandii* bajo condiciones controladas de oxígeno disuelto. En cultivos de *A. vinelandii* OP, se encontró que los cambios en el peso molecular promedio en peso (Mw) del biopolímero correlacionaron con la fase de cultivo de la bacteria; ya que, durante la fase exponencial de crecimiento, el Mw fue mayor (5,000-5,200 kDa) comparado con el Mw del P3HB producido durante la fase estacionaria (3,500-3,600 kDa). Estos valores de Mw máximo (5,200 kDa) del P3HB son los más altos reportados para un microorganismo nativo que produce este biopolímero. Durante la fase exponencial de crecimiento, mientras la actividad sintasa fue alta y la actividad depolimerasa baja, se favoreció la acumulación de P3HB de alto peso molecular. Por el contrario, durante la fase estacionaria temprana, aunque la actividad sintasa fue alta, la actividad P3HB depolimerasa aumentó y tuvo el principal efecto sobre el Mw del biopolímero, ocasionando una disminución de 1,200 kDa en el Mw del P3HB. Esto se demostró al utilizar una cepa *phbZ*$^{-}$ de *A. vinelandii* (que tiene inactivado el gen de la P3HB depolimerasa). Se encontró que en esta cepa el Mw del biopolímero permaneció constante (6,100 kDa) durante todo el cultivo, confirmando que la enzima P3HB depolimerasa tuvo un efecto negativo sobre el Mw del biopolímero acumulado por *A. vinelandii* OP. Los resultados obtenidos permiten plantear estrategias de cultivo que ayudan a tener control sobre el Mw del P3HB, para generar biopolímeros con propiedades deseadas dependiendo de su aplicación final.

Abstract

Poly-3-hydroxybutyrate (P3HB) is a carbon reserve biopolymer accumulated as intracellular inclusions by a variety of microorganisms, among them *Azotobacter vinelandii*. P3HB is a biodegradable and biocompatible thermoplastic that has properties similar to those of some petrochemical polymers. By these properties it has been proposed as a substitute for these polymers and also as support material in tissue engineering. The molecular mass of P3HB determines its thermomechanical properties and degradability. In *A. vinelandii* there are enzymes involved in the biosynthesis and degradation stages of the biopolymer; therefore, our hypothesis is that the molecular mass of P3HB is function of the balance between polymerization and depolymerization processes. That is why the aim of this study was to understand how the balance of the activities of synthesis and degradation of P3HB affect the molecular mass of P3HB in cultures of *A. vinelandii* under oxygen limited conditions. It was found that the changes in the weight-average molecular mass (Mw) of the biopolymer correlated with the growth phase of the bacterium; because, during the exponential growth phase, the Mw was higher (5,000-5,200 kDa) when compared with the Mw of P3HB produced during the stationary phase (3,500 to 3,600 kDa). These values of maximum Mw of P3HB (5,200 kDa) are the highest reported for a native microorganism producing this biopolymer. During the exponential growth phase, as synthase activity was higher and depolymerase activity was lower, the accumulation of high molecular mass P3HB was favored. On the contrary, during the early stationary phase, although P3HB synthase activity was high, P3HB depolymerase activity increased and had a strong effect on the MMM of biopolymer, causing a decrease of 1,200 kDa on the Mw of P3HB. This was confirmed when using an *A. vinelandii phbZ*$^{-}$ mutant (having inactivated gene P3HB depolymerase).We found that the Mw of biopolymer remained constant (6,100 kDa) throughout the fermentation, confirming that the P3HB depolymerase enzyme had a negative effect on the Mw of the biopolymer accumulated by the strain OP. The results allow proposing cultivation strategies that help to have proper control over the Mw of P3HB to generate biopolymers with desired properties depending on their final application.

1. ANTECEDENTES

1.1 Poli-3-hidroxibutirato (P3HB)

El poli-3-hidroxibutirato (P3HB) es el miembro mejor caracterizado de la familia de los polihidroxialcanoatos (PHAs) y está constituido por monómeros de 3-hidroxibutirato (Fig.1) unidos por medio de enlaces éster entre el grupo carboxilo e hidroxilo (Ashby *et al*., 1999; Suriyamongkol *et al*., 2007; Kung *et al.*, 2007; Peña *et al*., 2015). Es un biopolímero lineal que diversas bacterias y arqueas acumulan en su interior como material de reserva de carbono y energía (Kessler y Witholt, 2000; Khanna y Srivastava, 2005; Suriyamongkol *et al*., 2007; Peña *et al.*, 2014a).

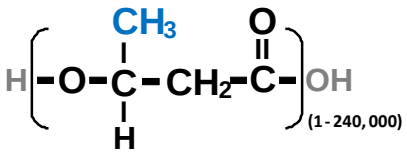


Figura 1. Estructura química del monómero constituyente del P3HB.

El P3HB tiene características térmicas y mecánicas similares a las de los plásticos derivados del petróleo, como el polipropileno y polietileno, con la ventaja de que puede ser sintetizado a partir de fuentes de carbono renovables; por lo tanto es un polímero totalmente biodegradable (pueden ser asimilados por muchos microorganismos ya sea de suelos, mares, lagos o aguas residuales) y biocompatible (no causan efectos tóxicos en organismos superiores). Estas propiedades le confieren una gran importancia como sustituto de los plásticos convencionales (Anderson y Dawes, 1990; Lee, 1996; Steinbüchel, 2001), además de que es idóneo para usarse en el área biomédica y farmaceútica (Chen y Wang, 2013).

1.1.1 Aplicaciones del P3HB

El poli-3-hidroxibutirato se encuentra en gránulos en estado amorfo, dentro de los microorganismos que lo acumulan. Después de su extracción con solventes orgánicos, el P3HB es un material parcialmente cristalino, rígido, frágil y de baja elasticidad, por lo que su uso industrial se encuentra limitado dentro del rango de posibles aplicaciones en el área de la ingeniería de tejidos como material para regeneración de nervios y tejidos (Taguchi y Doi, 2004; Suriyamongkol *et al.*, 2007), aunque sus características materiales, como la

elasticidad y resistencia, pueden ser modificadas cuando se mezcla con agentes plastificantes como polietilenglicol, glicerol o triacetato de glicerol (Hong *et al.*, 2013).

Por ser biodegradable, el P3HB puede ser empleado como una alternativa para reemplazar a los plásticos derivados del petróleo, con propiedades semejantes al polietileno y polipropileno (Steinbüchel, 2001; Philip *et al.*, 2007; Laycock *et al.*, 2013). Sin embargo, debido a la biocompatibilidad y biodegradabilidad del P3HB, las aplicaciones más atractivas se encuentran en el área farmacéutica y médica, donde las características químicas y pureza del producto son de gran importancia (Peña *et al.*, 2014a) (Tabla 1). Actualmente, este biopolímero es empleado como material de sutura, mallas quirúrgicas, hisopos, vendajes, sistemas de encapsulamiento de fármacos de liberación prolongada, en la fabricación de soportes para el desarrollo de tejidos cardiovasculares, nerviosos y cartilaginosos, así como soportes para huesos y regeneración de meniscos (Martin y Williams, 2003; Misra *et al.*, 2006, Chen y Wang, 2013).

Tabla 1. Aplicaciones biomédicas de P3HB con diferentes pesos moleculares (Adaptada de Peña *et al.*, 2014a)

Peso molecular promedio (kDa)	Aplicación biomédica	Referencia
144	Andamios para ingeniería de tejidos (huesos)	Ramier *et al.*, 2014
220	Andamios para crecimiento de osteoblastos	Shishatskaya *et al.*, 2006
230	Andamios para el crecimiento de células embrionarias de riñon (HEK293)	Domínguez-Díaz *et al.*, 2015
300	Microcápsulas para liberación de fármacos	da Silva-Valenzuela *et al.*, 2010
350	Nanopartículas para liberación de ácido retinoico	Errico *et al.*, 2009
890	Andamios para ingeniería de tejidos	Cao *et al.*, 2005
1143	Andamios para el crecimiento de células nerviosas	Chan *et al.*, 2014

1.1.2 Importancia del peso molecular del P3HB

Una característica que determina las propiedades fisicoquímicas y termomecánicas de cualquier polímero es el peso molecular. En el caso del P3HB, el peso molecular determina el comportamiento elástico, la resistencia mecánica y el grado de cristalinidad del material (Iwata, 2005; Kabe *et al.*, 2012; Peña *et al.*, 2014a). Esto se debe a la forma cristalina α o β que adopta el material cuando es procesado, favoreciéndose la forma β en biopolímeros con elevado peso molecular que presentan una mayor elongación y resistencia a la tracción. Por ejemplo, las fibras de P3HB con peso molecular promedio en peso (Mw) de 300 kDa presentan una resistencia a la tracción de 190 MPa y una elongación al rompimiento del 5 %; mientras que, cuando el Mw es de 5,300 kDa las fibras aumentan su resistencia a la tracción a 1,320 MPa y su elongación al rompimiento a un 58 % (Iwata, 2005; Ragan, 2007). Además, el P3HB con Mw menor a 1,000 kDa es un material frágil y presenta temperaturas de degradación térmica cercanas a su temperatura de fusión (Hong *et al.*, 2013). También la velocidad de degradación del material está determinada por el tamaño de las moléculas del biopolímero, observándose que cuando el peso molecular del P3HB es mayor, la velocidad de degradación disminuye (Bonartsev *et al.*, 2007 y 2012).

Es importante producir biopolímero con peso molecular según la aplicación final del producto, por lo que el control de esta característica durante el proceso de producción es de gran importancia, principalmente para el área biomédica (Peña *et al*., 2014a y 2015).

1.2 Microorganismos productores de polihidroxialcanoatos (PHAs)

Se han descrito más de 300 especies bacterianas capaces de acumular PHAs, siendo el P3HB el polihidroxialcanoato comúnmente sintetizado por los microorganismos. Entre las que destacan *Cupriavidus necator* (antes *Ralstonia eutropha), Alcaligenes latus, Pseudomonas oleovorans, Pseudomonas putida, Azotobacter chroococcum* y *Azotobacter vinelandii* (Choi y Lee, 1999; Peña *et al.*, 2014a y 2015).

También se han construido cepas recombinantes de *Escherichia coli* a las cuales se las han incorporado los genes de biosíntesis del P3HB, las cuales acumulan entre un 70 y un 80 % de biopolímero. Además, se ha encontrado que sintetizan P3HB con peso molecular de

hasta 20,000 kDa ± 4,000 kDa posiblemente debido a la ausencia de las enzimas encargadas de degradar el biopolímero (Kusaka *et al.*, 1997; Hiroe *et al.*, 2012).

En el caso de *A. vinelandii*, una bacteria Gram negativa que vive en el suelo, aerobia, fijadora de nitrógeno y capaz de sintetizar alginato (polisacárido extracelular), el P3HB acumulado representa hasta el 80 % del peso seco de la célula (Espín, 2002; Galindo *et al.*, 2007; García *et al.*, 2014). Es importante señalar, que hasta ahora *A. vinelandii* es el microorganismo nativo que sintetiza P3HB de ultra-alto peso molecular (Mw = 4,100 kDa) (Chen y Page, 1994).

En el genoma de *A. vinelandii* está presente el regulador transcripcional *algU*, el cual regula la expresión de genes involucrados en los procesos de respiración celular, biosíntesis de alginato y de P3HB. La inactivación del regulador *algU* en *A. vinelandii* dio origen a la cepa denominada OP, la cual es incapaz de sintetizar alginato (Martínez-Salazar *et al.*, 1996) y en cultivos en biorreactor, la acumulación de P3HB llega a ser de hasta el 80 % del peso seco de la bacteria (García *et al.*, 2014; Millán *et al.*, 2016).

1.2.1 Metabolismo del P3HB y el efecto del oxígeno disuelto sobre la síntesis del biopolímero

El ciclo del P3HB en *A. vinelandii* involucra dos etapas en las que participan una serie de enzimas que catalizan las reacciones de síntesis (primera etapa) y degradación o movilización intracelular (segunda etapa) del biopolímero (Fig.2) (Dawes, 1988; Braunegg *et al.*, 1998).

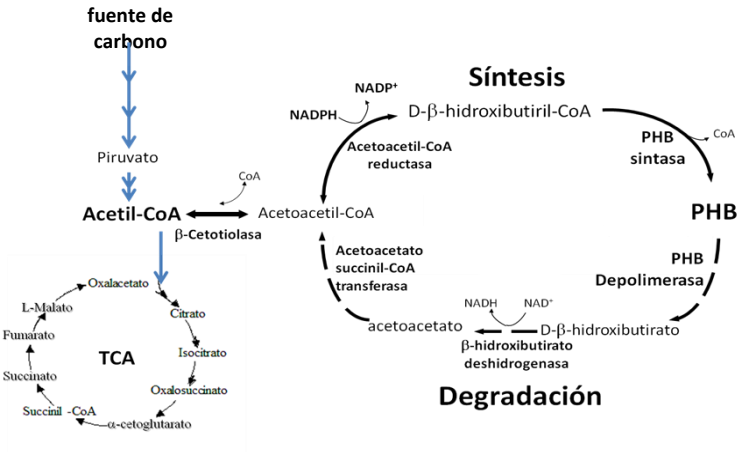


Figura 2. Ruta de biosíntesis y degradación del P3HB (Adaptado de Dawes, 1988)

1.2.1.1 Etapa de síntesis

La biosíntesis de P3HB inicia con la condensación de dos moléculas de acetil-CoA, que es catalizada por la enzima β-cetotiolasa, generando acetoacetil-CoA. Posteriormente, el acetoacetil-CoA es reducido por una acetoacetil-CoA reductasa dependiente de NADPH para producir β-hidroxibutiril-CoA. Finalmente, el β-hidroxibutiril-CoA es polimerizado por la P3HB sintasa, liberando CoA y produciéndose el P3HB (Senior y Dawes, 1973; Manchak y Page, 1994).

Los genes que codifican para estas tres enzimas de biosíntesis están incluidos en el operón *phbBAC*. Los genes *phbA*, *phbB* y *phbC* codifican para la β-cetotiolasa, la acetil-CoA reductasa y la P3HB sintasa, respectivamente (Segura *et al.*, 2000; Peralta-Gil *et al.*, 2002; Segura *et al.*, 2003).

1.2.1.2 Etapa de degradación

Cuando la fuente de carbono externa se agota, la bacteria recurre a la utilización de las reservas de P3HB, movilizando el polímero e incorporando los monómeros a su metabolismo para la producción de energía y precursores biosintéticos (Sudesh *et al.*, 2000; Steinbüchel y Hein, 2001). La degradación del P3HB inicia cuando el biopoliéster se fracciona en moléculas de 3-hidroxibutirato. Esta reacción es catalizada por la enzima P3HB depolimerasa, la cual está codificada por el gen *phbZ*. En la siguiente reacción, el 3-hidroxibutirato, es oxidado por la enzima hidroxibutirato deshidrogenasa dependiente de NAD^+, obteniéndose acetoacetato. Para finalizar, la enzima acetoacetato-succinil-CoA transferasa convierte el acetoacetato en acetoacetil-CoA, como se ilustra en la figura 2 (Dawes, 1988; Rehm, 2007). También se ha propuesto que el ciclo del P3HB regula el estado redox dentro de los microorganismos debido a que durante la etapa de síntesis del biopolímero se consume NADPH y en la etapa de degradación se libera NADH. Además se ha reportado que la síntesis y degradación son procesos simultáneos en los microorganismos que acumulan P3HB (Volova *et al.*, 2004; Ren *et al.*, 2009). Por lo tanto, nuestra hipótesis es que el peso molecular del P3HB es función de los procesos de polimerización (etapa de síntesis) y depolimerización (etapa de degradación o

movilización) que se presentan dentro de la bacteria durante la fase de acumulación del biopolímero.

1.2.1.3 Efecto del oxígeno disuelto sobre la síntesis del P3HB

Generalmente, las bacterias acumulan P3HB cuando existen condiciones de exceso de fuente de carbono en el medio y limitación de algún nutriente necesario para el crecimiento celular como nitrógeno, fósforo u oxígeno (Anderson y Dawes, 1990; Suriyamongkol *et al*., 2007; Peña *et al.,* 2015). El biopolímero es una fuente de reserva de carbono que se acumula en la célula y puede ser utilizado cuando existe escasez de la fuente de carbono externa. En el caso de las especies de *Azotobacter*, la acumulación del P3HB se presenta cuando el oxígeno disuelto en el medio limita el crecimiento de la bacteria (Senior y Dawes, 1973; Espín, 2002; Galindo *et al.,* 2007; Peña *et al.,* 2014a y 2015). En cultivos de *Azotobacter vinelandii*, la tensión de oxígeno disuelto (TOD) crítica es de 4 % de saturación (Lozano *et al*., 2011; García *et al.,* 2014), condiciones de oxígeno por debajo del valor de TOD crítica se denominan condiciones de limitación de oxígeno (Lozano *et al*., 2011). Cuando *A. vinelandii* se cultiva en condiciones de limitación de oxígeno (< 4 % TOD) se favorece la síntesis de P3HB; por el contrario, en condiciones de no limitación de oxígeno (> 4 % TOD) se favorece el crecimiento celular y disminuye la acumulación de P3HB (García *et al*., 2014; Peña *et al*., 2014b). Esto es debido a que, en condiciones de limitación de oxígeno el flujo de acetil-CoA que entra al ciclo de los ácidos tricarboxílicos (CAT) disminuye. Además, bajo estas condiciones, se producen elevadas concentraciones de NADPH, NADH y acetil-CoA, así como una baja disponibilidad de CoA libre. La acumulación de los cofactores NADH y NADPH inhibe a las enzimas citrato sintasa e isocitrato deshidrogenasa que participan en el CAT y la CoA libre regula de manera alostérica a la primera enzima (β-cetotiolasa) de la ruta de biosíntesis del P3HB, la cual incrementa su actividad cuando los niveles de CoA libre disminuyen, lo que favorece la acumulación del P3HB (Senior y Dawes, 1973; Anderson y Dawes, 1990).

1.3 Generalidades de las enzimas involucradas en el ciclo del P3HB

Los gránulos de P3HB más que simples inclusiones de biopolímero son estructuras subcelulares organizadas de una manera compleja (Jendrossek, 2009). Al parecer, están

rodeados por una gran variedad de proteínas, entre las cuales se encuentran: phasinas, depolimerasas intracelulares, proteínas regulatorias y P3HB sintasa, esta última se encuentra unida covalentemente al P3HB debido a que es necesario dicho enlace para realizar su actividad de polimerización. El biopolímero constituye el corazón del gránulo y las enzimas que lo rodean están implicadas en la formación, regulación, síntesis y movilización o degradación del P3HB (Rehm, 2003 y 2007; Jendrossek, 2009).

PHA sintasa

Esta enzima cataliza la polimerización de 3-hidroxibutiril-CoA a P3HB, liberando CoA. De acuerdo a las subunidades que las conforman y al tipo de monómeros que pueden polimerizar, estas enzimas se clasifican en cuatro clases (Tabla 2) (Rehm, 2007). En la bacteria *Cupriavidus necator* (antes *Ralstonia eutropha*), la sintasa está conformada por dos subunidades idénticas (PhaC) y la enzima solo polimeriza monómeros constituidos por 3 a 5 átomos de carbono (PHAs de cadena corta). Por el contrario, aunque las bacterias del género *Pseudomonas* presentan una sintasa conformada por dos subunidades idénticas (PhaC, pero no es la misma a la que se encuentra en *Cupriavidus necator*), la enzima polimeriza monómeros constituidos por 6 a 14 átomos de carbono (PHAs de cadena media).

Tabla 2. Clasificación de las PHAs sintasas reportadas hasta ahora (Adaptada de Rehm, 2007)

Clase	Subunidades	Especies	Sustrato
I	PhaC ~60-73 kDa	*Cupriavidus necator* **Azotobacter vinelandii**	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~≥C5)
III	PhaC ~40 kDa PhaE ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA ($3HA_{MCL}$-CoA [~C6-C8], 4HA-CoA, 5HA-CoA)
IV	PhaC ~40 kDa PhaR ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

En el caso del género *Bacillus*, la sintasa está conformada por dos subunidades diferentes (PhaC-PhaR) pero solo sintetiza PHAs de cadena corta. Por el contrario, en la bacteria *Allochromatium vinosum*, la sintasa está conformada por dos subunidades diferentes (PhaC-PhaE), pero la enzima polimeriza monómeros con 3 a 8 átomos de carbono (PHAs de cadena corta y media). *A. vinelandii* sintetiza P3HB y copolímeros (P3HB-HV) de cadena

corta (de 3 a 5 átomos de carbono), lo que indica que cuenta con una P3HB sintasa clase I similar a la enzima presente en *Cupriavidus necator* (Tabla 2).

Hasta el momento no se cuenta con la estructura tridimensional de la enzima y solo se han realizado alineamientos de las secuencias de aminoácidos de las 88 sintasas conocidas. La región N-terminal es muy variable entre las diversas clases de PHA sintasas, pero en la clase I está constituida por cerca de 100 aminoácidos. Se ha propuesto que esta región N-terminal podría determinar la especificidad de la enzima por el sustrato (Rehm, 2003 y 2007). Cuando se elimina la región N-terminal de la enzima, su actividad catalítica no es afectada, aunque mutaciones sitio específicas en dicha región permiten incrementar la actividad de la enzima. La región C-terminal constituida por cerca de 40 aminoácidos, es esencial para la actividad de la sintasa y parece ser bastante hidrofóbica lo que sugiere que esta región interactúa con el gránulo. Mutaciones en la región C-terminal originan la pérdida de la actividad de la enzima (Normi *et al.*, 2005; Zheng *et al.*, 2006; Rehm, 2007).

Diversas investigaciones han demostrado que el sitio activo de la enzima está constituido por una triada catalítica de residuos de Cisteína-Aspartato-Histidina (clase I: Cyst319-Asp480-Hys508) similar al que presentan otras esterasas (Jia *et al.*, 2000; Jendrossek, 2009). Las PHA sintasas pueden existir de manera soluble en el citoplasma y unidas covalentemente a los gránulos del biopolímero, donde presentan la mayor actividad enzimática en ensayos realizados *in vitro*. De hecho, se ha planteado que la enzima está presente en el citoplasma, mientras no haya acumulación de P3HB. Cuanto inicia la etapa de síntesis del biopolímero, la enzima unida a un monómero de 3-hidroxibutirato se dimeriza e inicia la etapa de polimerización del P3HB (Fig.3).

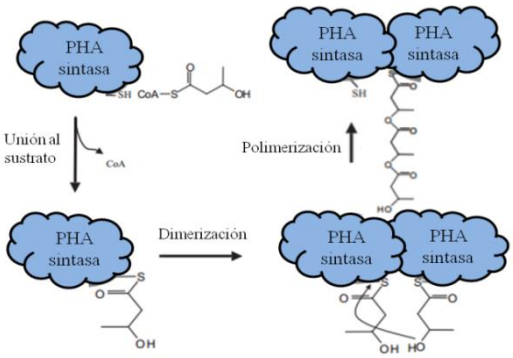


Figura 3. Modelo del mecanismo catalítico de las PHAs sintasas (Tomado de Rehm, 2007)

Recientemente se descubrió en *R. eutropha* una proteína denominada PhaM, la cual es el activador fisiológico de la P3HB sintasa, provocando su dimerización y formando un complejo enzimático conocido como sitio de nucleación, donde se lleva a cabo la formación de cadenas de polímero (Pfeiffer y Jendrossek, 2014).

β-cetotiolasa

Es la primera enzima de la vía de biosíntesis de P3HB, condensa dos moléculas de acetil-CoA para formar acetoacetil-CoA. Además cataliza la reacción de tiólisis (reacción inversa) del acetoacetil-CoA para formar dos moléculas de acetil-CoA. La reacción de condensación es inhibida en concentraciones de 10 µM de CoA o menos. Lo que sugiere que dependiendo de la disponibilidad de acetil-CoA y CoA libre en la célula, la reacción de condensación o de tiólisis pueden ocurrir, ya que la β-cetotiolasa es regulada alostericamente por CoA (Senior y Dawes, 1973).

Esta enzima se encuentra en el citoplasma, tiene un peso molecular de 190 kDa y está conformada por 4 subunidades idénticas, como se observa en el modelo de la figura 4 (Kim y Kim, 2014). Dos residuos de cisteína (Cys88 y Cys379 en *R. eutropha*) participan en la catálisis. En el primer paso de la reacción, el grupo tiol del residuo de cisteína Cys88 ataca nucleofilicamente al sustrato formando un enlace tioéster entre Cys88 y el acetil-CoA. Posteriormente, el nucleófilo Cys379 ataca el enlace tioéster formado entre Cys88-acetilCoA llevando a cabo la condensación de 2 moléculas de acetil-CoA para formar acetoacetil-CoA (Kim y Kim, 2014).

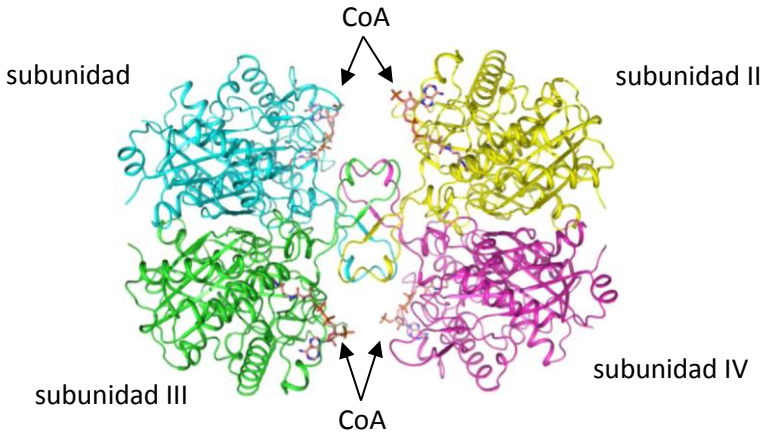


Figura 4. Modelo donde se representan las 4 subunidades que conforman a la enzima β-cetotilasa (Tomada de Kim y Kim, 2014).

Acetoacetil-CoA reductasa

Esta enzima realiza la reducción de acetoacetil-CoA a 3-hidroxibutiril-CoA y requiere del cofactor NADPH (aunque puede usar también NADH) para catalizar la reacción (Senior y Dawes, 1973). Sin embargo, se ha reportado la existencia de tres posibles reductasas (PhaB1, PhaB2 y PhaB3) en el genoma de *R. eutropha*, de las cuales una emplea NADPH y otra NADH en la reducción de acetoacetil-CoA (Budde *et al.*, 2010). Recientemente ha sido publicada la estructura cristalográfica de la acetoacetil-CoA reductasa presente en *R. eutropha* (Matsumoto *et al.*, 2013). Estos autores reportaron que la enzima funciona como un tetrámero y forma un complejo ternario junto con el acetoacetil-CoA y el $NADP^+$ (Fig.5).

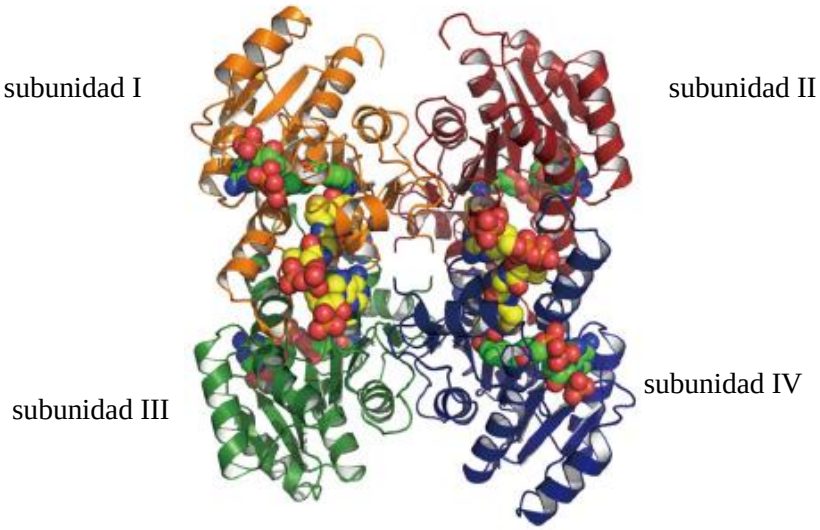


Figura 5. Modelo donde se representan las 4 subunidades que conforman a la enzima reductasa (Tomada de Matsumoto *et al*., 2013).

También reportaron dos mutaciones que ocasionaron un incremento en la actividad de la enzima debido a la modificación de la interacción de la proteína con el sustrato. Una fue la sustitución de la glutamina en la posición 47 por leucina (Gln47Leu) y la otra fue el cambio de la treonina en la posición 173 por serina (Thr173Ser). Dichas modificaciones afectaron la flexibilidad de la región que rodea al sitio activo y que tiene una función importante en el reconocimiento del sustrato.

PHA depolimerasa

Las enzimas presentes en microorganismos productores de PHAs que catalizan la depolimerización del P3HB previamente acumulado se denominan PHA o P3HB depolimerasas intracelulares y se encuentran ancladas a la superficie del gránulo (Rehm, 2007; Jendrossek, 2009). En *R. eutropha* se han reportado 7 posibles depolimerasas intracelulares y dos hidrolasas de oligómeros de 3-hidroxibutirato. Las depolimerasas intracelulares son específicas para hidrolizar biopolímero en estado nativo (amorfo) siendo incapaces de hidrolizar P3HB desnaturalizado (cristalino) (Jendrossek, 2007; Jendrossek y Pfeiffer, 2014).

También existen PHA depolimerasas extracelulares, presentes en microorganismos no productores de PHAs, pero que son capaces de degradarlo en estado desnaturalizado para usarlo como fuente de carbono y energía. Estas enzimas presentan un sitio catalítico análogo al que se encuentra en las esterasas/lipasas de serina donde la triada catalítica está integrada por serina-ácido aspártico-histidina (en *Penicillium funiculosum*: Ser39, Asp121, and His155; en *Paucimonas lemoignei*: Ser136, Asp242, y His306) (Hisano *et al.*, 2006; Papageorgiou *et al.*, 2008). También existen otras proteínas asociadas a los gránulos de P3HB llamadas phasinas. Hasta ahora, en *Ralstonia eutropha* se han reportado 7 phasinas, de las cuales PhaP1 es la proteína más abundante presente en los gránulos (Pfeiffer y Jendrossek, 2014). Aunque la función de estas phasinas no es del todo conocida, se ha propuesto que regulan el número y tamaño de los gránulos de P3HB, entre otras funciones. Por ejemplo, en *R. eutropha* una proteína (PhaM) con características similares a las phasinas que presenta sitios de unión al DNA, está implicada en la segregación de los gránulos de bioplástico, para garantizar la distribución uniforme de los gránulos entre la célula madre e hija durante la duplicación celular (Wahl *et al.*, 2012).

1.4 Influencia de la síntesis y degradación sobre el peso molecular del P3HB

En la literatura existen pocos trabajos sobre el estudio de los procesos de polimerización y depolimerización del P3HB en microorganismos que acumulan estos biopolímeros.

En 1997, Kusaka y colaboradores, utilizando una cepa recombinante de *Escherichia coli* (que tiene los genes de biosíntesis del polímero de *Ralstonia eutropha*), lograron obtener un bioplástico con peso molecular de 20,000 ± 4,000 kDa, el más alto reportado hasta ahora. Sin embargo, no realizaron estudios para determinar las causas y atribuyeron el resultado, a la ausencia en la cepa recombinante de un "factor de transferencia o terminación de la cadena" de P3HB, que estaría presente en *R. eutropha* y regularía el tamaño de las cadenas poliméricas. Sin embargo, no consideraron la posibilidad de que la falta de enzimas P3HB depolimerasas en *E. coli* haya favorecido la síntesis de P3HB de elevado peso molecular.

Volova y colaboradores (2004) fueron los primeros que determinaron la actividad de las enzimas del ciclo del P3HB en cultivos de *R. eutropha*. Encontraron que la actividad de las enzimas de síntesis (cetotiolasa, reductasa y sintasa) y degradación (deshidrogenasa y depolimerasa) fue mayor durante la etapa de acumulación del P3HB (la cual correspondió con la fase de crecimiento exponencial); disminuyendo cuando finalizó la acumulación y permaneciendo sin cambios hasta el final del cultivo. Sin embargo, no evaluaron el peso molecular del P3HB producido en los cultivos.

Para comprender de que forma el peso molecular del P3HB es afectado por las enzimas participantes en el metabolismo del biopolímero, Song y colaboradores (2012), empleando una cepa de *Methylosinus trichosporium* IMV3011 encontraron que el peso molecular del P3HB se incrementó durante la fase de crecimiento de la bacteria (Mw = 1,400 kDa: 4 días; Mw = 1,700 kDa: de 6 a 16 días). Además, observaron que el incremento del peso molecular estuvo asociado a una mayor actividad sintasa y depolimerasa así como a una menor actividad β-cetotiolasa y reductasa.

Por su parte, Hiroe y colaboradores (2012) estudiaron el efecto del rearreglo de los genes del operón de biosíntesis *phbCAB* de *R. eutropha* sobre el peso molecular del P3HB producido en una cepa de *E. coli* recombinante. El orden de transcripción afectó los niveles de expresión relativa de los genes de las enzimas de la biosíntesis del P3HB. Cuando la expresión del gen de la P3HB sintasa y su actividad fueron menores, se favoreció la síntesis de P3HB de elevado peso molecular promedio (Mw) entre 5,400 y 6,200 kDa), mientras que cuando la expresión del gen de la P3HB sintasa y su actividad fueron mayores, el Mw disminuyó (entre 3,400 y 4,200 kDa). Además, encontraron que una mayor actividad P3HB

sintasa aumentó la acumulación de P3HB en la célula, pero disminuyó el Mw del polímero acumulado. En contraste, una baja actividad sintasa disminuyó la acumulación de P3HB, pero aumentó el Mw del P3HB (Hiroe *et al.*, 2012).

Recientemente, Volova y colaboradores (2013) realizaron cultivos de *R. eutropha* y encontraron que el peso molecular del P3HB se incrementó durante la fase exponencial de crecimiento de 680 kDa, a las 16 h, a 967 kDa, a las 40 h de cultivo. Posteriormente, el peso molecular se redujo a 358 kDa a tiempos finales del cultivo (104 h) y no se observó una correlación con la actividad de las enzimas del ciclo del P3HB; ya que la actividad de las enzimas de biosíntesis y degradación disminuyó con respecto al tiempo de cultivo (Volova *et al.*, 2013).

Los resultados reportados hasta ahora no indican una correlación clara entre la actividad de las enzimas que participan en el ciclo del P3HB, a excepción de la actividad sintasa en cepas de *E. coli* recombinante donde se observó que una mayor actividad P3HB sintasa favorece la síntesis de una mayor cantidad de P3HB de bajo peso molecular (Hiroe *et al.*, 2012). Además, dichos resultados parecen ser específicos para cada microorganismo empleado y para las condiciones de cultivo evaluadas.

Hasta ahora no se han realizado estudios con cepas de *A. vinelandii*, un microorganismo capaz de producir P3HB de ultra-alto peso molecular (Chen y Page, 1994). En el genoma de *A. vinelandii*, mediante análisis bioinformáticos se han identificado 7 genes que codifican para posibles depolimerasas (Yanajara, 2015; Adaya *et al.*, 2017). Una de estas P3HB depolimerasas esta codificada por el gen *Avin03910*, y cuando se inactivó esta enzima en una cepa de *A. vinelandii* se obtuvo (en cajas Petri) un fenotipo "blanquecino", debido a una mayor acumulación de P3HB dentro de la célula (Yanajara, 2015). Sin embargo, no se ha estudiado la función de estas depolimerasas ni se ha evaluado el efecto que tendría la inactivación de estas enzimas en *A. vinelandii* (Adaya *et al.*, 2017) y su efecto sobre el Mw del P3HB producido en cultivos en biorreactor.

Por lo que el presente proyecto tiene como objetivo estudiar el efecto de los fenómenos de síntesis y degradación del poli-3-hidroxibutirato sobre el peso molecular del biopolímero acumulado por una cepa de *A. vinelandii* denominada OP (incapaz de sintetizar alginato) y

la cepa mutante de *A. vinelandii* denominada *phbZ*$^{-}$ (que tiene inactivado el gen *Avin03910*) bajo condiciones controladas de oxígeno disuelto en biorreactores agitados.

2. HIPÓTESIS

Hipótesis

En cultivos lote de *A. vinelandii* OP bajo condiciones controladas de oxígeno disuelto, la síntesis de P3HB de elevado peso molecular se presenta cuando existen condiciones de una alta actividad sintasa y una baja actividad depolimerasa.

3. OBJETIVOS

3.1 Objetivo general

Estudiar los procesos de polimerización y depolimerización del P3HB en cultivos de *A. vinelandii* bajo condiciones controladas de oxígeno disuelto en biorreactores agitados.

3.2 Objetivos específicos

- Estudiar en cultivos en biorreactor bajo condiciones controladas de limitación (1 %) y no limitación de oxígeno disuelto (15 %) los cambios en el peso molecular del P3HB producido por la cepa OP de *A. vinelandii*.
- Analizar la actividad de las enzimas que participan en la biosíntesis y degradación del P3HB en cultivos con la cepa OP *A. vinelandii* en condiciones de limitación de oxígeno.
- Analizar la actividad de las enzimas que participan en la biosíntesis del P3HB en cultivos de *A. vinelandii* utilizando una cepa mutante denominada *phbZ*$^{-}$.
- Estudiar los fenómenos de polimerización y depolimerización del P3HB que ocurren en *A. vinelandii* y su efecto sobre el peso molecular del biopolímero.

4. ESTRATEGIA EXPERIMENTAL

En la figura 6 se presenta un diagrama de la estrategia experimental llevada a cabo durante el desarrollo de este trabajo.

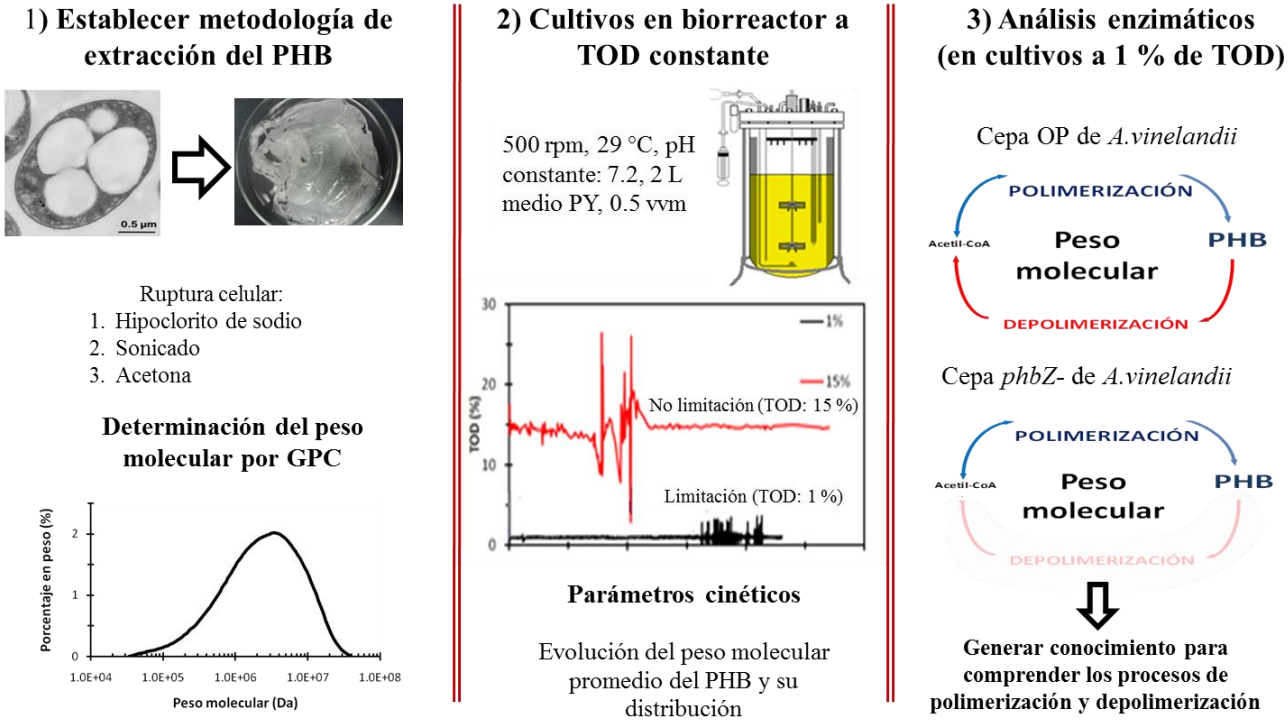


Figura 6. Estrategia experimental propuesta.

Durante la primera etapa del proyecto, se evaluaron tres métodos de ruptura celular empleando hipoclorito de sodio, acetona y sonicación. Esto para asegurarnos de contar con una técnica que ocasionara el menor daño posible al biopolímero y así garantizar que las diferencias observadas en términos del peso molecular fueran resultado de la condición de cultivo evaluada y no debido a un efecto del método de ruptura celular empleado en la extracción del P3HB. Además, se estableció la técnica de cromatografía de permeación en gel (GPC) para determinar el peso molecular del biopolímero. Esta técnica de análisis de pesos moleculares es una herramienta que nos permite obtener información completa sobre la distribución de pesos moleculares de una muestra y su polidispersión.

Posteriormente, se realizaron cultivos en biorreactor en condiciones controladas de tensión de oxígeno disuelto (TOD) en la zona de limitación (1 % de saturación) y no limitación de oxígeno disuelto (15 %). En cada condición de TOD evaluada, se realizó la cuantificación de proteína, biomasa, P3HB, consumo de sacarosa, y peso molecular del biopolímero. En la tercera etapa del proyecto se realizaron cultivos en condiciones de limitación de oxígeno disuelto (1 % de TOD) utilizando la cepa OP de *A. vinelandii* y una cepa mutante derivada de la cepa OP denominada *phbZ*$^{-}$, la cual tiene una mutación en el gen de una P3HB depolimerasa (gen: *Avin03910*). También se realizaron análisis enzimáticos para cuantificar la actividad de las enzimas de síntesis (β-cetotiolasa, acetoacetil-CoA reductasa y P3HB sintasa) y de degradación (P3HB depolimerasas) en muestras de los cultivos de ambas cepas. La información obtenida nos permitió entender de qué manera la actividad de las enzimas del ciclo del P3HB favorecen la polimerización y depolimerización del biopolímero y como el peso molecular del biopolímero se modificó en cultivos de *A. vinelandii*.

5. MATERIALES Y MÉTODOS

5.1 Mantenimiento de la cepa, medio de cultivo y preparación del inóculo

Se utilizaron las cepas OP (mutante espontánea no productora de alginato) de *A. vinelandii* y *phbZ*$^-$ (la cual tiene inactivado en gen *Avin03910* que codifica para una P3HB depolimerasa). Las cepas se cultivaron en medio PY, el cual contiene sacarosa (20 g L^{-1}), extracto de levadura (3 g L^{-1}) y peptona (5 g L^{-1}). Para los cultivos en cajas Petri (medio sólido), se adicionaron 18 g/L de agar bacteriológico al medio y el medio fue suplementado con 15 µg mL^{-1} de ácido nalidíxico para la cepa *A. vinelandii* OP. Para el caso de la cepa mutante de *A. vinelandii phbZ*$^-$ al medio sólido se adicionaron 15 µg mL^{-1} de ácido nalidíxico y 1 µg mL^{-1} de gentamicina. Las cepas fueron conservadas en tubos inclinados (*slants*) a 4° C, llevando a cabo su resiembra cada 30-45 días.

El inóculo se cultivó en matraces Erlenmeyer de 500 mL, conteniendo 100 mL de medio PY. A cada matraz se transfirieron 2 asadas de células previamente crecidas en cajas Petri incubadas a 29 °C durante 72 h. El inóculo se utilizó cuando alcanzó una densidad óptica entre 0.16-0.18 (determinada a 540 nm y aplicando una dilución 1/50), generalmente después de 20-24 h de incubación a 29 °C y 200 rpm. Entonces, el caldo de cultivo fue centrifugado a 12,860g durante 10 minutos y el paquete celular fue resuspendido en medio fresco para inocular el biorreactor y evitar que componentes del medio de cultivo agotado pudieran influir sobre las cinéticas de cultivo.

5.2 Cultivos lote en biorreactor

Los cultivos en lote se realizaron en un biorreactor Applikon (Holland) de 3 L, con un volumen de trabajo de 2 L, equipado con dos turbinas Rushton (diámetro impulsor/diámetro tanque = 0.35), de 6 paletas planas y un difusor de 7 orificios para efectuar aireación por burbujeo. El pH se controló a 7.2 ± 0.1 de forma *on-off*, adicionando automáticamente NaOH 2N durante la fase exponencial de crecimiento o HCl 2M en la fase estacionaria del cultivo. La tensión de oxígeno disuelto (TOD) fue determinada mediante un electrodo polarográfico de oxígeno (Metter-Toledo, Columbus OH). La señal fue transmitida a un amplificador de oxígeno (Ingold 2300, Columbus OH) y adquirida por una PC con la ayuda del software DataQ. Los cultivos se realizaron a 29 °C utilizando una

velocidad de agitación de 500 rpm y manteniendo la TOD constante en 1 % (1.09 ± 0.19) y 15 % (14.3 ± 2.6), de saturación mediante mezcla de gases. El flujo de entrada de la mezcla de gases (oxígeno y nitrógeno) fue de 1 L min^{-1}, y el porcentaje máximo de apertura del controlador de flujo másico de oxígeno fue 14 % para el cultivo de 1 % y 100 % para los cultivos desarrollados a 15 % de TOD.

5.3 Métodos analíticos

5.3.1 Determinación de biomasa por peso seco

La determinación de la concentración de biomasa se llevó a cabo mediante el método gravimétrico de peso seco. Para cada muestra se tomó una alícuota de 10 mL de caldo de cultivo y se centrifugó a 9,660g durante 10 minutos en una centrífuga marca Eppendorf (modelo 5804). Se separó el sobrenadante y el paquete celular fue filtrado al vacío a través de membranas *Millipore* de 0.22 μm de tamaño de poro, previamente pesadas. Las membranas con muestra se secaron por 24 h a 70 °C y posteriormente, se colocaron en un desecador hasta obtener peso constante. La cantidad de biomasa se obtuvo por diferencia de peso, expresada en g L^{-1} de medio de cultivo.

5.3.2 Determinación de proteína

El análisis de proteína se llevó a cabo mediante el método de Lowry *et al.,* (1951). En la reacción se genera un color azul cuya densidad óptica determinada a 625 nm es proporcional a la cantidad de proteína presente.

Soluciones: A) Na_2CO_3 2 % en NaOH 0.1 N; **B)** Tartrato de Na y K 2 % **C)** $CuSO_4$ 1%

Solución reactiva: Se preparó mezclando 1 mL de la solución C, 1 mL de la solución B y 98 mL de la solución A.

Reactivo de Folin-Ciocalteu: Se realizó una dilución 1:2 con agua destilada.

El procedimiento fue el siguiente: Se tomó 1 mL de la muestra del caldo de cultivo y se centrifugó a 9,660g durante 10 minutos. Entonces, el precipitado se suspendió en agua, aplicando una dilución de acuerdo a la cantidad de muestra. Una vez realizada la dilución adecuada, se tomaron 200 μL de la muestra y se adicionó 1 mL de la solución reactiva, se

agitó y dejó reposar por 10 minutos. Después, se añadieron 100 µL del reactivo de Folin-Ciocalteu (reactivo comercial, marca Sigma-Aldrich, no catálogo: F9252), se agitó y se dejó reposar en oscuridad por 30 minutos. Posteriormente, las muestras se centrifugaron por 5 minutos, para evitar la interferencia de sólidos disueltos (gránulos de P3HB) en la medición. Finalmente, se determinó la absorbancia a 625 nm en un espectrofotómetro (Genesys 10S UV-VIS marca Thermo Scientific) contra un blanco constituido de la mezcla de reactivos y utilizando como muestra agua destilada. Se realizó una curva patrón (Fig.7) utilizando albúmina bovina (marca Sigma-Aldrich), con concentraciones de 0.1, 0.2, 0.4, 0.6, 0.8 y 1.0 g/L.

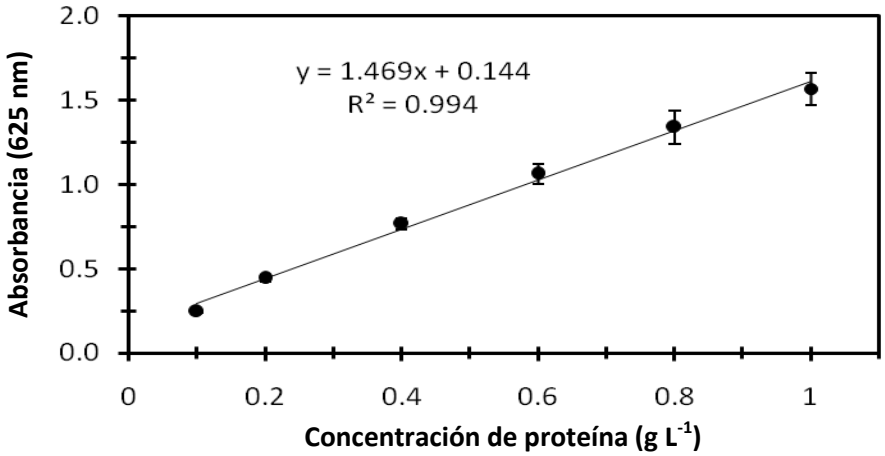


Figura 7. Curva estándar de proteína empleada para la cuantificación de la concentración de proteína.

Para realizar la cuantificación, se empleó la siguiente ecuación:

$$Proteína\ \left(\frac{g}{L}\right) = \left(\frac{Absorbancia - 0.144}{1.469}\right) * Dilución$$

5.3.3 Determinación de sacarosa por el método de β-fructofuranosidasa-DNS

El método se basa en la hidrólisis de la sacarosa usando la enzima invertasa y la posterior medición de los azúcares reductores libres por la reducción del ácido 3,5 dinitrosalicílico (DNS). En la reacción se forma un compuesto nitro-aminado colorido (amarillo), cuya

densidad óptica medida a 540 nm es proporcional a la concentración de grupos reductores (Miller, 1959).

Solución de DNS: Se preparó pesando 16 g de NaOH, 300 g de tartrato de sodio y 10 g de DNS, y disolviendo en 1 L de agua destilada. Nota: se adicionó lentamente el DNS para evitar su precipitación, de ser necesario se calentó la solución.

Buffer de citratos pH 4.6: Se preparó disolviendo 0.197 g de ácido cítrico en 50 mL de agua destilada. De forma independiente, se disolvieron 9.1 g de citrato de sodio en 50 mL de agua destilada. Posteriormente, se adicionó gota a gota el citrato de sodio al ácido cítrico hasta alcanzar un pH de 4.6.

Solución enzimática: Se mezclaron 2.5 mg de la enzima β-fructofuranosidasa (de Bakers Yeast, marca Sigma-Aldrich, no. cat. I-4504) con 1 mL de buffer de citratos (0.35 M, pH 4.6). Se tomó 1 mL del caldo de fermentación y se realizó una dilución 1/10-1/30 con agua destilada, para obtener lecturas de absorbancia dentro del intervalo de linealidad de la curva estándar de sacarosa (Fig.8).

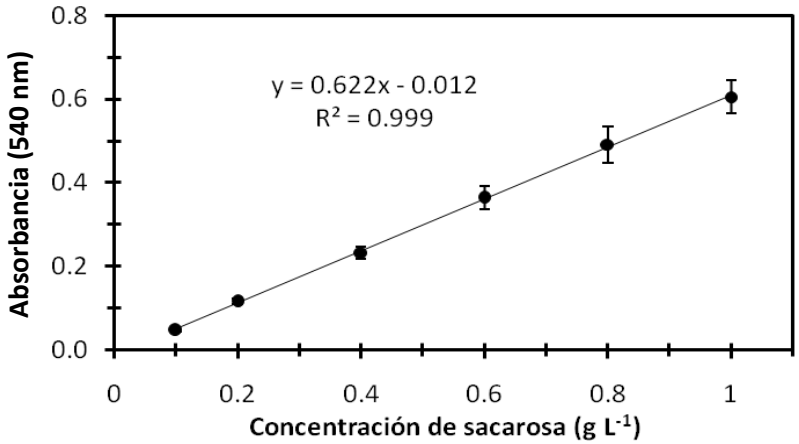


Figura 8. Curva estándar de sacarosa empleada para la cuantificación de la concentración de azúcares reductores.

Se colocó 0.9 mL de muestra en tubos de ensayo de vidrio y se mezcló con 0.1 mL de la solución enzimática. La mezcla se incubó a temperatura ambiente durante 10 minutos y, posteriormente, se adicionó 1 mL de la solución de DNS a cada muestra. Los tubos se colocaron en agua en ebullición durante 5 minutos, e inmediatamente después se enfriaron

en baño de hielo. A cada tubo se le adicionaron 10 mL de agua destilada. Finalmente se determinó la absorbancia a 540 nm en un espectrofotómetro (Genesys 10S UV-VIS, marca Thermo Scientific) contra un blanco constituido de la mezcla de reactivos y utilizando como muestra agua destilada.

El cálculo de la concentración de sacarosa se realizó con la siguiente ecuación:

$$Sacarosa\ \left(\frac{g}{L}\right) = \left(\frac{Absorbancia + 0.012}{0.622}\right) * Dilución$$

5.3.4 Cuantificación del contenido de P3HB en la biomasa

La cuantificación de P3HB se determinó mediante la conversión a ácido crotónico por tratamiento con H_2SO_4 concentrado (Law y Slepecky, 1961). Las muestras se analizaron por HPLC empleando una columna Aminex HPX-87H (Biorad), utilizando como fase móvil H_2SO_4 0.014 N, un flujo de 0.65 mL/min y una temperatura de análisis de 50 °C. El detector usado fue de arreglo de diodos (Waters 2996).

Se tomaron entre 2-3 mg de biomasa seca y se les adicionó 1 mL de H_2SO_4 concentrado. La mezcla se incubó a 90 °C y 700 rpm durante 1 h. Las muestras se dejaron enfriar y posteriormente se realizó una dilución 1/50 con agua MilliQ y se inyectaron 20 µL de la muestra al HPLC. El área bajo la curva del cromatograma se cuantificó a 220 nm y se empleó la siguiente ecuación, obtenida de soluciones de P3HB comercial a concentraciones conocidas entre 0.01-0.1 mg mL^{-1} (Castillo *et al.*, 2013).

$$PHB(\%) = \left[\frac{\left(\frac{Area\ bajo\ la\ curva + 7{,}600}{158{,}848{,}710}\right)}{Biomasa\ utilizada} * Dilución\right] * 100$$

5.3.5 Extracción del P3HB

Debido a que el P3HB se acumula intracelularmente en las células de *A. vinelandii*, es necesario llevar a cabo su extracción utilizando solventes orgánicos (acetona, cloroformo)

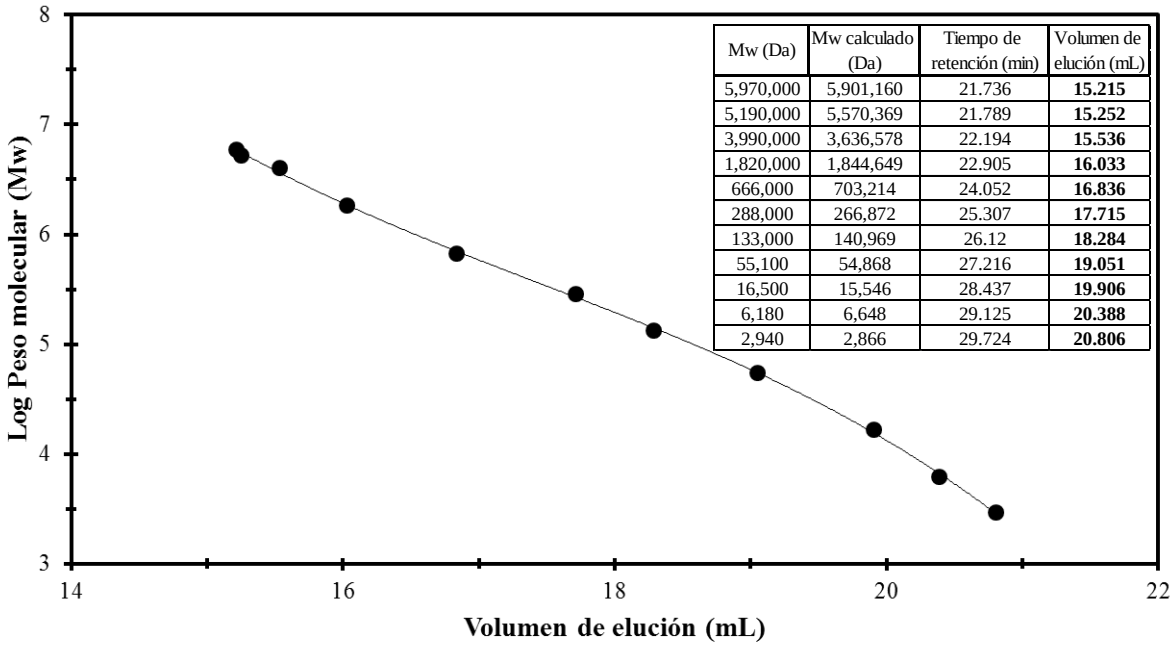
(Kunasundari y Sudesh, 2011). La metodología empleada es la descrita por Hahn *et al.*, (1994), con algunas modificaciones, el procedimiento fue el siguiente:

Se recuperó por centrifugación la biomasa celular contenida en 3 o 6 mL de muestra, dependiendo de su concentración (0.5-2 g L^{-1}). Posteriormente, la biomasa se lavó con 1 mL de agua destilada, se resuspendió y nuevamente se centrifugó a 9,660g por 10 minutos. Se adicionó 1 mL de acetona y el paquete celular se resuspendió en el solvente, dejando en contacto durante 5 minutos. Después de centrifugar a 9,660g por 5 minutos, la acetona se desechó y se adicionaron 2 mL de cloroformo para solubilizar el P3HB, dejando en contacto durante 20-24 h a temperatura ambiente. El P3HB disuelto en cloroformo se purificó mediante precipitación con 7 volúmenes de metanol. Posteriormente, la muestra se centrifugó y el precipitado se disolvió en cloroformo para los análisis de peso molecular.

5.3.6 Determinación del peso molecular del P3HB

La distribución de pesos moleculares se determinó por cromatografía de permeación en gel (GPC), usando dos columnas (Styragel HR 6 y Styragel HR 5E) conectadas en serie. Dichas columnas nos permiten analizar muestras de P3HB con pesos moleculares desde 2,000 hasta 10,000,000 de Dalton (Da). Dicho arreglo en serie se acopló a un equipo de HPLC (Waters Alliance 2695) con un detector de índice de refracción (Waters, 2414). Las condiciones de operación fueron: 30 °C y 0.7 mL min^{-1} de cloroformo como fase móvil. El volumen de inyección fue de 50 μL y el tiempo de corrida de 45 minutos. Se utilizaron estándares de poliestireno para la construcción de la curva de calibración con pesos moleculares entre 2.9×10^3 - 5.97×10^6 Da. Las muestras se prepararon a una concentración de 1-2 mg mL^{-1} y se disolvieron entre 12-18 h antes de su análisis. Cada muestra se filtró con membranas de 0.45 μm y se colocó en viales (Millán *et al.*, 2016).

Se utilizó el software *Empower* para el procesamiento y cuantificación de las muestras y la curva de calibración generada se presenta en la figura 9.



Mw (Da)	Mw calculado (Da)	Tiempo de retención (min)	Volumen de elución (mL)
5,970,000	5,901,160	21.736	**15.215**
5,190,000	5,570,369	21.789	**15.252**
3,990,000	3,636,578	22.194	**15.536**
1,820,000	1,844,649	22.905	**16.033**
666,000	703,214	24.052	**16.836**
288,000	266,872	25.307	**17.715**
133,000	140,969	26.12	**18.284**
55,100	54,868	27.216	**19.051**
16,500	15,546	28.437	**19.906**
6,180	6,648	29.125	**20.388**
2,940	2,866	29.724	**20.806**

Figura 9. Curva de calibración utilizada para la cuantificación del peso molecular de las muestras de P3HB empleando el software *Empower*.

El estándar con peso molecular de 5,970,000 Da corresponde a un volumen de elución de 15.21 mL, correspondiente a 21.73 minutos de corrida; mientras que, el de menor peso molecular (2,940 Da) presenta un volumen de elución de 20.8 mL, que corresponde a un tiempo de retención de 29.72 minutos.

Con la curva de calibración se obtuvo la siguiente ecuación que se empleó para estimar el peso molecular del P3HB dependiendo del volumen de retención:

$$\log Peso\ Molecular = 86.63 - 12.97V + 0.72V^2 - 0.0136V^3$$

Donde:

V: volumen de elución de la muestra (mL)

El error de la técnica cromatográfica fue menor al 5 %, y se incrementó a valores cercanos al 9 % cuando las columnas empezaron a saturarse y la presión del sistema se incrementó.

5.3.7 Obtención del extracto libre de células para análisis enzimáticos

Antes de la ruptura celular, las células fueron suspendidas en buffer de fosfatos 25 mM con sales pH 7.2.

Tabla 3. Composición del buffer de lisis utilizado para la ruptura celular.

Reactivo	**Concentración (g/L)**
KH_2PO_4	1.715
K_2HPO_4	2.160
NaCl (137mM)	8.0
KCl (20 mM)	1.491
EDTA (1 mM)	0.0372

Procedimiento: Las células almacenadas a -20 °C se resuspendieron en buffer de lisis (densidad óptica a 540 nm: 2)

Las células se rompieron por sonicación (3 ciclos de 15 s a 5-6 W de potencia). Posteriormente, se centrifugaron a 14,500g durante 10 minutos a 4°C y se recuperó el extracto libre de células (sobrenadante). Inmediatamente, el sobrenadante se mantuvo en hielo y se empleó para determinar la concentración de proteína y realizar los análisis enzimáticos.

5.3.8 Análisis enzimáticos

Se determinó la actividad de las enzimas involucradas en la síntesis (β-cetotiolasa, acetoacetil-CoA reductasa, P3HB sintasa) y degradación (depolimerasa) del P3HB. Las mediciones se realizaron de acuerdo a las técnicas previamente reportadas (Senior y Dawes, 1973; Segura *et al.*, 2000; Wu *et al.*, 2001; Volova *et al.*, 2004; Song *et al.*, 2012; Volova *et al.*, 2013).

β-cetotiolasa

La actividad de la enzima fue determinada mediante la reacción de tiólisis del acetoacetil-CoA donde se midió la disminución de la absorbancia a 303 nm debido al decremento de la concentración del complejo enol formado por el Mg^{2+} y el acetoacetil-CoA. El coeficiente de extinción molar del acetoacetil-CoA es de 17,260 $M^{-1}cm^{-1}$(Senior y Dawes, 1973; Segura *et al.*, 2000; Volova *et al.*, 2004).

La reacción de realizó en un buffer de fosfatos 25 mM, pH 7.8 a 29 °C en una celda de cuarzo con un volumen de trabajo de 1 mL. La celda de referencia contenía todos los componentes de reacción, excepto el cofactor coenzima A (CoA). La unidad fue definida como la actividad de conversión de 1 µmol de acetoacetil-CoA por minuto por mg de proteína.

Tabla 4. Concentración de los reactivos en la mezcla de reacción utilizada para medir la actividad de la enzima β-cetotiolasa.

Reactivo	**Concentración**
KH_2PO_4	25 mM
K_2HPO_4	25 mM
$MgCl_2*6H_2O$	40 mM
Ditiotreitol (DTT)	1 mM
Coenzima A	43.5 µM
Acetoacetil-CoA	30 µM
pH	7.8

Acetoacetil-CoA reductasa

La determinación de la actividad reductasa se realizó empleando un buffer de fosfatos 100 mM, pH 5.5 a 29°C en un mililitro de volumen de reacción como ha sido reportado previamente (Senior y Dawes, 1973; Volova *et al.*, 2004; Song *et al.*, 2012).

Tabla 5. Concentración de los componentes de reacción para medir la actividad de la enzima acetoacetil-coA reductasa.

Reactivo	**Concentración**
KH_2PO_4	100 mM
K_2HPO_4	100 mM
$MgCl_2*6H_2O$	12 µM
Ditiotreitol (DTT)	0.5 mM
NADPH	0.24 mM
Acetoacetil-CoA	20 µM
pH	5.5

La actividad fue determinada a través de la reacción de oxidación del NADPH, mediante mediciones de la disminución de la absorbancia a 340 nm y empleando un coeficiente de

extinción molar de 6,220 $M^{-1}cm^{-1}$. La celda de referencia contenía todos los componentes de reacción excepto el sustrato acetoacetil-CoA y la reacción inició al momento de adicionar el NADPH. La unidad fue definida como la actividad para reducir 1 µmol de acetoacetil-CoA por minuto por mg de proteína.

P3HB sintasa

La actividad de la enzima P3HB sintasa se determinó mediante la medición espectrofotométrica del ácido tionitrobenzoico (TNB). En la condensación de dos moléculas del monómero 3-hidroxibutiril-CoA, se libera CoA la cual reacciona con el 5,5`-ditio-bis-(2-ácido nitrobenzoico) (DTNB) y se produce el anión TNB de manera proporcional a la cantidad de CoA liberada. El coeficiente de extinción molar del TNB es de 13,600 $M^{-1}cm^{-1}$ a una longitud de onda de 412 nm (Burns *et al.*, 2007; Hiroe *et al.*, 2012; 2013). La reacción se realizó en una celda de cuarzo con 1 mL de buffer de fosfatos 25 mM a pH 7.0 y a 29 °C. La concentración final de los reactivos en la celda fue la siguiente:

Tabla 6. Concentración de los componentes de reacción para medir la actividad de la enzima P3HB sintasa.

Reactivo	Concentración
KH_2PO_4	25 mM
K_2HPO_4	25 mM
DNTB	0.1 mM
β-hidroxibutiril-CoA	55 µM
pH	7.0

La celda de referencia contenía todos los componentes de reacción excepto el sustrato β-hidroxibutiril-CoA y la reacción dio inicio al momento de adicionar el sustrato en la celda. La actividad de la enzima P3HB sintasa se midió tanto en la fracción soluble (sobrenadante) como en la fracción insoluble (enzima asociada a los gránulos de polímero). La unidad fue definida como la actividad requerida para polimerizar 1 µmol del monómero β-hidroxibutiril-CoA por minuto por mg de proteína.

P3HB depolimerasa (Volova *et al.*, 2013)

Para la determinación de la actividad P3HB depolimerasa fue necesario la recuperación de gránulos de biopolímero en estado nativo, debido a que la enzima presente en *A. vinelandii* solo es capaz de degradar cadenas de P3HB en estado amorfo (Jendrossek, 2007).

Recuperación de gránulos nativos

Las muestras de cultivo fueron almacenadas a -20 °C hasta su resuspensión en el buffer de lisis.

Tabla 7. Concentración de los componentes de reacción para medir la actividad de la enzima P3HB depolimerasa.

Reactivo	Concentración
NaH_2PO_4	50 mM
NaCl	300 mM
Imidazol	10 mM
Complete (inhibidor de proteasas)	1x
pH	8.0

La ruptura celular se realizó en una prensa French a 900 psi de presión máxima para evitar severos daños a la depolimerasa, la cual se encuentra anclada a los gránulos de biopolímero. El complejo gránulo-enzima (P3HB-depolimerasa) se empleó en la determinación de la actividad de la enzima.

Posteriormente, los gránulos de P3HB fueron purificados por ultracentrifugación (71,000g, 44 minutos a 4°C) en un gradiente de glicerol-Tris HCl 50 mM, pH 7.5 (87-40 % v/v). Los gránulos fueron recuperados de la interfase entre los gradientes de 80 y 60 % de glicerol. En seguida, la suspensión de los gránulos en el glicerol fue dializada durante 12 h a 4 °C en una membrana de diálisis (Spectra/Por 7) con tamaño de poro de 1,000 Da en buffer Tris-HCl 50 mM, pH 8.0 con agitación para acelerar la remoción del glicerol de la muestras.

Finalmente, se preparó una suspensión de gránulos (con una densidad óptica cercana a 1.0 medida a 600 nm) en el mismo buffer de diálisis para cada muestra. La medición de la actividad se realizó en una celda de 3 mL con 1.6 mL de volumen de trabajo, la cual se incubó a 37 °C y con agitación magnética. Para determinar la concentración de P3HB en la mezcla de reacción contenida en la celda, se midió la absorbancia a 600 nm a diferentes tiempos de reacción. Los valores de absorbancia se extrapolaron a una curva de calibración, la cual se construyó preparando soluciones de gránulos de P3HB a diferentes valores de

densidad óptica y se les determinó la concentración de P3HB por HPLC. La unidad fue definida como la actividad requerida para degradar 1 µg de P3HB por minuto por mg de proteína (Jendrossek, 2007; Volova *et al.*, 2013).

6. RESULTADOS Y DISCUSIÓN

6.1 Cinéticas de cultivos de *A. vinelandii* cepa OP en biorreactor bajo condiciones limitantes y no limitantes de oxígeno disuelto

Con el objetivo de conocer el comportamiento de *A. vinelandii* OP en cultivos en biorreactor, la bacteria se cultivó bajo condiciones controladas de tensión de oxígeno disuelto (TOD). Los cultivos se realizaron por triplicado en condiciones de TOD limitantes (1.09 ± 0.19 % de TOD) y no limitantes (14.7 ± 2.6 % de TOD), con control de pH y a una velocidad de agitación de 500 rpm. El control de la TOD se realizó a través de controladores de flujo másico que permitieron manipular la presión parcial de oxígeno en el sistema. En las cinéticas de crecimiento celular, producción de P3HB y consumo de sacarosa se observaron diferencias entre los cultivos realizados en condiciones de limitación (TOD ~ 1.0 %) y los realizados en condiciones de no limitación (TOD ~ 15 %) de oxígeno disuelto (Fig.10). En condiciones de limitación de oxígeno, el crecimiento celular fue 50 % menor, comparado con el observado en condiciones de no limitación de oxígeno (Fig. 10a). Este comportamiento fue similar cuando se cuantificó el crecimiento celular con base en la concentración de la biomasa residual (biomasa total menos P3HB). A 1 % de TOD, la velocidad específica de crecimiento (μ) fue de 0.08 h^{-1}, mientras que a 15 % de TOD, la μ fue de 0.18 h^{1} (Tabla 8). Respecto a la concentración y acumulación de P3HB, a 1 % de TOD se favorecieron tanto la concentración (2.4 g L^{-1}) como la acumulación (80 % del peso seco de la biomasa total) del polímero; mientras que a 15 % de TOD la concentración fue de 0.8 g L^{-1} y la acumulación de P3HB fue del 45 % (Fig. 10b-c) (Millán *et al.,* 2016). En términos del crecimiento celular y producción de P3HB, el comportamiento fue similar al observado para diversas cepas de *A. vinelandii* cultivadas en matraces y biorreactores agitados en condiciones controladas y no controladas de TOD, donde se ha reportado que en condiciones de mayor oxígeno se favorece el crecimiento de la bacteria con decremento en la acumulación del biopolímero y viceversa (Senior y Dawes, 1973; García *et al*., 2014; Peña *et al*., 2014b).

Bajo condiciones limitantes de TOD (1 %), la bacteria consumió solo el 40 % de la sacarosa disponible; mientras que, en los cultivos a 15 % de TOD la sacarosa que se consumió fue del 97 % al finalizar el cultivo (48 h).

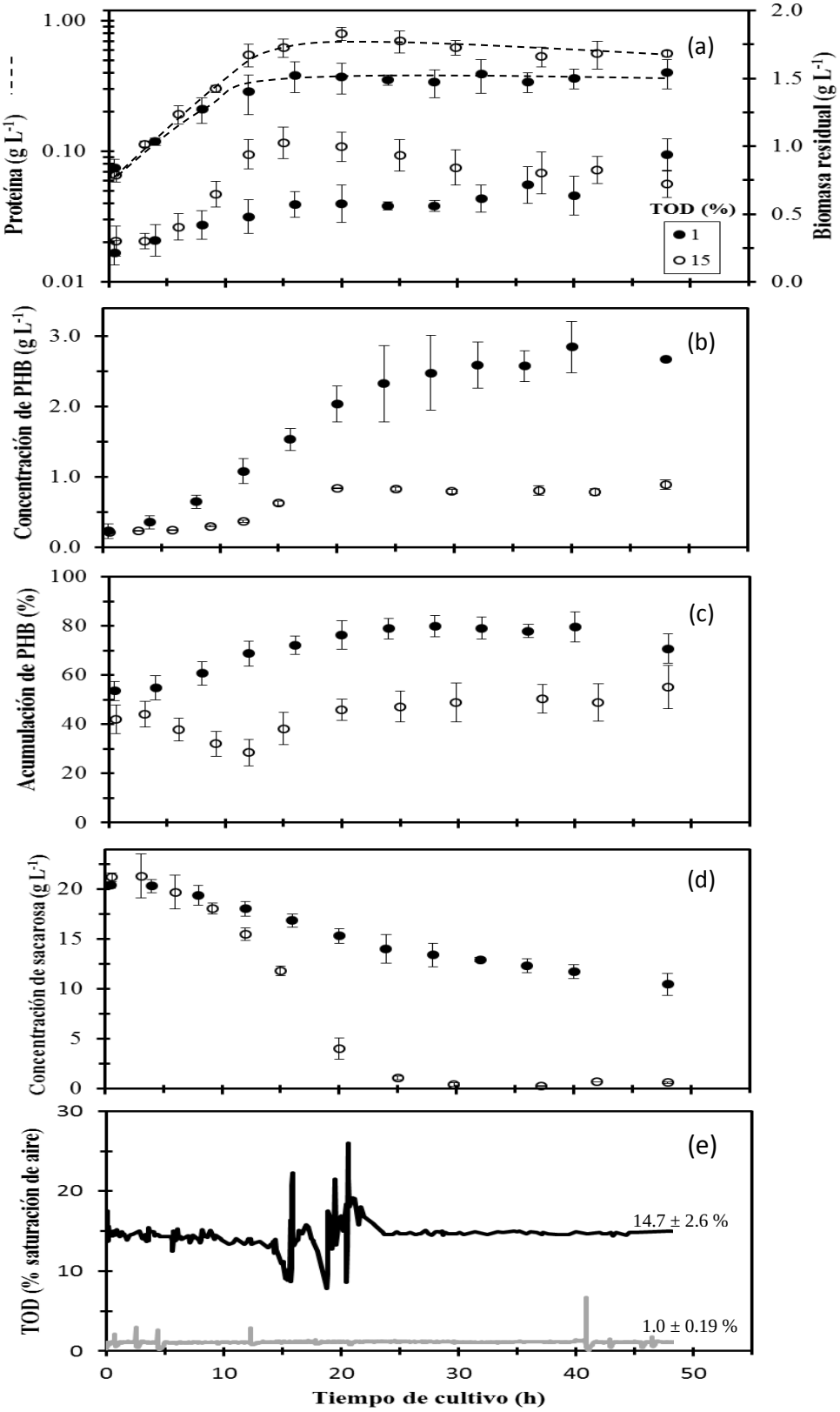


Figura 10. Cinéticas de (a) crecimiento celular, (b) concentración de P3HB, (c) acumulación de P3HB, concentración de sacarosa (d) y perfil de TOD (e) en cultivos de la cepa OP de *A. vinelandii* en condiciones de limitación (TOD ~ 1%) y no limitación (TOD ~ 15%) de oxígeno disuelto.

Durante la fase exponencial de crecimiento (hasta las 18-19 h de cultivo), se observó la mayor velocidad de consumo de sacarosa (0.97 g_{sac} L^{-1} h^{-1}) a 15 % de TOD y fue debido a mayor velocidad de transferencia de oxígeno (VTO = 32 mmol L^{-1} h^{-1}), comparado con lo observado en la condición de 1 % de TOD, donde la velocidad de consumo de sacarosa fue tres veces menor (0.32 g_{sac} L^{-1} h^{-1}) y la VTO fue de 6 mmol L^{-1} h^{-1} (Tabla 8). Sin embargo, el mayor consumo de sacarosa en condiciones de no limitación de oxígeno no se vio reflejado en una mayor producción de P3HB (Millán *et al.*, 2016).

Mediante un análisis teórico del flujo de moles de carbono se encontró que en los cultivos con 15 % de TOD, el 86 % de los moles de carbono se dirigió principalmente hacia la producción de CO_2, el 9 % al crecimiento bacteriano (medido como biomasa total) y el 4.5 % hacía la producción de P3HB. Por el contrario, bajo condiciones de limitación de oxígeno, el 49 % de los moles de carbono se dirigió hacia la producción de CO_2, el 13 % al crecimiento bacteriano y el 38 % hacia la síntesis de P3HB (Millán *et al.*, 2016).

Tabla 8. Parámetros cinéticos y de cultivo obtenidos en biorreactores bajo condiciones de limitación (TOD ~1%) y no limitación (TOD ~ 15%) de oxígeno en cultivos de *A. vinelandii* cepa OP.

TOD (%)	**1**	**15**
Concentración de proteína (g L^{-1})	0.3 ± 0.04	0.75 ± 0.05
Velocidad específica de crecimiento (μ; h^{-1})	0.08 ± 0.01	0.18 ± 0.01
$Biomasa_{máx}$ (g L^{-1})	3.12 ± 0.28	1.70 ± 0.12
$PHB_{máx}$ (g L^{-1})	2.43 ± 0.44	0.81 ± 0.07
% PHB (peso seco)	80 ± 4.3	45 ± 5.2
Biomasa residual (g L^{-1})	0.60 ± 0.09	0.95 ± 0.12
Sacarosa consumida (g L^{-1})	7.5 ± 2.9	20 ± 0.27 (30 h)
Poductividad específica de PHB (g_{PHB} g_{prot}^{-1} h^{-1})	0.26	0.03
Rendimiento PHB/sac (g_{PHB} g_{sac}^{-1})	0.32	0.04
Consumo específico de O_2 (mmol O_2 g_{prot}^{-1} h^{-1})	18	39
Velocidad de consumo de sacarosa (g_{sac} L^{-1} h^{-1})	0.25	0.9

En los cultivos a 15 % de TOD, el porcentaje de acumulación del biopolímero en la célula no fue mayor al 45 % del peso seco en la biomasa (Fig. 10c y Tabla 8). Esta menor acumulación de P3HB en la célula fue debido a que en condiciones de no limitación de oxígeno, la fuente de carbono se dirige hacia el ciclo de los ácidos tricarboxílicos (CAT), por lo que la disponibilidad de acetil-CoA que se dirige hacia la acumulación de P3HB es menor (Castillo *et al*., 2013). A 15 % de saturación de oxígeno se obtuvo el menor rendimiento de P3HB por gramo de sacarosa consumida (0.04 g_{P3HB} g_{sac}^{-1}) y la menor productividad específica de P3HB (0.03 g_{P3HB} $g_{proteína}^{-1}$ h^{-1}) comparado con el rendimiento obtenido (0.32 g_{P3HB} g_{sac}^{-1}) y la productividad específica (0.26 g_{P3HB} $g_{proteína}^{-1}$ h^{-1}) en los cultivos a 1 % de TOD (Millán *et al.*, 2016).

En resumen, en cultivos de *A. vinelandii* OP se encontró que las condiciones de oxígeno disuelto influyeron sobre el crecimiento celular y la producción de P3HB. Cuando la bacteria fue cultivada en condiciones de limitación de oxígeno (1 % de TOD), la acumulación de P3HB se incrementó y el crecimiento celular disminuyó; mientras que, en condiciones de no limitación de oxígeno el crecimiento celular se incrementó y la acumulación del polímero disminuyó. Estos resultados concuerdan con lo reportado previamente (Senior y Dawes, 1973; García *et al*., 2014; Peña *et al*., 2014b).

6.2 Análisis del peso molecular del P3HB sintetizado bajo condiciones de limitación y no limitación de oxígeno.

La cuantificación del peso molecular del P3HB extraído de la biomasa celular se realizó como se describió en la sección de materiales y métodos. El procedimiento de extracción se detalla en el anexo B. En la figura 11 se presenta la variación del peso molecular promedio en peso (Mw) del P3HB con respecto al tiempo de cultivo, sintetizado por la cepa OP de *A. vinelandii* cultivada en condiciones de limitación (1 %) y no limitación (15 %) de oxígeno. El P3HB producido por la bacteria presentó pesos moleculares promedio similares, tanto en condiciones de limitación como de no limitación de oxígeno. El peso molecular fue mayor (entre 4,800 y 5,200 kDa) durante la fase de crecimiento (hasta las 18 h de cultivo). Posteriormente, durante el inicio de la fase estacionaria (después de 20 h de cultivo) en ambas condiciones de TOD, el peso molecular del polímero disminuyó a valores entre 3,500 - 3,800 kDa.

Los cambios en el Mw del P3HB con respecto al tiempo de cultivo fueron similares en ambas condiciones de TOD evaluadas (1 y 15 % de saturación de oxígeno). Estas evidencias nos indican que el peso molecular del polímero no fue determinado ni por la condición de oxígeno disuelto imperante en el biorreactor, ni por el contenido de P3HB dentro de la bacteria, ya que en la condición de 1 % de TOD el porcentaje de P3HB dentro de la bacteria fue del 80 %, mientras que a 15 % de TOD la bacteria acumuló el 45 %. Sin embargo, la evolución del peso molecular promedio fue similar en ambas condiciones de TOD (Millán *et al*., 2016).

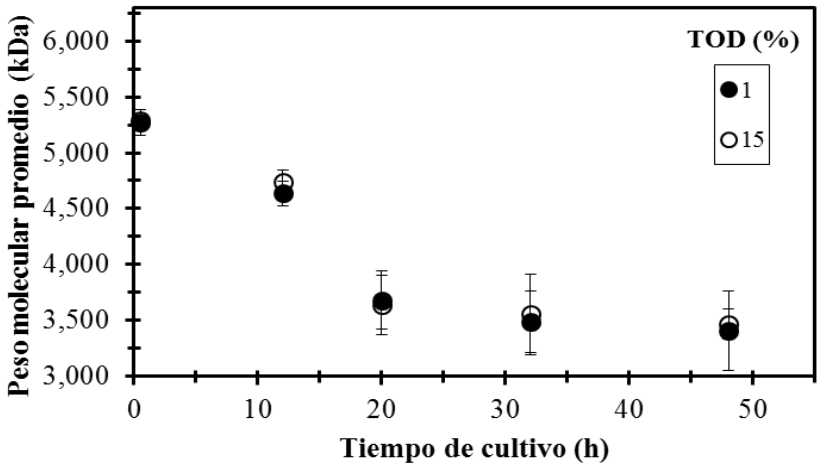


Figura 11. Evolución del peso molecular promedio del P3HB producido por la cepa OP de *A. vinelandii* bajo condiciones de limitación (1 % de TOD) y no limitación (15 % de TOD) de oxígeno disuelto.

Debido a que los polímeros están compuestos por cadenas de diferente longitud, se analizó la distribución de pesos moleculares de las muestras. Este análisis se realizó a las muestras de polímero acumulado en fase exponencial de crecimiento (12 h de cultivo), fase estacionaria temprana (20 h) y fase estacionaria tardía (48 h de cultivo). Las muestras de P3HB obtenidas en fase exponencial (12 h de cultivo) bajo las dos condiciones de oxígeno disuelto (1 y 15 % de saturación) presentaron moléculas con pesos moleculares entre 30 y 20,000 kDa (Fig.12a). Se observó que entre el 86 y 89 % de la muestra de biopolímero corresponde a fracciones de pesos moleculares entre 100 y 10,000 kDa (Fig.12a y Tabla 9), mientras que el P3HB que se recuperó en la fase estacionaria temprana (20 h) presentó moléculas con Mw bajos (menores a 10 kDa) y el 85 % de la muestra estuvo constituida por moléculas con pesos moleculares entre 100 y 10,000 kDa (Fig.12b y Tabla 9).

La disminución en el Mw del biopolímero en la fase estacionaria temprana fue debido a la disminución del porcentaje de 1) moléculas de P3HB con pesos moleculares entre 1,000 - 10,000 kDa (10-12 %) y 2) moléculas de peso molecular mayor a 10,000 kDa (2.4-6.6 %).

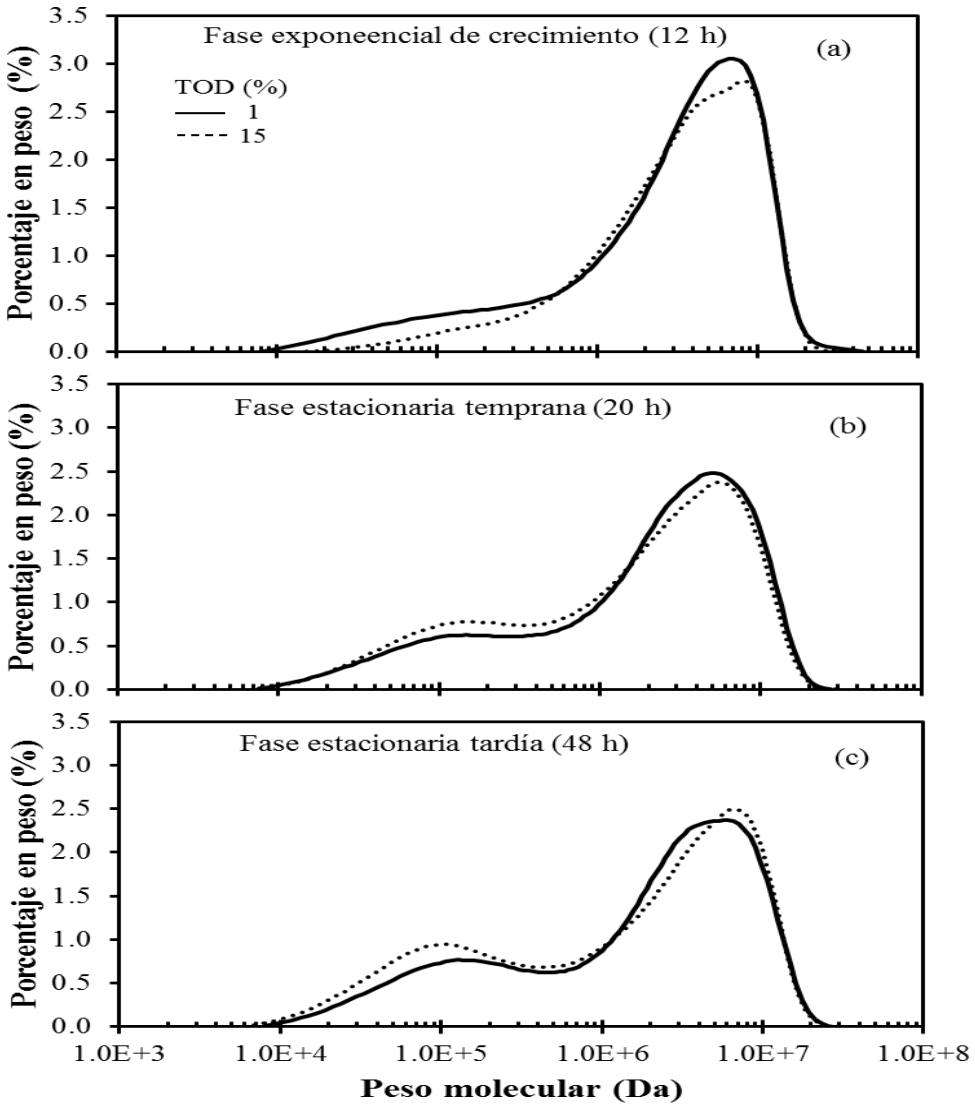


Figura 12. Distribución del peso molecular del P3HB acumulado en condiciones de TOD constante de 1 y 15 % de saturación a diferentes fases de cultivo. Fase exponencial de crecimiento (a), fase estacionaria temprana (b) y fase estacionaria tardía (c).

Es interesante señalar que bajo las dos condiciones de TOD (1 y 15 %) se duplicó la fracción de pesos moleculares del P3HB en el rango de 100 a 1,000 kDa; sin embargo, ese incremento solo hizo la diferencia en el Mw de 140-160 kDa. Dicho fenómeno es interesante, ya que se presentó tanto en los cultivos realizados en condiciones limitantes y de no limitación de oxígeno. Entre las 12 y 20 h de cultivo se observó la disminución del

Mw, tanto en la condición a 1 como a 15 % de TOD; sin embargo, en ambas condiciones la bacteria acumuló P3HB (Fig.10c) y no se observó una disminución en el porcentaje de acumulación del polímero. Las fracciones de P3HB con pesos moleculares en el rango de 10 a 400 kDa se incrementó durante la fase estacionaria tardía (48 h) y dicha fracción representó el 20 % del total de la muestra (Fig.12c) en ambas condiciones de TOD.

Tabla 9. Porcentaje de diversas fracciones de moléculas de P3HB presentes en las muestras de 12 y 20 h de cultivo a 1 y 15 % de TOD.

Tiempo de cultivo (h)	Tensión de oxígeno disuelto (%)	$< 1 \times 10^5$	1×10^5-1×10^6	1×10^6-1×10^7	$> 1 \times 10^7$
12	1	1.0	12.9	76.5	9.6
	15	1.7	15.1	71.2	12.0
20	1	7.2	21.0	64.6	7.2
	15	8.3	24.9	61.4	5.4

Los resultados obtenidos indican que las condiciones de limitación y no limitación de oxígeno, no tienen efecto sobre la distribución del peso molecular del P3HB, ni sobre su peso molecular promedio. Por el contrario, los cambios observados en el Mw del biopolímero y su distribución bajo las condiciones evaluadas hasta ahora se presentan durante la fase exponencial de crecimiento e inicio de la fase estacionaria de cultivo. Probablemente, el agotamiento de nutrientes o la acumulación de subproductos en el caldo de cultivo provocaron el cese del crecimiento celular y estas condiciones tuvieron influencia sobre la actividad de las enzimas de síntesis y degradación del P3HB, modificando la relación de los procesos de polimerización y depolimerización del polímero y por consecuencia la disminución del peso molecular. Pero no sabemos si la disminución del peso molecular y el incremento de moléculas de peso molecular menor a 1,000 kDa se deben a la síntesis de moléculas de menor peso molecular o a la degradación de las moléculas de P3HB de alto peso molecular (Millán *et al*., 2016).

6.3 Análisis de las enzimas que participan en el ciclo del P3HB

La actividad de las enzimas β-cetotiolasa, acetoacetil-CoA reductasa, P3HB sintasa y P3HB depolimerasa se midió con el objetivo de entender de qué manera estas enzimas están

modificando el peso molecular del P3HB producido por la cepa OP de *A. vinelandii*. Estos análisis se realizaron en muestras de cultivo bajo la condición de limitación de 1 % de saturación (alta producción de P3HB) y los resultados presentados son el promedio de dos cultivos independientes (Fig.13).

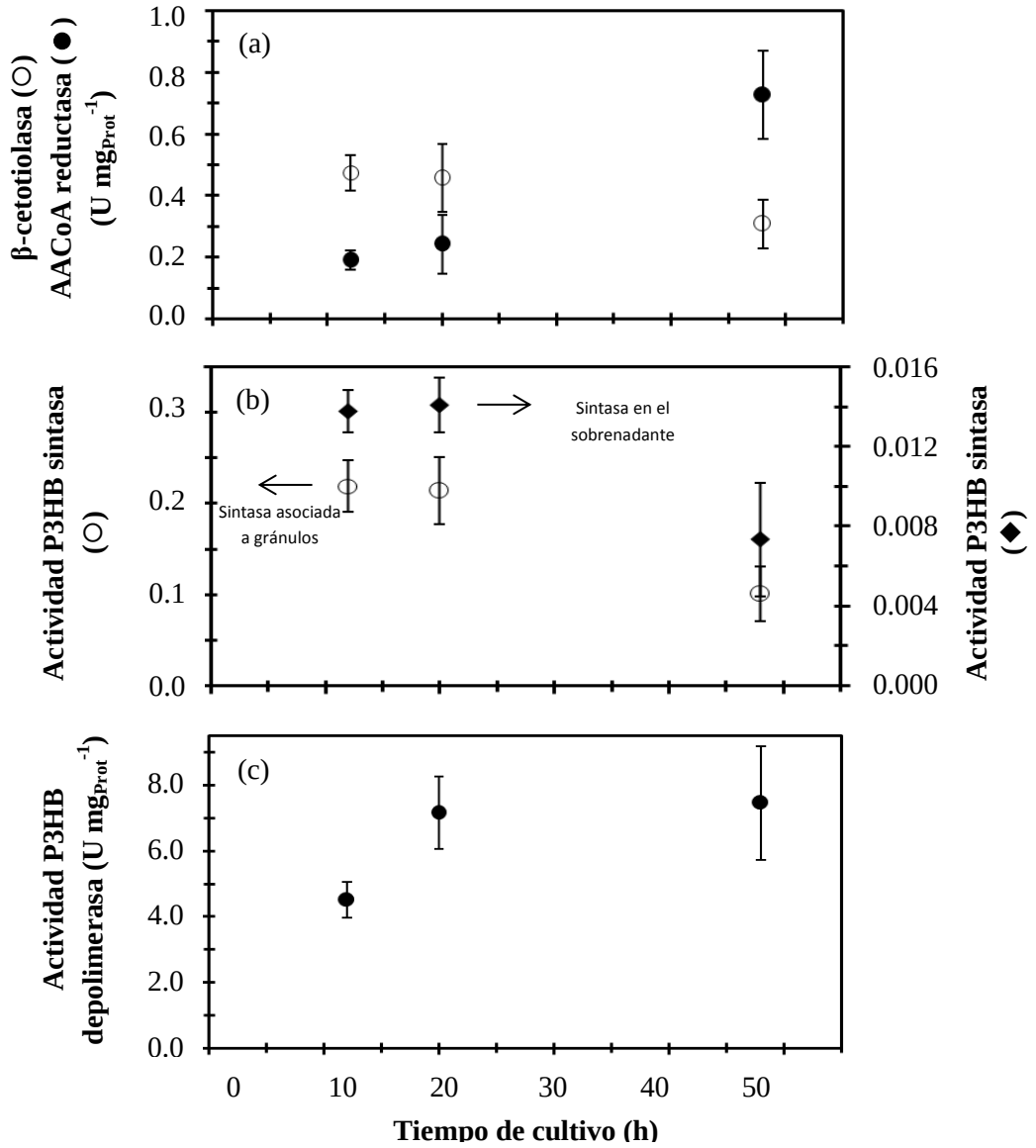


Figura 13. Actividades de las enzimas β-cetotiolasa (a), acetoacetil-CoA reductasa (a), P3HB sintasa (b) y P3HB depolimerasa (c) en cultivos de *A. vinelandii* cepa OP realizados en condiciones controladas de TOD a 1 %.

Para la actividad β-cetotiolasa se encontró que esta permanece en valores 0.46 ± 0.07 U mg_{prot}^{-1} (µmol/min mg proteína) entre las 12 y 20 h de cultivo y posteriormente (48 h de cultivo) la actividad de la enzima disminuyó a 0.31 ± 0.05 U mg_{prot}^{-1} (Fig.13a). La actividad de esta enzima estuvo asociada a la acumulación de P3HB, ya que mientras la bacteria

acumuló el polímero la actividad de la enzima fue mayor y cuando la bacteria dejó de acumular P3HB, la actividad de la enzima disminuyó un 33 % a las 48 h de cultivo (fase estacionaria tardía).

El comportamiento de la enzima acetoacetil-Coa reductasa (la cual es dependiente del cofactor NADPH) fue diferente al de la enzima β-cetotiolasa (Fig.13a). La enzima acetoacetil-Coa reductasa presentó una menor actividad durante las primeras 20 h de cultivo (0.19 ± 0.03 U mg_{prot}^{-1}: 12 h; 0.24 ± 0.08 U mg_{prot}^{-1}: 20 h) con respecto a su actividad en la fase estacionaria tardía (0.73 ± 0.1 U mg_{prot}^{-1} a las 48 h de cultivo). Interesantemente, durante el tiempo en el que ocurren los cambios en el peso molecular del P3HB, la actividad de la enzima acetoacetil-Coa reductasa fue muy similar y solo a las 48 h de cultivo, cuando el peso molecular del polímero fue menor, la enzima incrementó su actividad al menos tres veces.

La actividad de la enzima P3HB sintasa se determinó tanto en el sobrenadante (fracción soluble), como en los gránulos (fracción insoluble) (Fig.13b). El comportamiento de la actividad P3HB sintasa fue similar al observado para la β-cetotiolasa, tanto en sobrenadante como en gránulos. La actividad de la enzima en sobrenadante fue al menos un orden de magnitud menor a la actividad cuantificada en los gránulos de P3HB. En las primeras 20 h de cultivo, la actividad de la enzima fue de 0.23 ± 0.05 U mg_{prot}^{-1} y de 0.013 ± 0.002 U mg_{prot}^{-1} en los gránulos y en el sobrenadante, respectivamente. A las 48 h de cultivo, cuando finalizó la acumulación del polímero, la actividad P3HB sintasa disminuyó a la mitad, tanto en gránulos como en sobrenadante (0.10 ± 0.04 U mg_{prot}^{-1} y 0.008 ± 0.003 U mg_{prot}^{-1}, respectivamente).

Para la enzima P3HB depolimerasa (Fig.13c), se encontró que esta actividad se presentó baja (4.5 ± 0.5 μg_{P3HB}/min mg proteína) en la fase de crecimiento (hasta las 17 h) y se incrementó un 60 % cuando finalizó el crecimiento celular y ocurrió el fenómeno de caída del peso molecular del P3HB, permaneciendo muy activa y prácticamente sin cambios durante la fase estacionaria (7.45 ± 1.2 μg_{P3HB}/min mg proteína). Durante el crecimiento de la bacteria, mientras ocurrió la acumulación de P3HB, la actividad P3HB sintasa y β-cetotiolasa fueron mayores que en la fase estacionaria tardía cuando finalizó la acumulación del polímero. Por su parte, la actividad acetoacetil-Coa reductasa fue baja cuando el peso

molecular del P3HB disminuyó y solo se incrementó al finalizar el cultivo. En cuanto a la actividad P3HB depolimerasa fue baja a las 12 h de cultivo cuando el peso molecular fue mayor y se incrementó al finalizar el crecimiento celular, permaneciendo constante hasta el final del cultivo. Estos resultados sugieren que la disminución del peso molecular del P3HB probablemente es el resultado de un incremento de la actividad depolimerasa, la cual ocurre desde el inicio de la fase estacionaria de cultivo, coincidiendo con la disminución del peso molecular.

Con el objetivo de confirmar si el incremento de la fracción de P3HB con pesos moleculares menores a 100 kDa observado durante la fase estacionaria de los cultivos fue debido a la degradación de polímeros de alto peso molecular, se purificaron gránulos nativos de P3HB de las muestras del cultivo a 1 % de TOD. Dichos gránulos con la P3HB depolimerasa asociada a estos, se sometieron a un ensayo de degradación *in vitro* a 37 °C durante 60 h y se midió el peso molecular de las muestra de 12 y 20 h de cultivo antes y después de la degradación de los gránulos (Fig.14).

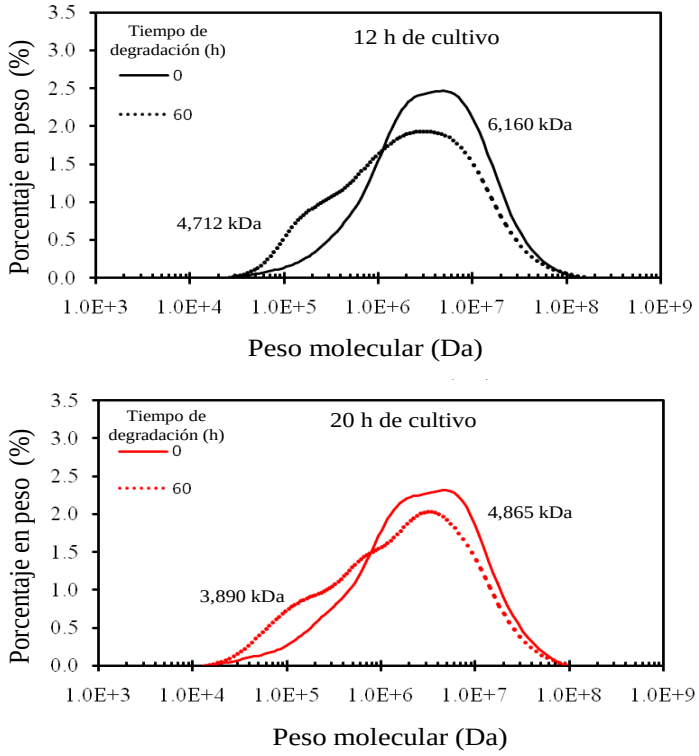


Figura 14. Distribución de pesos moleculares de los gránulos de P3HB antes y después del ensayo de degradación *in vitro* obtenidos de cultivos a 1 % de TOD a 12 y 20 h de cultivo.

El Mw del P3HB en los gránulos recuperados de las muestras de 12 h de cultivo (fase de crecimiento) fue de 6,160 kDa y para las muestra de 20 h de cultivo fue de 4,865 kDa (Fig.14 y tabla 10). Después de 60 h de degradación, se observó que el Mw del P3HB disminuyó a 4,700 kDa en la muestra de 12 h de cultivo y a 3,890 kDa en la muestra de 20 h de cultivo (Fig.14). Interesantemente, en ambas muestras de cultivo (12 y 20 h) la fracción de P3HB con pesos moleculares mayores a 10,000 kDa disminuyó y la fracción de P3HB con pesos moleculares menores a 100 kDa incrementó. Además, las fracciones de P3HB con pesos moleculares entre 30 y 1,000 kDa se incrementaron después de 60 h de degradación (Tabla 10).

Tabla 10. Porcentaje de moléculas de P3HB con diferentes pesos moleculares que presentan los gránulos de polímero sometidos a degradación *in vitro* y sin degradación.

Tiempo de cultivo (h) DOT = 1 %	Tiempo de degradación *in vitro* (h)	Porcentaje de moléculas de P3HB con diferentes peso molecular (kDa)			
		< 100	100 - 1,000	1,000 – 10,000	> 10,000
12	0	0.1	22.7	60.7	16.5
	60	**3.1**	**35.6**	**49.4**	**11.9**
20	0	2.7	27.3	58.3	11.7
	60	**6.9**	**35.8**	**47.4**	**9.9**

Estos resultados apoyan la hipótesis de que el incremento de la fracción de P3HB con bajos pesos moleculares fue debido a la mayor actividad depolimerasa que se observó en la fase estacionaria del cultivo (después delas 20 h de cultivo).

6.4 Caracterización cinética y análisis del peso molecular del P3HB producido por la cepa mutante *phbZ*⁻ de *A. vinelandii*

Con el objetivo de entender cuál es la contribución de las enzimas de síntesis y de degradación sobre el peso molecular del P3HB, y debido a que en los cultivos con la cepa OP no fue posible determinar si el incremento en la fracción de pesos moleculares menores a 200 kDa fue resultado de la síntesis de cadenas de polímero de bajo peso molecular o fue resultado de la degradación de cadenas de P3HB de alto peso molecular, se empleó una cepa mutante de *A. vinelandii* denominada *phbZ*⁻, la cual presenta una mutación (inactivación por inserción) en el gen (*Avin03910*) que codifica para una P3HB depolimerasa (Adaya *et al.,* 2017). Los cultivos de esta cepa se realizaron en condiciones

limitantes de oxígeno disuelto y se caracterizó la cinética de crecimiento, producción de P3HB, consumo de sacarosa, así como el peso molecular del polímero y la actividad de las enzimas de síntesis y degradación del P3HB.

El crecimiento celular (medido como concentración de proteína) de la cepa *phbZ*⁻ fue similar al observado para la cepa OP de *A. vinelandii* (Fig.15a).

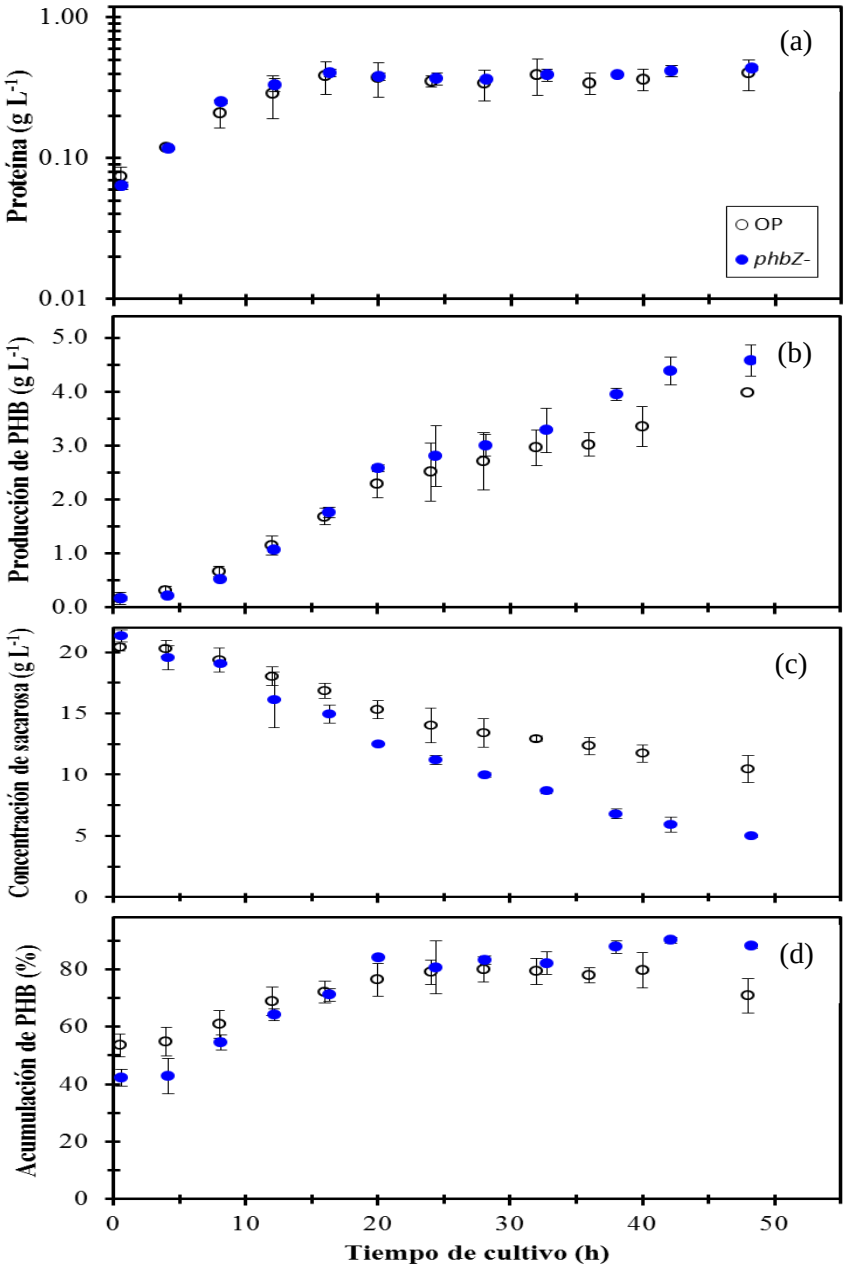


Figura 15. Cinéticas de (a) crecimiento celular, (b) concentración de P3HB, (c) concentración de sacarosa y (d) acumulación de P3HB en cultivos de la cepas OP y la mutante *phbZ*⁻ de *A. vinelandii* en condiciones de limitación (1 % de TOD) de oxígeno.

La velocidad específica de crecimiento (μ) obtenida fue similar para ambas cepas (0.09 ± 0.01 h^{-1} para la cepa *phbZ*$^-$ y 0.08 ± 0.01 h^{-1} para la cepa OP) (fig. 15a y Tabla 11).

En los cultivos con la cepa mutante, la concentración de P3HB fue 60 % mayor con respecto a lo obtenido con la cepa OP y el porcentaje de acumulación de polímero fue 90 ± 3.0 % de su peso seco con la cepa mutante; mientras que, con la cepa OP la acumulación máxima de P3HB fue del 80 ± 4.3 %. Esta mayor producción de P3HB con la cepa mutante fue debido a un mayor consumo de sacarosa, obteniéndose un rendimiento de P3HB con base en sacarosa de 0.27 ± 0.01 g_{P3HB} g_{sac}^{-1}, menor al observado para la cepa OP donde el rendimiento fue de 0.32 ± 0.01 g_{P3HB} g_{sac}^{-1} (Tabla 11).

Tabla 11. Parámetros cinéticos y de cultivo obtenidos de los cultivos de las cepas *phbZ*$^-$ y OP de *A. vinelandii* en condiciones de limitación de oxígeno (TOD ~1 %).

Parámetro	*phbZ*$^-$	OP
Velocidad específica de crecimiento (μ) (h^{-1})	0.09 ± 0.01	0.08 ± 0.01
$[Proteína]_{máx}$ ($g\ L^{-1}$)	0.41 ± 0.04	0.3 ± 0.04
$PHB_{máx}$ (%)	90 ± 3.0	80 ± 4.3
$[Biomasa]_{máx}$ ($g\ L^{-1}$)	4.5 ± 0.3	3.1 ± 0.2
$[PHB]_{máx}$ ($g\ L^{-1}$)	4.0 ± 0.4	2.4 ± 0.4
Sacarosa consumida ($g\ L^{-1}$)	15.2 ± 1.2	7.5 ± 2.9
Rendimiento PHB /sac ($g_{PHB}\ g_{sac}^{-1}$)	0.27 ± 0.01	0.32 ± 0.01

El Mw máximo del P3HB producido por la cepa mutante fue de 6,100 ± 177 kDa y permaneció constante durante todo el cultivo a diferencia de lo observado con la cepa OP de *A. vinelandii* (Fig.16a). Estas diferencias en las cinéticas del Mw del P3HB producido por las cepas OP y *phbZ*$^-$ de *A. vinelandii* son el resultado de la mutación en el gen de la P3HB depolimerasa en la cepa *phbZ*$^-$, lo que permite la síntesis de polímero de alto peso molecular y evita la degradación del P3HB de alto peso molecular, ya que en la cepa OP, la mayor actividad depolimerasa en la fase estacionaria promueve la degradación del polímero, incrementándose la fracción de P3HB de bajos pesos moleculares (menores a 400 kDa), la cual representó al menos el 20 % del total de la muestra en la fase estacionaria del cultivo (Fig.16c) (Millán *et al*., 2016; Adaya *et al*., 2017).

En la distribución de pesos moleculares del P3HB producido por la cepa mutante se observó que la muestra de P3HB contiene fracciones con pesos moleculares en el rango de 300 a 30,000 kDa; mientras que, el polímero producido por la cepa OP presentó fracciones con pesos moleculares entre 10 y 300 kDa.

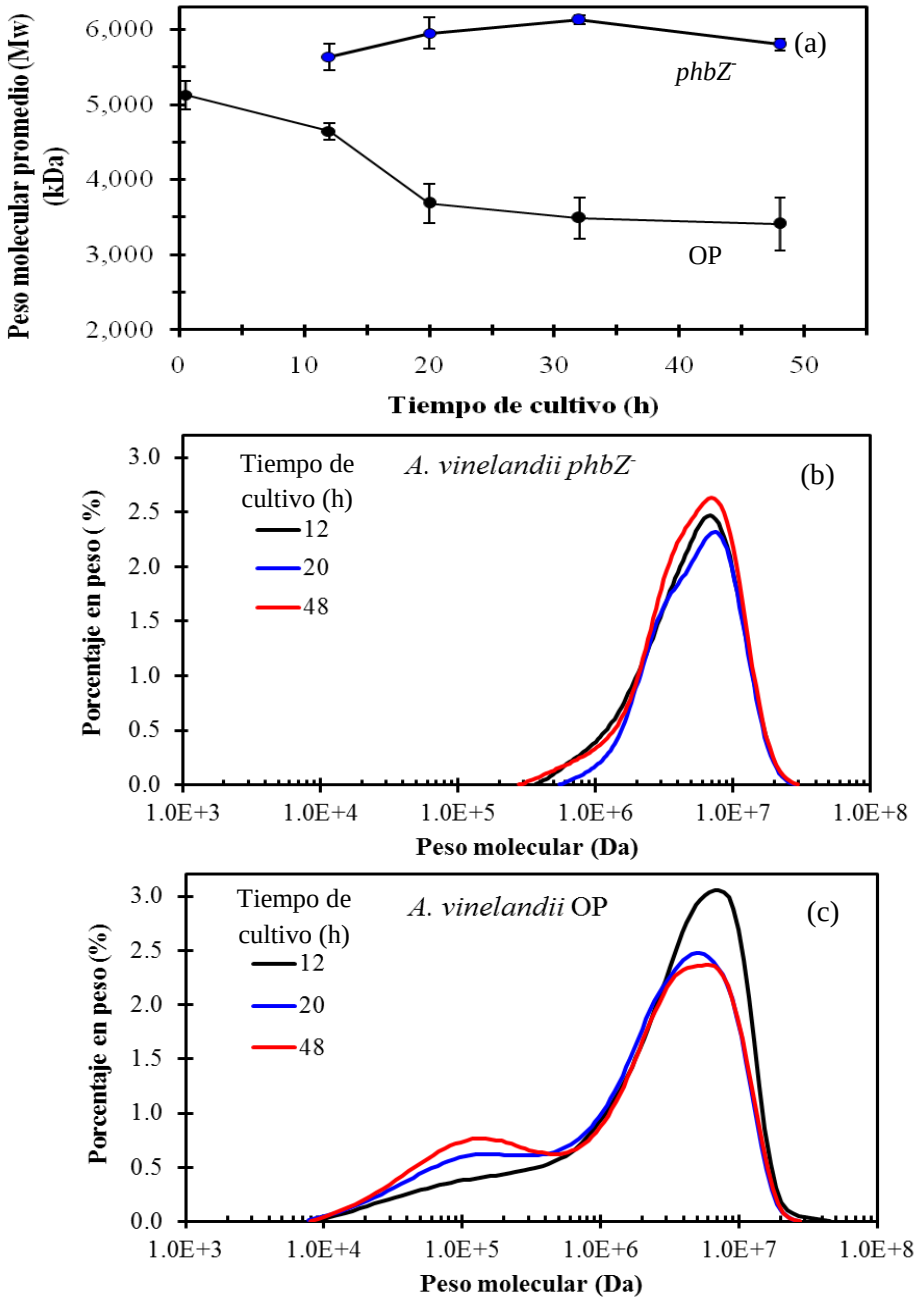


Figura 16. Peso molecular promedio (a) y distribución de pesos moleculares (b-c) del P3HB producido por las cepas OP y *phbZ*⁻ de *A. vinelandii* en condiciones de limitación de oxígeno.

Esta fracción de 10 a 300 kDa representó el 9 % de la población en las muestras de P3HB producido por la cepa OP a las 12 h de cultivo y en las muestras de 48 h de cultivo dicha fracción representó el 20 % de la población de moléculas de P3HB. En el caso de las muestras de P3HB producido por la cepa mutante, no se observaron cambios en la distribución de los pesos moleculares del polímero con respecto al tiempo. Esto nos indica que la bacteria acumuló polímero con el mismo peso molecular durante el tiempo de cultivo, y no fue dependiente de la fase de crecimiento de la bacteria como ocurrió en el caso del polímero acumulado por la cepa OP de *A. vinelandii* (Fig.16a), lo que demuestra que la disminución del peso molecular se debe a la actividad de degradación de la P3HB depolimerasa (Millán *et al*., 2016; Adaya *et al*., 2017).

6.5 Análisis de las enzimas que participan en el ciclo del P3HB en cultivos de la cepa mutante phbZ- de A. vinelandii

La actividad de la enzimas de biosíntesis del P3HB se determinó en los cultivos con la cepa *A. vinelandii phbZ*$^-$. También se determinó la actividad de la enzima P3HB depolimerasa; sin embargo, no se detectó la actividad de esta enzima mediante el ensayo utilizado en este trabajo. Los resultados obtenidos en términos del peso molecular promedio del P3HB así como su distribución sugiere que la P3HB depolimerasa codificada por el gen *Avin03910* es la depolimerasa principal que determina el peso molecular del biopolímero acumulado por *A. vinelandii* en las condiciones de cultivo evaluadas y no se activaron otras depolimerasas presentes en *A. vinelandii* (Adaya *et al.*, 2017). Para el caso de la enzima β-cetotiolasa se observó que su actividad fue de 0.91 ± 0.07 U mg_{prot}^{-1} durante la fase de crecimiento de la bacteria y en la fase estacionaria, la actividad de ésta enzima disminuyó a la mitad, permaneciendo constante el resto del cultivo (Fig. 17b). Interesantemente, no se observó una relación entre la acumulación de polímero y la actividad de la enzima β-cetotiolasa, ya que después de 20 h de cultivo, la bacteria acumuló un 10 % más de P3HB, periodo en el cual la actividad de la enzima fue de 0.45 ± 0.09 U mg_{prot}^{-1} (Fig.17b).

La actividad de la enzima P3HB sintasa presentó un comportamiento similar al observado en los cultivos con la cepa OP (Fig. 17c). En la fase de crecimiento (12 h), la actividad de la P3HB sintasa fue de 0.27 ± 0.02 U mg_{prot}^{-1} y disminuyó con respecto al tiempo de cultivo. Estos resultados indican que la actividad sintasa en *A. vinelandii* es la responsable de la

síntesis de P3HB de elevado peso molecular (hasta 6,100 ± 177 kDa) en la cepa mutante y en la cepa OP (5,200 ± 113 kDa) de *A. vinelandii* (Fig. 17a). Durante la fase de crecimiento de la bacteria (12 h), la actividad P3HB favoreció la síntesis de P3HB con mayor peso molecular comparado con el polímero acumulado por la cepa OP. En los cultivos de *A. vinelandii* OP se observó que el Mw disminuyó durante la fase de crecimiento de la bacteria, mientras que en la muestras del biopolímero acumulado por *A. vinelandii phbZ*$^-$ el Mw se incrementó de 5,500 a 6,000 kDa (Fig 17a).

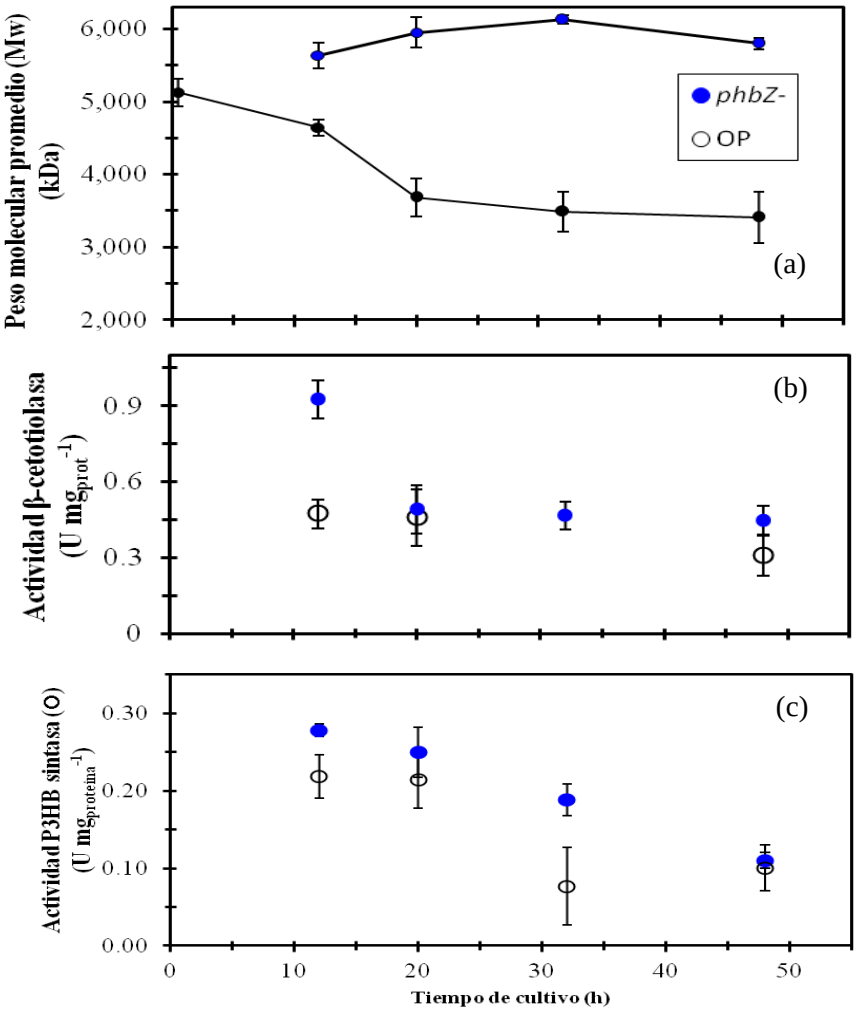


Figura 17. Peso molecular promedio (a) y actividad de las enzimas β-cetotiolasa (b) y sintasa (c) del P3HB producido por las cepas OP y *phbZ*$^-$ de *A. vinelandii* en condiciones de limitación de oxígeno.

En cuanto no estuvo presente (muestras de cultivo de la cepa mutante) o no se incrementó la actividad P3HB depolimerasa (muestras de cultivo de la cepa OP durante la fase de

crecimiento) el peso molecular del polímero fue mayor (5,200 ± 113 kDa con OP y 6,100 ± 177 kDa con *phbZ*$^-$) (Fig.17 y 18) comparado con el valor obtenido cuando se incrementó la actividad depolimerasa (3,600 ± 275 kDa en la fase estacionaria de los cultivos con la cepa OP).

A pesar de la disminución de la actividad de la P3HB sintasa en los cultivos de *A. vinelandii phbZ*$^-$ durante la fase estacionaria del cultivo, el Mw del biopolímero no disminuyó (Fig. 17 y 18).

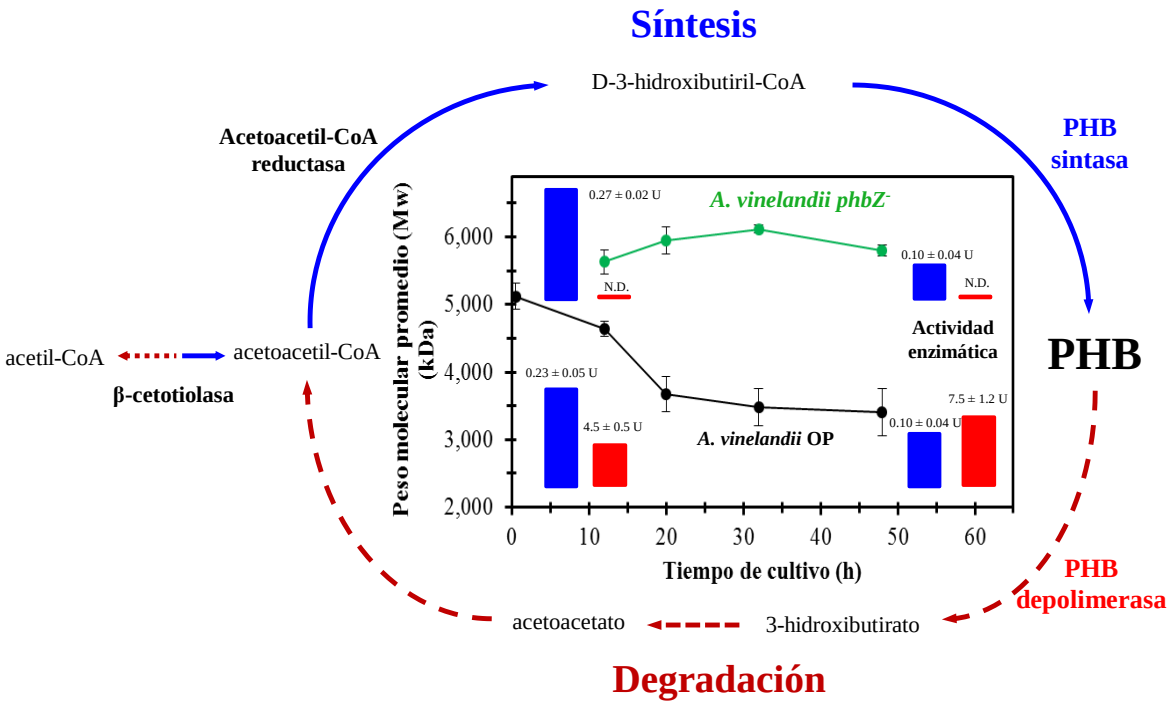


Figura 18. Esquema del ciclo del P3HB donde se presenta la actividad P3HB sintasa (barras azules) y P3HB depolimerasa (barras rojas) en la fase de crecimiento y estacionaria en los cultivos de las cepas OP y *phbZ*$^-$ de *A. vinelandii* bajo condiciones de limitación de oxígeno disuelto.

Es interesante señalar que la inactivación de la P3HB depolimerasa no afectó la actividad de la enzima P3HB sintasa, pero si se favoreció la cantidad de polímero acumulado y el peso molecular del mismo. Aún falta mucho por entender acerca de los procesos de polimerización y depolimerización del P3HB en cultivos de *A. vinelandii*, ya que queda la incertidumbre de que factores externos (condiciones de cultivo) o internos (enzimáticos, fisiológicos) están regulando la actividad de las enzimas del ciclo del P3HB en *A. vinelandii*. Probablemente, enzimas como las phasinas (por ejemplo PhaM descubierta en

Cupriavidus necator) estarían involucradas en el control de los procesos de polimerización y depolimerización que ocurren dentro de la bacteria (Jendrossek y Pfeiffer, 2014), pero que hasta ahora no han sido estudiadas para elucidar su participación en dichos procesos.

Todos los resultados en conjunto apoyan la idea que la disminución del peso molecular del P3HB en los cultivos con la cepa OP fue debido al incremento de la actividad depolimerasa *phbZ* (codificada por el gen *Avin03910*), ya que esta disminución del Mw del polímero no se observó en los cultivos con la cepa *phbZ*¯, donde la actividad P3HB depolimerasa no se detectó con el ensayo utilizado en este trabajo (Millán *et al*., 2016; Adaya *et al*., 2017).

7. CONCLUSIONES

- Las condiciones controladas de limitación (1 %) y no limitación de tensión de oxígeno disuelto (15 %) influyen significativamente sobre la acumulación de P3HB (1 % de TOD = 80 % de acumulación, 15 % de TOD = 45 % de acumulación) en cultivos de *A. vinelandii* cepa OP; sin embargo, no tienen efecto sobre el peso molecular del P3HB acumulado por la bacteria.

- El peso molecular del P3HB acumulado por la cepa OP de *A. vinelandii* estuvo asociado a la fase de crecimiento de la bacteria, y fue mayor durante la fase exponencial de crecimiento (4800-5,200 ± 113 kDa) comparado con el obtenido en la fase estacionaria (3,600 ± 275 kDa).

- Durante la fase de crecimiento de la bacteria se observó la disminución del peso molecular del P3HB y esa disminución del peso molecular del P3HB fue asociada al aumento de la actividad P3HB depolimerasa en el inicio de la fase estacionaria temprana del cultivo.

- El peso molecular promedio (Mw) del P3HB acumulado por la cepa mutante *phbZ*$^-$ de *A. vinelandii* permaneció prácticamente constante durante todo el cultivo (5,800 - 6,100 ± 177 kDa) y fue 17 % mayor comparado con el Mw máximo producido por la cepa *A. vinelandii* OP (4800-5,200 ± 113 kDa).

- La enzima sintasa fue la responsable de la síntesis de P3HB con pesos moleculares entre 5,200 ± 113 y 6,100 ± 177 kDa en los cultivos de *A. vinelandii* OP y *phbZ*$^-$, respectivamente.

- La acumulación de P3HB con Mw de 5,200 ± 113 – 6,100 ± 177 kDa se favoreció cuando la actividad sintasa fue 0.23 ± 0.05U mg_{prot}^{-1} y la actividad depolimerasa fue de 4.5 ± 0.5 U mg_{prot}^{-1} o nula (cepa mutante *phbZ*$^-$). Mientras que por el contrario, condiciones de mayor actividad depolimerasa (7.45 ± 1.2 U mg_{prot}^{-1}) favorecen la degradación del polímero disminuyendo el peso molecular del P3HB a 3,600 ± 275 kDa.

8. APORTACIONES Y PERSPECTIVAS

APORTACIONES

Las principales aportaciones en este trabajo fueron:

- Se estableció una metodología para la extracción del P3HB con el menor detrimento en el peso molecular del polímero, así como la técnica del análisis de pesos moleculares por cromatografía de permeación en gel.
- Se demostró que el oxígeno disuelto en el rango de 1 a 15 % de saturación no tiene efecto sobre el peso molecular del P3HB producido por *A. vinelandii* OP.
- Se encontró que la actividad de las enzimas de síntesis y degradación del P3HB en cultivos de *A. vinelandii* afectan el peso molecular del polímero acumulado, siendo la enzima depolimerasa *phbZ*$^-$ (*Avin03910*) la causante de la disminución del peso molecular en la fase estacionaria del cultivo.
- Se caracterizó la cepa mutante *phbZ*$^-$ de *A. vinelandii*, la cual fue capaz de acumular hasta el 90 ± 3.0 % de P3HB (con base en el peso seco de la bacteria). Además el polímero acumulado por esta cepa fue de elevado peso molecular (6,100 kDa).

PERSPECTIVAS

- Realizar estudios de síntesis y degradación utilizando medios de cultivo químicamente definidos donde se ha observado que el peso molecular del P3HB es menor comparado con el peso molecular del P3HB producido en medios complejos.
- Identificar los componentes de degradación solubles en sobrenadante de los cultivos para correlacionarlos con la actividad depolimerasa.
- Investigar cuales son las condiciones fisiológicas o de cultivo celular (*ej*. contenido de P3HB en el inóculo) que promueven el aumento de la actividad de las enzimas P3HB depolimerasas y podrían afectar el peso molecular del P3HB en *A. vinelandii*.

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10. ANEXOS

ANEXO A. Productos Generados

Artículo de investigación

Millán, M., Segura, D., Galindo, E., Peña, C. (2016). Molecular mass of poly-3-hydroxybutyrate (P3HB) produced by *Azotobacter vinelandii* is determined by the ratio of synthesis and degradation under fixed dissolved oxygen tension. **Process Biochemistry**. 51: 950-958.

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Molecular mass of poly-3-hydroxybutyrate (P3HB) produced by *Azotobacter vinelandii* is determined by the ratio of synthesis and degradation under fixed dissolved oxygen tension

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ABSTRACT

Poly-3-hydroxybutyrate (P3HB) is an intracellular polyester produced by numerous bacteria, including *Azotobacter vinelandii*. Thermo-mechanical properties and biomedical applications of P3HB depend on its molecular mass (MM), which in turn is controlled by the balance between synthesis and degradation of the polymer during its biosynthesis. The aim of this study was to determine the activity levels of enzymes involved in the synthesis and degradation of P3HB and their effect on the molecular mass of the polymer produced by *A. vinelandii* strain OP under the conditions of dissolved oxygen tension of 1 and 15%. The results show that the MM of P3HB changed between the exponential and stationary growth phases, under both oxygen conditions. During the exponential growth phase, the mean molecular mass (MMM) of P3HB was high (4800 kDa), coincident with a high activity P3HB synthase and with a low activity of P3HB depolymerase. In contrast, during the stationary phase, the P3HB MM decreased to 3600 kDa, because of the increased activity of the P3HB depolymerase.

1. Introduction

Poly-3-hydroxybutyrate (P3HB) is a homopolymer of 3-hydroxybutyrate that belongs to the family of polyhydroxyalkanoates (PHAs), accumulated as carbon and energy storage material, forming intracellular granules in several bacteria, including *Azotobacter vinelandii* [1]. P3HB has some mechanical properties similar to those of conventional plastics, such as polypropylene or polyethylene, although it exhibits a high degree of crystallinity, leading to brittleness and low elongation at break [2]. P3HB is a fully biodegradable and biocompatible material, and due to these characteristics, this polymer is a potential candidate as a substitute for petrochemical plastics. However, due to the high production costs of P3HB and other PHAs, the most economically attractive applications are in the biomedical field, as scaffolds for the cellular growth of cartilage and bone; for medical devices, such as sutures, adhesion barriers and valves to guided tissue repair; and for regeneration devices such as cardiovascular patches [1,3–5].

Physicochemical properties and applications of P3HB are affected by its molecular mass, as this feature determines the elastic behavior of the material and its mechanical resistance [6]. For example, fibers of P3HB with a molecular mass of approximately 300 kDa have a tensile strength of 190 MPa and an elongation at break of 5%. In contrast, when the molecular mass is of 5300 kDa, the fibers increase their tensile strength 7-fold (1320 MPa), and the elongation at break is 57% [6].

It has been reported that the molecular mass of P3HB is affected by various culture parameters such as the culture medium composition, pH, temperature and aeration conditions of the bioreactor used for the production of the polymer [7–10]. In addition, the molecular mass of P3HB has been shown to be affected by other factors, such as the relative level of expression of the biosynthetic enzymes, which can be affected by the order of the biosynthetic genes (*phbA, phbB* and *phbC*) within the *phb* operon, and the level of activity of P3HB synthase [11], the type of PHA synthase present [12,13] and mutations on the P3HB synthase enzyme [14].

Among the bacteria that are able to accumulate large amounts of P3HB, *Azotobacter spp* have been reported to synthesize a polymer of ultra-high molecular mass [1,7,10,15]. It is important to note that the process of intracellular accumulation of the polymer is the result of two stages of its metabolism: one is the P3HB synthesis, and the other is the degradation of the polymer. P3HB

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synthesis starts with the condensation of two molecules of acetyl-CoA, which is catalyzed by the β-ketothiolase (encoded by the *phbA* gene) to generate acetoacetyl-CoA. Subsequently, acetoacetyl-CoA is reduced by an NADPH dependent acetoacetyl-CoA reductase (encoded by the *phbB* gene) to produce β-hydroxybutyryl-CoA. Finally, β-hydroxybutyryl-CoA is polymerized by the P3HB synthase (encoded by the *phbC* gene), releasing CoA and producing the P3HB [16,17]. When the external carbon source is depleted, the bacterium uses the P3HB reserves, mobilizing this polymer for energy production [18,19]. P3HB degradation starts when the polymer chains are converted into β-hydroxybutyrate or P3HB oligomers by P3HB depolymerase (*phbZ* gene) enzyme. Later, β-hydroxybutyrate is oxidized by NAD^+ dependent β-hydroxybutyrate dehydrogenase enzyme, producing acetoacetate. Finally, succinyl-CoA acetoacetate transferase converts acetoacetate to acetoacetyl-CoA [20,21]. Because synthesis and degradation occur simultaneously [22,23], the molecular mass of P3HB accumulated by the bacterium could be affected by the ratio of both stages, but in the case of *A. vinelandii* it is unknown how the stage of synthesis and degradation influence the molecular mass of the polymer.

There are very few reports in which the changes in the molecular mass of P3HB have been studied during the cultures of *A. vinelandii* under fixed dissolved oxygen conditions. Thus far, it is not known whether the level of activity of the enzymes involved in the process of intracellular accumulation influence the molecular mass of polymer produced. In a recent study [10], it was reported that the molecular mass (MM) of P3HB produced by *A. vinelandii* was influenced by the aeration conditions and the strain used. In that study, when *A. vinelandii* OPN mutant and its parental strain OP were cultivated during 60 h under low aeration conditions, they produced polymers with a molecular mass of 2020 kDa and 1650 kDa, respectively. However, when both strains were cultivated under a condition of higher aeration, the molecular mass of P3HB decreased to 1010 kDa and 551 kDa for OPN and OP strains, respectively. It should be noted that study was performed in shaken flasks where culture parameters, such as the pH and the dissolved oxygen tension (DOT), were not controlled. Changes in these parameters during the cultivation might influence the molecular mass of P3HB, as it was reported in cultures of recombinant *Escherichia coli* and *Azotobacter chroococcum* [7,9,24]. Additionally, in that study, they did not determine the activity of the enzymes that participated in the synthesis and degradation of the P3HB.

There are some reports in the literature about the analysis of the enzyme activities of both synthesis and degradation during the process of accumulation of P3HB in other microorganisms [11,25–27]. However, it is not clear how the activities of the enzymes that participate in both the synthesis and degradation influence the molecular mass of P3HB. In *A. vinelandii,* this has not been studied yet. Therefore, the aim of this work was to study the role of the level of enzymatic activities involved in the synthesis and degradation of P3HB on its molecular mass in a bioreactor under controlled dissolved oxygen conditions in cultures of *A. vinelandii* OP.

2. Materials and methods

2.1. Microbial strain, culture medium and inoculum preparation

A. vinelandii OP (ATCC 13705) was used in this study. This strain is unable to produce alginate (an extracellular polysaccharide) because it has an insertion sequence in the *algU* gene, which encodes a transcriptional factor that regulates genes involved in synthesis of alginate and mobility [28]. The strain was cryopreserved at −70 °C in a 40% glycerol solution and maintained by monthly subculture on PYís agar slopes and stored at 4 °C [1]. *A. vinelandii* OP was cultured in PY medium with the following composition in g L^{-1}: sucrose 20.0 (J.T. Baker); yeast extract 3.0 (Difco); peptone 5.0 (Difco). The pH of medium was adjusted to 7.2 with addition of a 2 N solution of NaOH, and the medium was sterilized at 121 °C for 20 min. The inoculum was incubated on a rotatory shaker (New Brunswick Scientific Co., Model G 25, shaking radius = 2.5 cm) at 200 rpm and 29 °C, to an absorbance (measured at 540 nm, after performing a dilution 1:50 before the measurement) between 0.16 and 0.18 (corresponding to a cell dry weight between 0.08 and 0.1 g L^{-1}). A total of 200 mL of the broth culture was centrifuged at 12,860*g* for 10 min at 4 °C. The supernatant was discarded, and the cells were suspended in 200 mL of fresh PY medium and transferred to the bioreactor containing 1.8 L of medium.

2.2. Culture conditions in the bioreactor

The cultures were grown in an Applikon bioreactor containing 2 L of PY medium at 29 ± 0.5 °C, at 500 rpm. The stirred tank bioreactor was equipped with two Rushton turbines ($D_T/T = 1/3$), where D_T is the turbine diameter and T is the bioreactor diameter. The pH was measured with an Ingold probe (Applikon, ADI 1010) and controlled to 7.2 ± 0.1 by an on/off system using a peristaltic pump and by adding 2 N NaOH or HCl solutions. The dissolved oxygen tension (DOT) was measured with an Ingold polarographic probe, controlled through a gas mixture (O_2 and N_2) [29]. *A. vinelandii* is a strict aerobic bacterium that has a critical value of DOT at 4% [30–32]. It is known that when this bacterium is grown under oxygen limited conditions (below the critical oxygen concentration), the P3HB accumulation process is favored. In contrast, under non-oxygen-limited conditions (DOT higher of 4%) cell growth is promoted, followed by the accumulation of the polymer. In this study, conditions of oxygen limitation (DOT = 1 ± 0.2% equivalent to 0.0021 mmol O_2 L^{-1}) and non-oxygen limitation (DOT = 15 ± 0.65% equivalent to 0.0137 mmol O_2 L^{-1}) were evaluated. All experiments were conducted in triplicate, and the results presented are the average of independent runs.

2.3. Oxygen transfer rate and specific oxygen uptake rate determinations

Equations used for determination of oxygen transfer rate (OTR) and specific oxygen uptake rate (q_{O_2}) were as follows:

$$C^* = \frac{\left[\frac{F_{O_2}}{F_T}(P_{atm})\right]}{H} \quad (1)$$

$$OTR = k_L a (C^* - C_L) \quad (2)$$

$$q_{O_2} = \frac{OTR}{X} \quad (3)$$

where C^* (mmol L^{-1}) is the dissolved oxygen concentration at equilibrium, calculated based on the proportion of the flux of oxygen at the inlet (F_{O_2}: L h^{-1}) of the system and the total flux of gas at the inlet (F_T: L h^{-1}), P_{atm} is the pressure in the system (0.825 atm), and H is Henryís constant at 29 °C in water for oxygen. OTR (mmol L^{-1} h^{-1}) is the oxygen transfer rate, $k_L a$ (h^{-1}) is the volumetric oxygen transfer coefficient, C_L (mmol L^{-1}) is the dissolved oxygen concentration, q_{O_2} (mmol g^{-1} h^{-1}) is the specific oxygen uptake rate, and X (g L^{-1}) is the protein concentration.

2.4. Analytical determinations

2.4.1. Biomass, protein, sucrose and poly-3-hydroxybutyrate (P3HB) concentration

Cell dry weight was determined gravimetrically using 6 mL of culture broth, which was centrifuged at 9660*g* during 10 min. The pellet was isolated, mixed in distilled water and filtered through

previously weighted Millipore filters (0.45 μm pore size). The filters were dried at 80 °C to constant weight. Protein concentration was determined by the Lowry method [33] using bovine serum albumin as the standard. The specific growth rate (μ) was calculated based on the measured protein concentrations using the logistic model reported previously [34]. Sucrose was assayed for reducing power with the DNS reagent. Samples were previously hydrolyzed by using β-fructofuranosidase (from Bakers Yeast, Sigma-Aldrich, St Louis MO, USA) to generate glucose and fructose and then assayed for reducing power with the DNS reagent [35]. The quantification of P3HB was made measuring the crotonic acid concentration. P3HB inside the biomass was hydrolyzed with concentrate sulfuric acid at 90 °C for 1 h, causing the formation of crotonic acid [36], and measured using a high performance liquid chromatography (HPLC) system with a UV detector (Waters 2996, USA) and an Aminex HPX-87H ion-exclusion organic acid column at 220 nm. Elution was performed with 0.014 N of H_2SO_4 at a flow rate of 0.65 mL min^{-1} and 50 °C, as described previously [10,37].

2.5. Recovery of P3HB and analysis of molecular mass

An aliquot of 6–8 mL of broth culture was centrifuged at 9660*g* during 10 min. The pellet was separated and washed with distilled water. After this, the pellet was suspended in acetone for 20 min. This mixture was centrifuged at 9660*g* for 10 min. Then, the pellet was mixed with 2 mL of chloroform and incubated at room temperature for 20–24 h. Later, the samples were centrifuged at 9660*g* for 10 min, and the P3HB-chloroform phase was separated. The P3HB was precipitated using 8 vols of cold methanol, and the precipitate was suspended in chloroform during 3 h before the analysis of P3HB molecular mass.

The molecular mass analysis was performed by gel permeation chromatography (GPC) using two columns in series (Styragel HR5E and HR6, Waters) in an HPLC system (Waters Alliance 2695, USA) coupled with a refractive index detector (Waters 2414, USA). The mobile phase was chloroform at 30 °C at a flow rate of 0.7 mL min^{-1}. A calibration curve was constructed with polystyrene standards (2.94×10^3–5.97×10^6 g/g_{mol}). The samples were dissolved in chloroform at a concentration of 2–3 mg mL^{-1} and were filtered through a 0.45 μm GHP membrane (PALL Acrodisc, cat. number 21854606) before being injected into the HPLC [10].

2.6. Enzyme activity assays

Bacteria were harvested by centrifugation at 9660*g* during 15 min at 4 °C and stored at −20 °C. Cells were suspended in 25 mM potassium phosphate buffer pH 7.2 with salts (137 mM NaCl, 20 mM KCl and 1 mM EDTA), then the cells were disrupted by ultrasonic treatment at 4 °C (four pulses for 15 s at 6 W) (VirSonic 60, Virtis) and centrifuged at 9660*g* during 15 min at 4 °C. The resulting centrifuged cell-free extracts were used for the following enzymatic assays.

2.6.1. β-Ketothiolase activity

The specific activity was determined by the rate of consumption of the magnesium chelated enolate of acetoacetyl-CoA (thiolysis of acetoacetyl-CoA) measured at 29 ± 0.1 °C and 303 nm (ε_{303}: 17,260 M^{-1} cm^{-1}). A quartz cuvette contained 1 mL of reaction mixture: 25 mM potassium phosphate buffer pH 7.8 with 40 mM $MgCl_2 \cdot 6H_2O$ and 1 mM dithiothreitol. After 2 min, 43.5 μM coenzymeA (20 μL) and 50 μM acetoacetyl-CoA (20 μL) were added. The reaction was initiated by the addition of 10 μL of crude cell extract (protein concentration: between 7.2 and 13 μg). One unit was defined as the activity for consuming 1 μmol of Mg^{2+} per minute [38–40].

2.6.2. Acetoacetyl-CoA reductase activity

Specific activities were determined in the forward enzymatic direction using a spectrophotometric method which followed the decrease in absorbance resulting from β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADPH) consumption at 29 ± 0.1 °C at a wavelength of 340 nm. The reaction mixture contained 100 mM potassium phosphate buffer pH 5.5 with 12 μM $MgCl_2 \cdot 6H_2O$ and 0.5 mM dithiothreitol. Later, 20 μL of 0.24 mM NADPH were added in the cuvette and immediately 20 μL of 40 μM acetoacetyl-CoA was poured. Finally, 10 μL of crude cell extract containing between 16 and 28 μg of protein was placed in the cuvette and the reaction started. One unit was defined as the activity for consuming 1 μmol of NADPH per minute (ε_{340}: 6220 M^{-1} cm^{-1}) [11,39,40].

2.6.3. P3HB synthase activity

The specific activity of the P3HB synthase was determined using a spectrophotometric assay that detected the increase of thionitrobenzoic acid (TNB) corresponding to the CoA released as a result of the condensation of monomers measured at 29 °C and wavelength of 412 nm. P3HB synthase activity was determined both in the soluble fraction of crude cell extract as in the insoluble fraction (granule-bound synthase). In a cuvette were added: 25 mM potassium phosphate buffer pH 7.0, 0.1 mM of 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 20 μL) and 55 μM of the substrate β-hydroxybutyryl-CoA (20 μL). The reaction was initiated with the addition of 10 μL of soluble or insoluble fraction that contained the enzyme (protein concentration: between 36 and 55 μg for supernatant and between 19 and 22 μg for granules). One unit was defined as the activity for producing 1 μmol of TNB anion per minute (ε_{412}: 13,600 M^{-1} cm^{-1}) [13,40].

2.6.4. P3HB granules purification and depolymerase activity assay

Granule-bound P3HB depolymerase activity was determined in native polymer granules purified from *A. vinelandii* OP. First, the cells were suspended in a lysis buffer pH 8.0 (NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 10 mM and complete 1X), disrupted in a French press (three passes at 900 psi) and centrifuged in a discontinuous glycerol gradient (87–40% v/v) at 71,000*g*, for 40 min at 4 °C. Later, the granule layer remaining between 50 and 60% glycerol fractions was recovered and the glycerol was removed by dialysis against 50 mM Tris-HCl pH 8.0 during 12 h at 4 °C. Then, the suspension of granules (containing between 7 and 10 μg of protein determined by Lowry method [33] in a suspension of granules 10 fold concentrated) in 50 mM Tris-HCl buffer pH 8.0 was prepared and the absorbance of the suspension was adjusted to ∼1.0 (measured at 600 nm).

P3HB depolymerase specific activity was measured as granule degradation through changes in the absorbance of the granule suspension incubated at 37 °C with magnetic agitation during 36 h. The values of absorbance measured in the assay were extrapolated in a calibration curve (absorbance values *vs* P3HB concentration measured by HPLC) which was set up using P3HB granules suspension at different absorbance values (0.2–1.5 measured at 600 nm). One unit of activity P3HB depolymerase was defined as the amount of polymer degraded per minute (mg P3HB min^{-1}) [27,41]. All reagents used in the enzyme activity assays were purchased from Sigma-Aldrich (St. Louis MO, USA).

2.7. Degradation in vitro of P3HB

P3HB granules degradation *in vitro* assay was carried out in order to evaluate the changes in the molecular mass of polymer in 50 mM Tris-HCl buffer pH 8.0. The complex P3HB granules-depolymerase (previously purified in a discontinuous glycerol gradient) was incu-

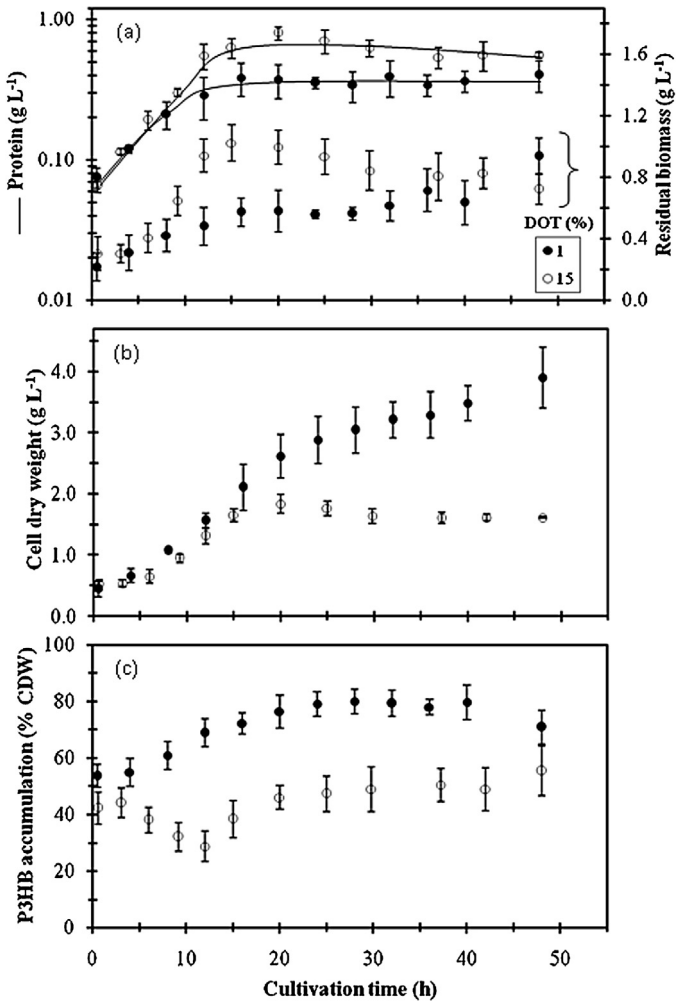

Fig. 1. Growth kinetics, measured as protein, residual biomass (a) and cell dry weight (b) and P3HB accumulation (c) under oxygen-limited and non oxygen-limited conditions. Determinations were carried out in triplicate, and the average and standard deviation for each point is shown.

bated in plastic cells for 60 h at 37 °C with magnetic agitation. At the end of the experiment, the samples were centrifuged at 9660*g* during 10 min at room temperature. Later, the P3HB granules were washed with distilled water, centrifuged at the same conditions and suspended in acetone during 10 min. Later, the samples were centrifuged and then the precipitate was suspended in chloroform between 20 and 24 h at room temperature and filtered through a 0.45 μm GHP membrane (PALL Acrodisc, cat. number 21854606) before being injected into the HPLC [10]. For these experiments, samples from 12 and 20 h of cultivation were analyzed.

3. Results

3.1. Growth and P3HB production of *A. vinelandii* OP under conditions of limitation and non-limitation of oxygen

A. vinelandii OP is a strain able to produce P3HB because it is unable to synthesize alginate [28]. When this strain was cultivated in a bioreactor under controlled dissolved oxygen conditions, the cell growth (measured as protein) was 50% lower under oxygen-limited conditions (1% of saturation of DOT) compared to the cell growth observed under non oxygen-limited conditions (15% of saturation) (Fig. 1a). The specific growth rate (μ) in the cultures grown at 1% of DOT was 0.08 h^{-1}, whereas at 15% of DOT, the μ was 0.18 h^{-1} (Table 1), confirming the important effect that oxygen tension has on bacterial growth [32]. As shown in Fig. 1a, in the cultures developed at 15% of DOT (non-limitation oxygen), the exponential growth phase lasted for 17 h, and the stationary phase started after this time. In contrast, in the cultures grown at 1% of DOT, the cell growth was not exponential but linear, due to the oxygen-limited conditions. In the cultures at 15% of DOT the growth cell stopped at approximately 17 h, probably due to nutrient limitation (phosphates or trace elements). In the case of cultures developed at 1% of DOT the residual biomass remains practically constant from 17 to 40 h and only in the period from 40 to 48 h a slight increase in residual biomass was observed (Fig. 1a).

The residual biomass (cell dry weight without P3HB) evolution was similar to that observed for the protein concentration in both oxygen conditions evaluated (1 and 15%). For the cultures at 1% of DOT, the residual biomass was 0.6 g L^{-1} and for 15% of DOT, this parameter was of 1.0 g L^{-1}. In addition, P3HB accumulation was clearly influenced by the oxygen tension of the culture, considerably affecting the cell dry weight. For the cultures at 1% of DOT, the maximum cell dry weight was of 3.0 g L^{-1}, due to a high P3HB accumulation (80% of cell dry weight). In contrast, in the cultures developed at 15% of DOT, both cell dry weight and P3HB accumulation were lower (1.7 g L^{-1} and 45%, respectively; Fig. 1b, c).

As shown in Table 1, the yield of P3HB (based on sucrose) was almost an order of magnitude higher in the cultures at 1% of DOT compared to those developed at 15% of DOT. The sucrose concentration was of 12.1 g L^{-1} at the end of the cultures developed at 1% of DOT and 0.60 g L^{-1} in the cultures at 15% of DOT (Table 1). In both DOT conditions evaluated, the sucrose was not the limiting substrate because these values were higher than 0.1 g L^{-1}, which is the value of the saturation constant (*Ks*) reported for *A. vinelandii* cultures utilizing sucrose as carbon source [42]. For the condition of non-limitation oxygen (15% of DOT), although the bacterium consumed almost all of the carbon source, this was not mobilized for the synthesis of P3HB or cell growth. A theoretical analysis showed that under this culture condition, 86% of carbon moles was directed towards the formation of CO_2.

3.2. Mean molecular mass of the P3HB accumulated under oxygen limited and non-oxygen limited conditions

It has been reported that *Azotobacter* spp. synthesize P3HB with an ultra-high molecular mass [7,10,15], and it was previously reported [10] that in *A. vinelandii*, the aeration conditions in shake flasks influence the molecular mass of the P3HB produced. Therefore, we analyzed the size of the polymer produced under 1 and 15% DOT. As shown in Fig. 2, the mean molecular mass (MMM) of P3HB was between 5500–4800 kDa during the exponential growth phase (before 17 h) for both oxygen conditions tested. Later, during the stationary growth phase (after 17 h), the MMM of P3HB decreased to approximately 3600 kDa and remained nearly constant until the end of the culture. Interestingly, the changes in the molecular mass of P3HB were very similar between oxygen-limited (1% DOT) and non-oxygen-limited (15% DOT) conditions. These results indicated that the dissolved oxygen tension, between 1 and 15% of DOT, did not determine the mean molecular mass of the P3HB produced by *A. vinelandii* OP. However, it was interesting to observe that the MMM of polymer changed over time depending on the culture growth phase.

3.3. Molecular mass distribution of the P3HB accumulated by A. vinelandii OP during exponential and stationary growth phases

A more detailed analysis of the molecular mass distribution of the P3HB along the culture revealed that the changes observed in

Table 1
Kinetics parameters calculated for oxygen-limited (DOT 1%) and non oxygen-limited (DOT 15%) conditions evaluated in this study. The values of yield of P3HB in bold were determined considering the carbon provided from the peptone and the yeast extract.

Parameter	Oxygen condition (% of saturation)	
	1	**15**
Specific growth rate (μ) (h^{-1})	0.08	0.18
P3HB accumulation (% cell dry weight)[a]	80.0	45.0
Cell dry weight (CDW: $g\,L^{-1}$)[a]	3.0	1.7
P3HB concentration ($g\,L^{-1}$)[a]	2.4	0.8
Residual biomass (CDW minus P3HB: gL^{-1})[a]	0.60	0.94
Residual sucrose ($g\,L^{-1}$)	12.1	0.6
Yield P3HB/sucrose ($g_{P3HB}\,g_{sucrose}^{-1}$)	0.32 [**0.22**]	0.04 [**0.03**]
Maximum specific oxygen uptake rate (qO_2) (mmol O_2 $g_{protein}^{-1}\,h^{-1}$)	18	39

[a] Values were obtained in the early stationary phase (between 20 and 24 h).

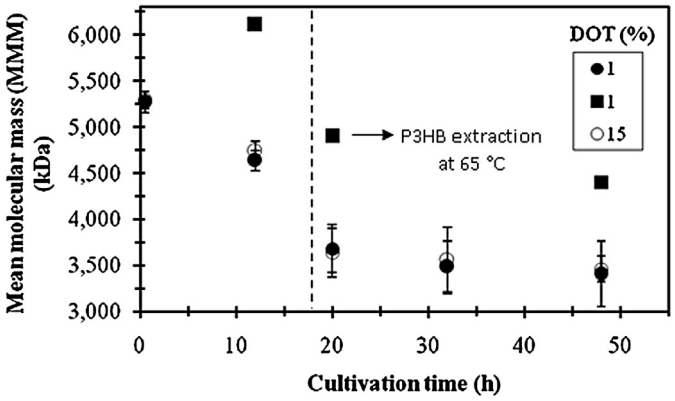

Fig. 2. Evolution of mean molecular mass of P3HB isolated from the cultures at 1% and 15% of DOT. Determination of molecular mass was carried out in triplicate and the average is shown. The square symbols are P3HB samples extracted during 2 h at 65 °C. The error bars represents standard deviation and the dashed line indicate the transition between exponential growth and stationary phases.

the MMM between the exponential growth phase (before 17 h) and the stationary phase (after 17 h) were due to an increase in the percentage of P3HB molecules with a molecular mass lower than 1.0×10^6 Da (Fig. 3). During the exponential growth phase under both oxygen conditions evaluated (1 and 15% DOT), the percentage of P3HB molecules having a molecular mass lower than 1.0×10^5 Da was between 1.0 and 2.0% of all of the fractions of polymer produced (Fig. 3a and Table 2). On the other hand, the percentage of molecules with a size from 1.0×10^5 to 1.0×10^6 Da was between 13 and 15% in the polymer obtained from the two oxygen conditions tested (Table 2). During the stationary phase (20 h), the percentage of molecules in the small size range (1.0×10^5–1.0×10^6 Da) increased almost two times. Simultaneously, the percentage of molecules with a molecular mass higher than 1.0×10^6 Da decreased between 15 and 18% compared to the percentage obtained in the exponential growth phase (12 h), under both oxygen conditions evaluated (Table 2). These changes in the molecular mass distribution of the P3HB molecules were reflected in the mean molecular mass (Fig. 2).

Table 2
P3HB percentage of different molecular mass classes at 1 and 15% of DOT.

Culture time (h)	Dissolved oxygen condition (% of saturation)	$<1 \times 10^5$	1×10^5–1×10^6	1×10^6–1×10^7	$>1 \times 10^7$
12	1	1.0	12.9	76.5	9.6
	15	1.7	15.1	71.2	12.0
20	1	7.2	21.0	64.6	7.2
	15	8.3	24.9	61.4	5.4

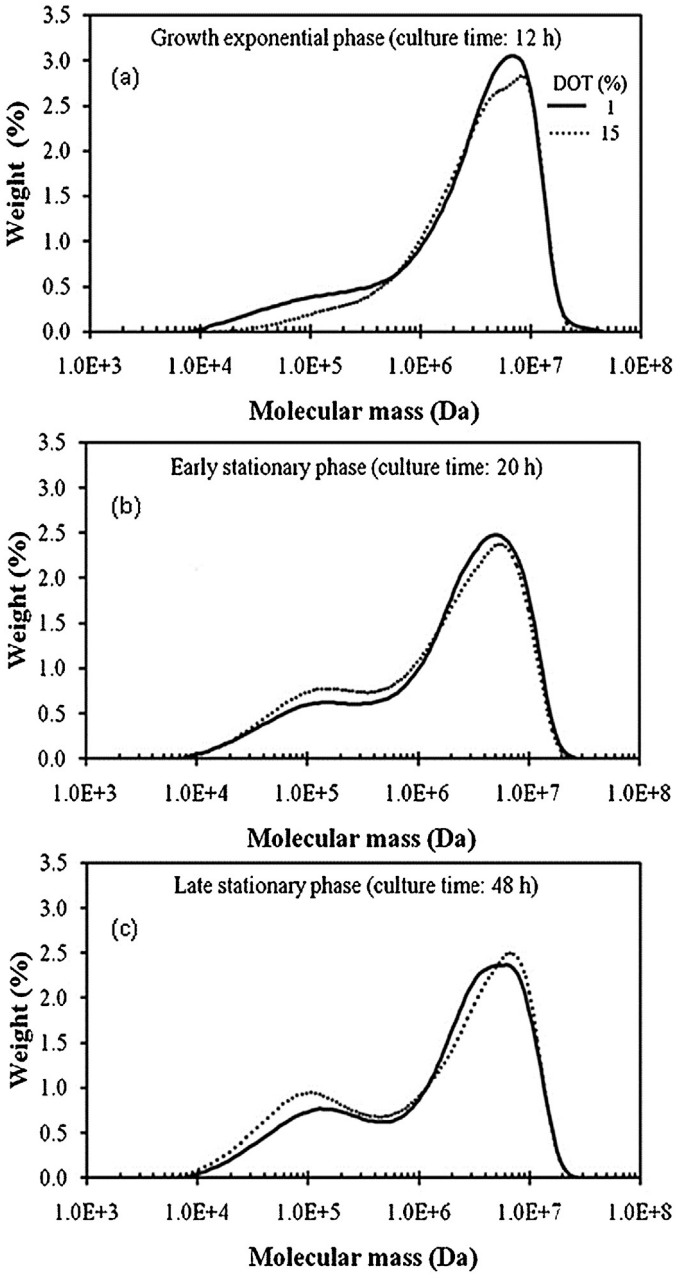

Fig. 3. Molecular mass distributions of P3HB isolated from *A. vinelandii* OP cultures under oxygen-limited (DOT: 1%, solid lines) and non oxygen-limited (DOT: 15%, dashed lines) conditions.

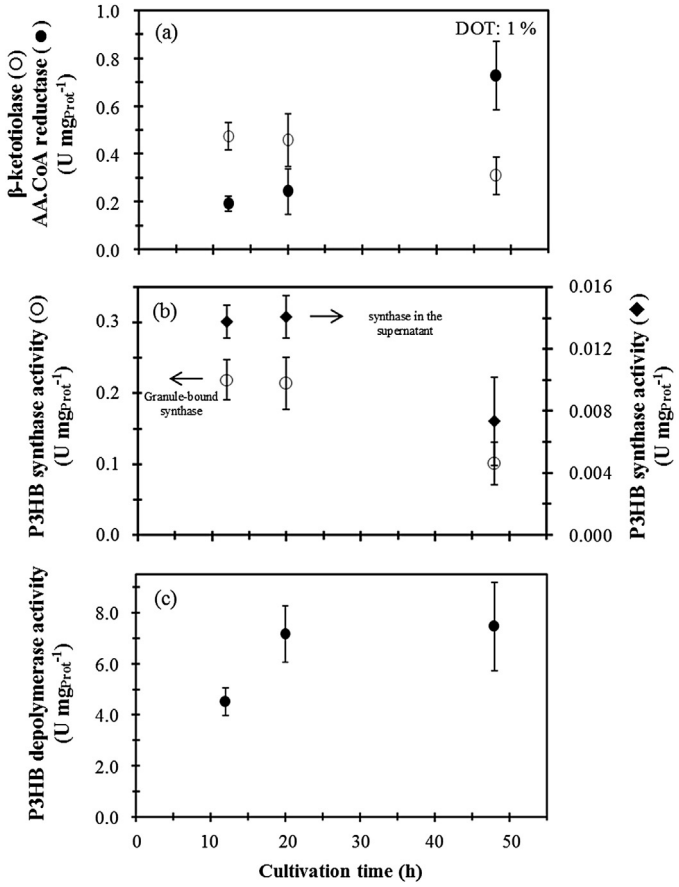

Fig. 4. Enzyme activities that participate in the P3HB cycle during exponential growth and stationary phases in cultures of *A. vinelandii* OP under oxygen-limited (DOT = 1%) conditions. For β-ketothiolase (a) acetoacetyl-CoA reductase (a) and P3HB synthase (b) 1 unit (U) was defined as μmol min^{-1} and for P3HB depolymerase (c) 1 unit (U) was defined as μg min^{-1}.

3.4. Determination of the activity of the enzymes involved in P3HB cycle

To analyze whether the changes in the molecular mass of the P3HB could be explained by changes in the activities of the metabolic cycle of this polymer and because the evolution of the mean molecular mass of the polymer and its distribution were very similar in the cultures grown at 1 and 15% of DOT, the activities of the enzymes β-ketothiolase, acetoacetyl-CoA reductase, P3HB synthase and depolymerase were determined only from the cultures grown under 1% of DOT.

The enzymatic activities were measured at 12, 20 and 48 h of cultivation. As shown in Fig. 4a, the β-ketothiolase activity maintained a constant value of approximately 0.47 U mg_{Prot}^{-1} during the first 20 h of cultivation; later, at the end of the stationary phase (48 h), the activity decreased to 0.31 U mg_{Prot}^{-1}. A similar behavior was found for the P3HB synthase measured in both the supernatants and that associated with the polymer granules (Fig. 4b). It is important to note that the high activity of the P3HB synthase was measured in the insoluble fraction (P3HB granules) and was an order of magnitude higher (0.22 U mg_{Prot}^{-1}) than the soluble fraction (0.014 U mg_{Prot}^{-1}).

Both the β-ketothiolase activity and P3HB synthase activity were increased during the exponential growth phase. The high activity of the P3HB synthase enzyme during the exponential growth phase was consistent with the high molecular mass of P3HB obtained (between 5500 and 4800 kDa) during this stage (Figs. 2 and 4). Meanwhile, high β-ketothiolase activity corresponded with the stage of P3HB accumulation (until 24 h of cultivation), and the enzyme activity decreased in the late stationary phase, when the percentage of polymer within of the cell remained unchanged (Fig. 1c).

Contrary to what was observed for these two enzymes (β-ketothiolase and P3HB synthase), the activity of the acetoacetyl-CoA reductase (Fig. 4a) remained unchanged (0.21 U mg_{Prot}^{-1}) between 12 and 20 h (exponential growth phase) but increased almost three times at the end of the stationary phase (48 h: 0.71 U mg_{Prot}^{-1}). This increase was related to the increase in the percentage of molecules of polymer with a molecular mass less than 1.0×10^6. The behavior of this enzyme neither corresponded with the mean molecular mass nor with the percentage of accumulation of P3HB produced by *A. vinelandii* OP.

The P3HB depolymerase activity was determined using native granules because these types of enzymes are found to be attached to the P3HB granules [41]. It is interesting to observe in Fig. 4c that in the exponential growth phase (12 h), the P3HB depolymerase activity was low (4.5 U mg_{Prot}^{-1}), but when the stationary phase started (after 17 h), the enzyme activity increased to 7.3 U mg_{Prot}^{-1} (Fig. 4c). This increase of P3HB depolymerase activity correlates with the decrease in the mean molecular mass of polymer and with the changes in the molecular mass distribution. Although the P3HB synthase activity was high at the beginning of the stationary phase (after 17 h), the molecular mass of the polymer decreased during this phase (Fig. 2), probably due to an increase in the P3HB depolymerase activity (20 h, Fig. 4c).

4. Discussion

The results of the present study have shown that DOT conditions between 1 and 15% influence the growth of cells (as measured by protein and residual biomass concentration) and influence P3HB accumulation and sucrose uptake in cultures of *A. vinelandii* in bioreactor. However, the P3HB molecular mass was not influenced by the DOT of the culture. In contrast, the mean molecular mass (MMM) of P3HB changed with the stage of the culture, reaching a high value during the exponential growth phase, and revealing that the culture growth phase strongly influences the degree of synthesis and degradation of P3HB. In contrast to what we observed in the present study, previous studies [10] have reported that aeration conditions affected the molecular mass of P3HB produced by *A. vinelandii* OP cultured in shaken flasks, finding that low aeration conditions promoted the synthesis of P3HB with a high molecular mass. The differences observed in the behavior of *A. vinelandii* OP cultivated in shaken flasks and in a bioreactor, under controlled conditions of dissolved oxygen, could be due to the lack of control of culture parameters in shake flask cultures, such as the pH and DOT of the medium. In addition, the oxygen transfer rates (OTR_s) evaluated in both culture systems were different. For example, in shaken flasks, the maximum OTR_s estimated using the equation proposed by [43] were between 2.8 and 5.5 mmol L^{-1} h^{-1} [44,45]. Whereas, in the bioreactor, the maximum OTR measured was 6.4 mmol L^{-1} h^{-1} for the 1% of DOT condition and 32.2 mmol L^{-1} h^{-1} for the 15% of DOT condition. It is possible that low values of OTR positively affect the molecular mass of P3HB accumulated by *A. vinelandii* OP, as it has been previously reported for the exopolysaccharide alginate produced by *A. vinelandii* ATCC 9046 under non-controlled oxygen conditions [44,46–48].

Fig. 2 and 3 show that in cultures of *A. vinelandii* grown under limited and non-limited oxygen conditions, the MMM was high (between 5500 and 4800 kDa) during the exponential growth phase until 17 h of cultivation and that this value decreased to nearly 3600 kDa during the entire stationary growth phase (between 17 and 48 h). This decrease in the MMM, likely caused by degrada-

tion of the polymer, was not due to depletion of the carbon source, as sucrose concentration remained above the limiting concentrations (12.1 and 0.6 g L^{-1} for DOT of 1 and 15%, respectively). It has been previously reported that under conditions of stress (pH, temperature, nutrients limitation), the native producers of P3HB start the degradation or mobilization of polymer within the cell [27,38,49,50]. Because *A. vinelandii* is able to fix nitrogen [51] and because the culture is continually fed by the atmospheric nitrogen in the gas mixture, it is unlikely that the reduction of the molecular mass of the P3HB was due to nitrogen limitation. A hypothesis is that the limitation of other elements, such as calcium, magnesium, potassium, iron and phosphate ions, could stop the growth of the bacteria after 17 h of cultivation time and could affect the P3HB molecular mass, as it was previously observed in *Methylosinus spp.* [52,53]. On the other hand, the limitation of these elements could be affecting the activity or expression of the enzymes that participate in P3HB synthesis and degradation, as is the case for depolymerase, which plays an important role in determining the molecular mass of the polymer.

The MMM of P3HB (between 5500 and 4800 kDa) is the highest reported thus far in the literature for *A. vinelandii* strains. These values are in line with the behavior reported by other authors for cultures of *Azotobacter* spp. [7,10,15]. When the polymer was extracted at high temperatures (65 °C), the MMM of P3HB increased between 25 and 30% (Fig. 2). It has been reported previously that the mutant strain *A. vinelandii* named UWD (ATCC 53799), which has a mutation in respiratory NADH oxidase, produced P3HB with a MMM near 4200 kDa when this strain was cultivated in a minimum medium supplement with 5% (w/v) of beet molasses under controlled DOT at 5 and 20% of saturation [15]. These authors found that the molecular mass increased during the first 16 h of cultivation, changing from 2000 kDa (12 h of cultivation) to 4200 kDa (16 h of cultivation) and later remained unchanged at values near 4200 kDa.

In the present study, we showed for the first time that the activity of the enzymes involved in the synthesis (i.e., synthase) and degradation (i.e., depolymerase) of P3HB depend on the culture growth phase and that these values correlate with the molecular mass of the polymer (Fig. 4). In the light of this evidence, it is clear that the synthesis and degradation occur simultaneously, as previously reported [22,23,26,27]. The P3HB molecular mass was higher during the exponential growth phase, probably due to the high activity of P3HB synthase and low P3HB depolymerase activity. Later, the P3HB molecular mass decreased at the beginning of the stationary phase, and this decrease in the MMM of P3HB was associated with the increase in the P3HB depolymerase activity. The reduction of MMM of P3HB during the stationary phase resulted from the increase in the percentage of P3HB molecules of smaller size (lower to 1×10^6 Da) (Fig. 3, Table 2). At the end of the stationary phase (48 h), the molecular mass class of P3HB in the interval of 1×10^4–2×10^5 increased almost three-fold compared with that observed in the exponential growth phase (12 h of cultivation) (Fig. 3). During this period (48 h), the acetoacetyl-CoA reductase activity increased nearly three times and this likely provided high 3-hydroxybutyryl-CoA concentration, which would modify the monomer concentration/P3HB synthase ratio, favoring the production of P3HB with high molecular mass by the synthase. However, in the light of evidences of the present study, this hypothesis is not supported, because the production of smaller P3HB molecules was observed (Fig. 3c). It is important to point out that it was not possible to determine if these smaller molecules of polymer were the result of the P3HB depolymerase activity (i.e., degradation) or P3HB synthase activity (i.e., synthesis). Therefore further experimental work is required to understand this phenomenon in *A. vinelandii* cultures. Despite the formation of P3HB molecules of smaller size, the accumulation percentage did not decrease because these chains were not excreted into the culture medium but remained within the bacteria.

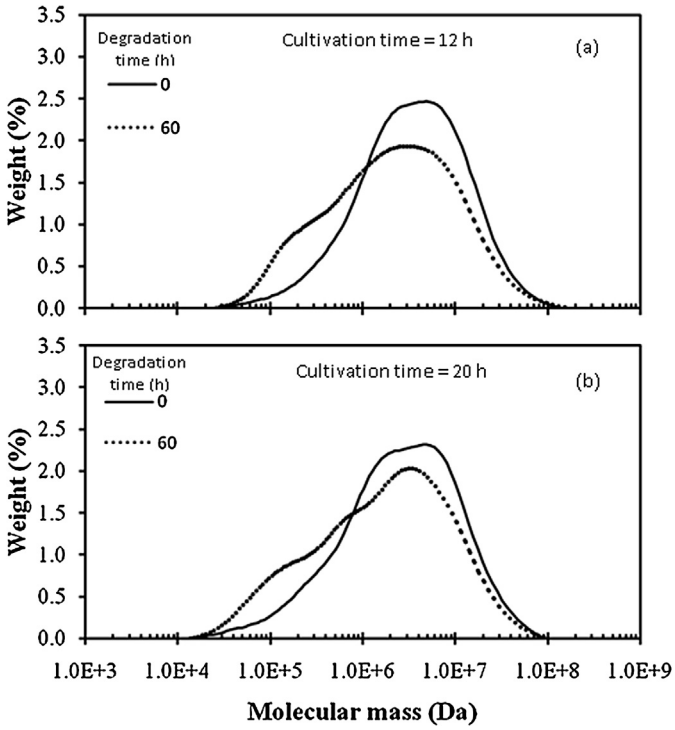

Fig. 5. P3HB molecular mass distribution before and after of degradation assay *in vitro*. The P3HB granules used correspond at 12 h (a) and 20 h (b) of cultivation time. The samples were degraded for 60 h at 37 °C.

With the aim of identifying the main products of P3HB degradation by the depolymerase, P3HB granules were hydrolyzed *in vitro* for 60 h, and the mean molecular mass of polymer was measured by GPC. In this assay, it was observed that the MMM of P3HB decreased between 25 and 30% compared with the initial molecular mass of the polymer (Table 3 and Fig. 5). In both of the samples from 12 and 20 h of cultivation, the P3HB chains with molecular masses higher than 1×10^7 decreased during the assay. In addition, in the samples from 12 and 20 h of cultivation time, the percentage of P3HB molecules with molecular mass between 1×10^4 and 1×10^6 increased. Although the water-soluble products of degradation were not measured, the mean molecular mass distribution indicated that the depolymerase enzyme generates mainly P3HB chains with a smaller molecular mass (1×10^4–1×10^5). It is likely that the oligomer and monomer products represent a minor percentage of the samples because more than 90% of the product mass, which originally was used in the reaction *in vitro*, are water-insoluble polymers (Fig. 5).

On the other hand, the behavior observed by the β-ketothiolase activity corresponded with the accumulation of polymer within of the bacterium. During the first 24 h of cultivation (i.e., the exponential growth and early stationary phases), the percentage of P3HB increased, correlating with an increase in the β-ketothiolase activity. Conversely, during the stationary phase of the culture, when the polymer accumulation stops, the β-ketothiolase activity decreased (Fig. 4a). In the case of acetoacetyl-CoA reductase activity, the behavior did not correlate with the P3HB accumulation in the bacteria and, in contrast, the activity increased in the stationary phase, when the accumulation of polymer stopped. Then, the P3HB accumulation was determined mainly by the β-ketothiolase activity because this was the first enzyme controlling the flux of acetyl-CoA toward the P3HB cycle [16,38].

Table 3
P3HB percentage of different molecular mass classes produced by P3HB depolymerase during *in vitro* assay in samples from 12 and 20 h of cultivation time.

Culture time (h)	Degradation time	Mean molecular mass	P3HB percentage of different molecular mass classes			
DOT = 1%	*in vitro* (h)	kDa	$<1 \times 10^5$	1×10^5- 1×10^6	1×10^6- 1×10^7	$>1 \times 10^7$
12	0	6160	0.1	22.7	60.7	16.5
	60	4712	3.1	35.6	49.4	11.9
20	0	4865	2.7	27.3	58.3	11.7
	60	3890	6.9	35.8	47.4	9.9

There are some reports in the literature [11,23,25–27,53] that evaluate the activity of the enzymes involved in the P3HB cycle in other microorganisms. However, very few studies have focused in the evaluation of the effect of the activity of those enzymes on the molecular mass of P3HB. For example, [27] reported that when *Cupriavidus necator* B5786 (formerly *Ralstonia eutropha*) was cultivated under autotrophic conditions, the P3HB molecular mass increased during the exponential growth phase, reaching a maximum of 967 kDa at 40 h of cultivation. Later, during the stationary phase (104 h of cultivation), the MMM of P3HB decreased to values near 350 kDa. In these experiments, the authors found that the activities of all of the enzymes evaluated from the P3HB cycle (β-ketothiolase, acetoacetyl-CoA reductase, P3HB synthase, P3HB depolymerase and 3-hydroxybutyrate dehydrogenase) were higher at the beginning of the culture (8 h). Later, in the stationary phase (i.e., between 64 and 72 h of culture) the activity of these enzymes decreased. During P3HB degradation (i.e., from 72 to 104 h of cultivation), only acetoacetyl-CoA reductase and 3-hydroxybutyrate dehydrogenase activity increased. Therefore, the authors did not find a relationship between the P3HB cycle enzymes activities and the molecular mass of the polymer.

On the other hand, in a study using *Methylosinus trichosporium* IMV3011, [26] it was found that the mean molecular mass of P3HB increased to values of 1700 kDa when P3HB synthase and depolymerase activities were maximized. In contrast, the molecular mass of the polymer was low (i.e., 1400 kDa) when the β-ketothiolase and acetoacetyl-CoA reductase activities were at the maximum, suggesting that a high P3HB molecular mass is dependent on simultaneous P3HB synthase and depolymerase activities. The results found in *C. necator*, *M. trichosporium* and in our recent study in *A. vinelandii* indicate that the molecular mass of P3HB is determined by the activity of the enzymes involved in the synthesis and degradation of the polymer and that this is dependent on the type of microorganism used, as previously was suggested [53].

In the case of the cultures of *A. vinelandii* OP, the observed behavior, specifically, the changes in molecular mass and its relationship with enzymatic activity, could be due to different factors, such as the concentration of active P3HB synthase, the concentration of substrate for the enzyme and the ratio of P3HB synthase/depolymerase [49]. This is the first time that the changes in the molecular mass of P3HB have been related to the analysis of the activity of the enzymes involved in the synthesis and degradation process in cultures of *A. vinelandii* OP.

Finally, Hiroe et al. [11] modified the order of the genes of *C. necator phbCAB* operon, affecting the relative levels of P3HB synthase, β-ketothiolase and acetoacetyl-CoA reductase. They built recombinant strains of *E. coli* containing the operons with different orders of the biosynthetic genes *phbA, phbB, phbC,* and therefore affecting their expression levels. Their results [11] demonstrated that P3HB synthase activity negatively correlates with the molecular mass of polymer and positively correlates with cellular P3HB content [11,54]. Our results were opposite to those found in recombinant *E. coli* cultures because it was found that in cultures of *A. vinelandii,* the molecular mass was higher when P3HB synthase activity was increased. These differences in the behavior observed for a recombinant or native P3HB producer could be due to a lack of P3HB depolymerase enzymes in recombinant *E. coli* strains, in which the production of polymer with ultra-high molecular mass is favored. However, it is interesting to note that despite the presence of P3HB depolymerases, *A. vinelandii* OP was able to produce P3HB with ultra-high molecular mass.

In summary, our study has shown that in cultures of *A. vinelandii* OP under fixed dissolved oxygen conditions, the DOT (between 1 and 15%) did not affect the mean molecular mass of P3HB accumulated. The values of the MMM observed are associated to the culture stage (exponential growth and stationary phase), which in turn seems to define the MMM of the polymer. This study has shown that the high molecular mass of P3HB during the exponential growth phase in the cultures at 1% of DOT was associated with the high activity P3HB synthase and that the decrease in the molecular mass of the polymer at the end of the stationary phase correlated with a higher P3HB depolymerase activity. In addition, the β-ketothiolase activity was high, whereas the P3HB accumulation increased and the activity of the enzyme decreased when the accumulation of polymer within of the cell was stopped. From a technological point of view, the understanding of how the molecular mass of the polymer is determined by the activity of enzymes involved in the P3HB cycle would allow the establishing culture strategies to produce polymers with suitable properties for specific applications in medical fields.

Acknowledgements

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Artículo de revisión

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Minireview

Biotechnological strategies to improve production of microbial poly-(3-hydroxybutyrate): a review of recent research work

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Summary

Poly-(3-hydroxybutyrate) [P(3HB)] is a polyester synthesized as a carbon and energy reserve material by a wide number of bacteria. This polymer is characterized by its thermo-plastic properties similar to plastics derived from petrochemical industry, such as polyethylene and polypropylene. Furthermore, P(3HB) is an inert, biocompatible and biodegradable material which has been proposed for several uses in medical and biomedical areas. Currently, only few bacterial species such as *Cupriavidus necator*, *Azohydromonas lata* and recombinant *Escherichia coli* have been successfully used for P(3HB) production at industrial level. Nevertheless, in recent years, several fermentation strategies using other microbial models such as *Azotobacter vinelandii, A. chroococcum*, as well as some methane-utilizing species, have been developed in order to improve the P(3HB) production and also its mean molecular weight.

Introduction

Poly-(3-hydroxybutyrate) [P(3HB)] is produced and intracellularly accumulated as a carbon and energy reserve material. It can be produced by various bacteria, such as *Cupriavidus necator*, several species of *Pseudomonas, Bacillus, Azotobacter* and also recombinant *Escherichia coli*, expressing the P(3HB) biosynthetic genes from *C. necator* and *A. vinelandii* (Centeno-Leija *et al.*, 2014). Since its discovery, P(3HB) has been used as substitute for bulk plastics, such as polyethylene and polypropylene, in the chemical industry. More recently, and based on its properties of biocompatibility and biodegradability, new attractive applications for P(3HB) have been proposed in the medical and pharmaceutical fields, where chemical composition and product purity are critical (Williams and Martin, 2005). In the medical field, P(3HB) has been used in artificial organ construction, drug delivery, tissue repair and nutritional/ therapeutic uses (Chen and Wang, 2013).

In all these applications, the molecular mass of P(3HB) is a very important feature to consider, because this determines the mechanical properties of the polymer, and in turn, the final applications. From a biotechnological point of view, the manipulation of the molecular mass of P(3HB) by means of the use of new strains and manipulating the culture conditions, seems to be a convenient method that could considerably improve the properties of P(3HB), expanding the potential application of this polymer, especially in the medical field.

Poly-(3-hydroxybutyrate) is produced by fermentation, either in batch, fed batch or continuous cultures using improved bacterial strains, cultured on inexpensive carbon sources such as beet and cane molasses, corn starch, alcohols and vegetable oils, combined with multistage fermentation systems (Lee, 1996; Chen and Page, 1997; Chen, 2009; 2010; Chanprateep, 2010; Peña *et al.*, 2011). All these strategies have been attempted to improve both the yields and process productivity in order to have a more competitive process.

There are several reviews regarding the properties and applications of P(3HB); as well as about the different microorganisms producing P(3HB) (Byrom, 1987; Sudesh *et al.*, 2000; Chen, 2009; 2010; Grage *et al.*, 2009; Chanprateep, 2010; Peña *et al.*, 2011); however, there are not recent reviews about the fermentation strategies for improving the P(3HB) production.

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This review aims to summarize the recent trends in the bacterial production of P(3HB) using novel fermentation strategies combined with the use of genetic engineering to improve productivity and quality (in terms of its molecular weight) of P(3HB) that could be applied for its commercial production.

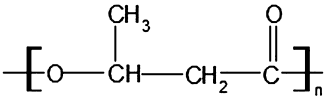
P(3HB): structure and properties

Polyhydroxyalkanoates (PHAs) are linear polyesters conformed by hydroxyacyl units. They can be found as homopolymers or as copolymers containing combined 2-, 3-, 4-, 5- or 6-hydroxyacids (Sudesh *et al*., 2000; Kessler and Witholt, 2001; Chen, 2010). Polyhydroxyalkanoates classification depends on the number of carbon atoms present in their monomers as short-chain-length PHAs (scl-PHA; three to five C-atoms) and medium-chain-length PHAs (mcl-PHA; with six or more C-atoms) (Pan and Inoue, 2009).

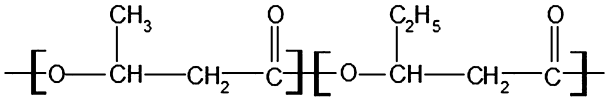
Interest in these polymers has increased in the last decades due to their thermoplastic properties, which make them a biodegradable and environmentally friendly alternative to petroleum based plastics, such as polyethylene and polypropylene. Although PHAs include a broad number of polymers of diverse monomeric composition, only few of them have been incorporated into the large-scale production: P(3HB); poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] and poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] (Chen, 2009; Chanprateep, 2010; Fig. 1).

Poly-(3-hydroxybutyrate) is the homopolymer of (R)-3-hydroxybutyrate units. It can be obtained within a wide range of molecular masses fluctuating from 200 to up to 20 000 KDa (Kusaka *et al*., 1998; Sudesh *et al*., 2000). The thermoplastic properties of P(3HB) and its biodegradability, without generation of toxic by-products, make it a sustainable alternative to petroleum-based plastics. In addition, this polymer is produced by biotechnological strategies allowing the control of its chemical composition, and therefore its physicochemical properties. Besides, this polymer shows interesting properties such as a high biocompatibility with mammalian cells, making them suitable for medical applications (Chen, 2009; 2010; Grage *et al*., 2009; Pan and Inoue, 2009; Shishatskaya *et al*., 2011; Bornatsev *et al*., 2013).

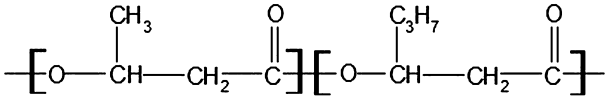
P(3HB) is a semi-crystalline polymer, characterized by a polymorphic crystallization, that is able to crystallize into two forms, α and β (Pan and Inoue, 2009). The α-form which consists in lamellar crystals, being the most common conformation for P(3HB) crystals (Pan and Inoue, 2009; Kabe *et al*., 2012) and the β-form characterized as a planar zigzag conformation which has been reported in films and fibres with high tensile strength



Poly-(3-hydroxybutyrate)



Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate)



Poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate)

Fig. 1. Chemical structure of poly-(3-hydroxybutyrate), poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate).

(Iwata, 2005; Pan and Inoue, 2009; Kabe *et al*., 2012). It must be emphasized that the crystallization process affects the thermal and mechanical properties, as well as biodegradability of biopolymers (Pan and Inoue, 2009).

The thermoplastic and crystallization properties of P(3HB) are highly dependent of its molecular mass. Poly-(3-hydroxybutyrates) of low molecular masses ($< 1 \times 10^3$ kDa) are characterized by their brittleness and an early thermal degradation, near their melting temperature (above 180°C) (Hong *et al*., 2013). This behaviour has been explained as a result of its α-form crystallization (Kabe *et al*., 2012); however, increasing P(3HB) molecular mass improves the mechanical properties of films and fibres by promoting the β-form crystallization (Kabe *et al*., 2012). In this line, using P(3HB) of ultra-high molecular weight (UHMW; $M_w = 5.3 \times 10^3$ kDa), Iwata (2005) reported that the tensile strength of the polymer could be manipulated from 38 to 1320 MPa, only by modifying the drawing method. This last value (1320 MPa) is higher than the tensile strength reported for polyethylene, polypropylene, polyvinyl alcohol and polyglycolic acid used at industrial level (Iwata, 2005).

However, up to now the UHMW-P(3HB) production has been restricted only for cultivations of low cell density, such as the cultures of recombinant *E. coli* XL-1 Blue (pSLY105), harbouring the *Cupriavidus necator* P(3HB) biosynthetic genes *phbCAB* (Kusaka *et al*., 1998; Iwata, 2005; Murakami *et al*., 2007; Kabe *et al*., 2012), mixed cultures of methane-utilizing bacteria (Helm *et al*., 2008) and *Azotobacter* cultivations (Peña *et al*., 2014).

Table 1. Thermo-mechanical properties of P(3HB) and its composites with UHMW-P(3HB) or LMW-P(3HB).

Compound	Drawn ratio	T_g (°C)	T_c (°C)	T_m (°C)	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)	Crystallinity (%)	Reference
P3HB	12[a]	1.8	53	170	161	45	2.8	78	Kabe *et al*., 2012
UHMW	10[a]	2.4	57	172	191	56	1.6	73	Kabe *et al*., 2012
UHMW/P3HB (5/95)	12[a]	2.2	53	170	242	88	1.5	75	Kabe *et al*., 2012
UHMW	60[b]	n.d.	n.d.	n.d.	1320	35	18.1	n.d.	Iwata, 2005
P3HB/LMW (87.5/12.5)	None	–2.6	93	162.3	23.4	4.2	n.d.	44.8	Hong *et al*., 2013
P3HB/LMW (83.3/16.6)	None	–4.8	82	160.5	24.3	9.8	n.d.	40.4	Hong *et al*., 2013
P3HB/LMW (75/25)	None	–7.3	76	155.8	11.6	3.8	n.d.	37.8	Hong *et al*., 2013

a. Processed by cold drawing.
b. Processed by cold drawing/two step drawing.
T_g, temperature to glass transition; T_c, crystallization temperature; T_m, melting temperature; n.d., not described.

Therefore, several strategies have been designed to improve the thermo-mechanical properties of P(3HB) including: P(3HB) composites with other PHAs [P(3HV) or P(3HHx)] or other biopolymers (i.e.: cellulose, chitosan; Rajan *et al*., 2012), the addition of chemical plasticizers (i.e.: polyethylene glycol, glycerol, glycerol triacetate, 4-nonylphenol; Hong *et al*., 2013), as well as the blending of P(3HB) of different molecular masses (Kabe *et al*., 2012; Hong *et al*., 2013).

As shown in Table 1, it is possible to modify and improve the thermo-mechanical properties of P(3HB) for specific applications by combining P(3HB) of medium molecular weight with UHMW-P(3HB) (Sharma *et al*., 2004; Kabe *et al*., 2012) or P(3HB) of very low molecular weight [LMW-P(3HB); $M_w = 1.76$ kDa] (Hong *et al*., 2013). In this line, blending P(3HB) of medium molecular weight with only 5% of UHMW-P(3HB) increased the tensile strength and elongation at break up to 33% and 48%, reaching values similar to those of conventional plastic films (Kabe *et al*., 2012). In contrast, addition of LMW-P(3HB) reduces polymer crystallinity, as well as the melting and crystallization temperature of P(3HB), but positively affects elongation at break and degradation rate (Hong *et al*., 2013), being this last characteristic of great interest for biomedical applications.

Biomedical applications of P(3HB)

Previous reviews have focused on novel applications of P(3HB) and other PHAs in several biomedical areas (Chen and Wu, 2005; Chen, 2009; Grage *et al*., 2009; Peña *et al*., 2011; Chen and Wang, 2013) which can be described as follows: material for sutures and tissue engineering, including heart valves, bone scaffolding, scaffolds for skeletal myotubes and nerve tissue (Grage *et al*., 2009; Ricotti *et al*., 2012; Masaeli *et al*., 2013); nano or micro beads for drug delivery and target-specific therapy for treatment of illness such as cancer and tuberculosis (Grage *et al*., 2009; Parlane *et al*., 2012; Althuri *et al*., 2013); and finally, its possible application as biomarker or biosensor (Grage *et al*., 2009). Table 2 summarizes some of the more recent attempts to apply P(3HB) in these fields, mainly as tissue engineering scaffolds and micro or nanoparticles for drugs delivery. It must be emphasized that, for these applications, P(3HB)s of a wide range of molecular weights (MW) have been used. For applications such as nano- or microparticles, the MW did not affect the production yield of particles (Shishatskaya *et al*., 2011). On the other hand, P(3HB) used for tissue engineering, in some cases requires to be mixed with materials such as chitosan (Cao *et al*., 2005; Medvecky *et al*., 2014; Mendonca *et al*., 2013), other PHAs (Masaeli *et al*., 2013), polyethylene glycol (PEG) (Chan *et al*., 2014), hydroxyapatite (Shishatskaya *et al*., 2006; Ramier *et al*., 2014) or even cell growth inductors (Filho *et al*., 2013). Addition of those materials allows to improve not only the mechanical properties of P(3HB) but also its degradability, hydrophilicity and its cell attachment capabilities.

Producers of P(3HB)

The ability to synthesize and accumulate P(3HB) and other PHAs as a carbon and energy reserve material is widespread among the prokaryotes. More than 300 species, mainly of bacteria, have been reported to produce these polymers (Olivera *et al*., 2001; Chanprateep, 2010). However, not all of these microorganisms have been shown to accumulate sufficient P(3HB) for large-scale production. Among the bacteria that are able to accumulate large amounts of PHA are *C. necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Azohydromonas lata* (also known as *Alcaligenes latus*), *Pseudomonas oleovorans*, *Pseudomonas putida, Aeromonas hydrophila*, *Paracoccus denitrificans*, *Methylobacterium extorquens*, *Bacillus spp*., *Azotobacter vinelandii* and recombinant *E. coli*, expressing the P(3HB) biosynthetic genes from *C. necator, A. lata* or *A. vinelandii* (Lee, 1996; Olivera *et al*., 2001; Chen, 2009; Centeno-Leija *et al*., 2014).

Table 2. Biomedical applications of P(3HB) with different molecular weights.

	Applications	P(3HB) MW (kDa)	Preparation procedure	Reference
P(3HB) LMW	Osteoblast scaffolds	220	P3HB and hydroxyapatite were mixed using mechanical and physical methods	Shishatskaya *et al.*, 2006
	Scaffolds	89–110	Blends of P3HB and chitosan at different ratios were evaluated	Medvecky *et al.*, 2014
	Nanofibrous scaffolds for bone tissue engineering	144	Electrospinning/electrospraying, P3HB and hydroxyapatite	Ramier *et al.*, 2014
P(3HB)	Nanoparticles for retinoic acid (RA) delivery	350	50 nm particles of P3HB/RA were prepared by dialysis	Errico *et al.*, 2009
	Microcapsules for drugs delivery	300	Microcapsules of 0.5–1.5 μm with P3HB and smectite clays were formed	da Silva-Valenzuela *et al.*, 2010
	Scaffolds of PHB and otholits (osteoinductor) for bone tissue regeneration	300	Solutions of P3HB and otholits (1% w/w) were electrospinning	Filho *et al.*, 2013
	Scaffolds 3D for osteoblasts engineering	524	P3HB and chitosan blends were evaluated	Mendonca *et al.*, 2013
	Scaffolds for tissue engineering	300	P3HB scaffolds were prepared by salt leaching and electrospinning	Masaeli *et al.*, 2012
	Nanofibrous scaffolds nerve tissue engineering	437	Blends of P3HB (50)/PHBV (50) were treated by electrospinning	Masaeli *et al.*, 2013
P(3HB) UHMW	Scaffolds for tissue engineering	890	Chitosan and P3HB films were prepared by emulsion blending	Cao *et al.*, 2005
	Scaffolds for nerve cells	1143	P3HB was treated with PEG reducing 10 fold-times its MW but promote cell growth	Chan *et al.*, 2014

Figure 2 shows *A. vinelandii* cells with granules of P(3HB). From the microorganisms mentioned, the more successful species for production at pilot or large scale are *C. necator*, *A. lata* and recombinant *E. coli*, being able to accumulate up to 80% of the polymer from a final dry cell weight of up to 200, 60 and 150 g l^{-1} respectively (Chen, 2009).

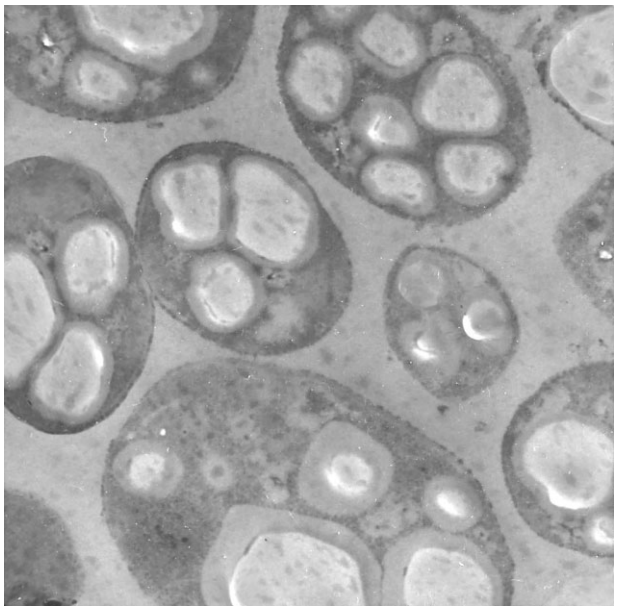


Fig. 2. Transmission electron micrograph of a thin section of *A. vinelandii* containing P(3HB) granules (white inclusions).

Many species of Archaea have also been shown to be PHA producers, particularly members of *Haloarchaea* (Legat *et al.*, 2010; Poli *et al.*, 2011). These organisms could present important advantages as PHA producers because they can utilize cheap carbon sources (Huang *et al.*, 2006), they do not need strict sterilization (they are able to grow in hypersaline conditions, in which very few organisms can survive), and because they can release the polymer produced easily because they lyse in distilled water, facilitating its isolation and lowering the production costs (Hezayen *et al.*, 2000; Poli *et al.*, 2011). The carbohydrate-utilizing species *Haloferax mediterranei* is particularly interesting because it accumulates large amounts of P(3HB) on glucose or starch, it grows optimally with 25% (w/v) salts and accumulates 60–65% of polymer (w/w) (Rodriguez-Valera and Lillo, 1992). *H. mediterranei*, shows the highest potential for industrial application because it can reach cell concentrations of 140 g l^{-1}, with a PHA content of 55.6% reaching a PHA concentration of 77.8 g l^{-1} in a repeated fed-batch fermentation (Huang *et al.*, 2006), and it is also able to produce a P(3HB-co-P3HV) copolymer (10.4 mol% 3HV) from enzymatic extruded starch (Chen *et al.*, 2006).

Metabolic pathways and genetics involved in production of P(3HB)

The biosynthetic pathway for P(3HB) (Fig. 3) starts with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. The enzyme catalyzing this reaction

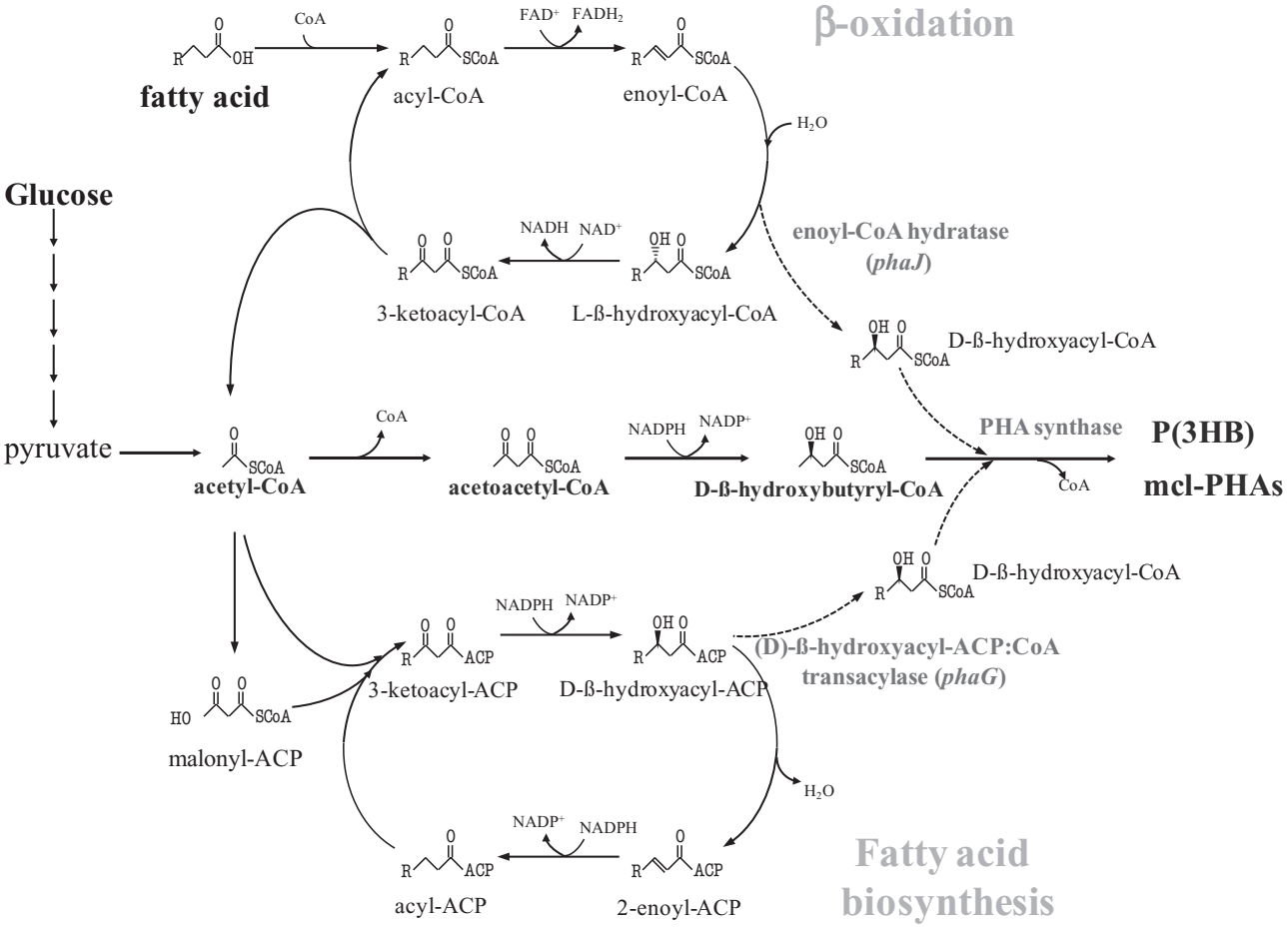


Fig. 3. Metabolic pathways and genetics involved in the production of P(3HB).

is 3-ketothiolase, encoded by the *phbA* gene. An acetoacetyl-CoA reductase (gene *phbB*) coverts the acetoacetyl-CoA to 3-hydroxybutyryl-CoA using NADPH. Finally, the enzyme PHA synthase (encoded by *phbC*) polymerizes the 3-hydroxybutyryl-CoA monomers to P(3HB), liberating CoA (Rehm, 2003; Stubbe *et al*., 2005) (Fig. 3). In some species, the P(3HB) biosynthetic genes *phbA*, *phbB* and *phbC* are clustered and are presumably organized in one operon *phbCAB* (Reddy *et al*., 2003); although this gene order varies from species to species, and the genes can also be unlinked. More than 60 PHA synthase genes (*phbC* or *phaC*) from eubacteria have been cloned and sequenced, and many more have been revealed in the bacterial genomes sequenced (Steinbüchel and Lütke-Eversloh, 2003). Other genes whose products are also involved in PHA metabolism and their specific metabolic roles have been reviewed by Chen (2010).

Besides P(3HB), other PHAs containing 150 different monomers have been reported. This PHA diversity is due to the broad substrate range exhibited by the PHA synthases, the PHA polymerizing enzymes (Steinbüchel and Lütke-Eversloh, 2003; Stubbe *et al*., 2005; Volova *et al*., 2013). The different PHAs are synthesized depending also on the carbon source provided; the metabolic routes present to convert that carbon source into the hydroxyacyl-CoA monomers, and the specificity of the PHA synthase of that particular organism. The biosynthetic pathways reported up to date have been reviewed recently (Lu *et al*., 2009; Chen, 2010; Panchal *et al*., 2013), so we only present a brief description of the routes involved. For the synthesis of PHAs composed of 3-hydroxyalkanoic acids of C6–C16 (referred to as mcl-PHAs) the hydroxyacyl-CoA precursors are derived from fatty acid metabolism (Fig. 3). These precursors can be obtained either from ß-oxidation of alkanes, alkanols or alkanoic acids (De Smet *et al*., 1983; Brandl *et al*., 1988; Lagaveen *et al*., 1988), mainly by an enantioselective enoyl-CoA hydratase (encoded by *phaJ*) that produces the (R)-hydroxyacyl-CoA (Tsuge *et al*., 2003), or from fatty acid *de novo* biosynthesis using an (R)-3-hydroxyacyl-ACP:CoA transacylase (encoded by *phaG*) to produce the substrates for the PHA synthase from a non-related carbon source, such as carbohydrates (Rehm

et al., 1998; Hoffmann *et al*., 2000a,b; Matsumoto *et al*., 2001).

Molecular strategies to improve P(3HB) production

Although many of the P(3HB) production systems use non-genetically modified bacterial strains, some efforts have been undertaken to increase the production of these polymers by genetic manipulation. These efforts include mainly the modification of the metabolism to favour P(3HB) synthesis, the modification of regulatory systems controlling P(3HB) synthesis and recombinant *phb* gene expression.

The P(3HB) biosynthetic routes compete for precursors with central metabolic pathways, such as the tricarboxylic acid (TCA) cycle, fatty acid degradation (ß-oxidation) and fatty acid biosynthesis. They also compete with other biosynthetic pathways that use common precursors. Three examples of genetic modifications that favour P(3HB) synthesis by metabolism modification of the producer strain were reported in *A. vinelandii*. Page and Knosp (1989) reported a strain (UWD), which has a mutation in the respiratory NADH oxidase that resulted in the ability to accumulate P(3HB) during the exponential phase without the need of nutrient limitation. The second example is found in the inactivation of pyruvate carboxylase, the anaplerotic enzyme catalyzing the ATP-dependent carboxylation of pyruvate, to generate oxaloacetate that replenishes the TCA cycle (Segura and Espín, 2004). This mutation increased three times the specific production of P(3HB) ($g_{P(3HB)}$ $g_{protein}^{-1}$), in contrast with the wild type strain *A. vinelandii* UW136, probably as a result of a diminished flux of acetyl-CoA into TCA cycle, leaving it available for P(3HB) synthesis. In the same bacterium, a mutation blocking the synthesis of alginate, an exopolysaccharide produced by this organism, increased the P(3HB)-specific production up to five times, depending on the growth conditions evaluated, with a higher yield based on glucose as compared with the wild type strain ATCC9046. The mutation not only increased the capacity of the bacterium to produce P(3HB) per biomass unit, but also allowed an increased growth, raising the volumetric production of the polymer up to 10-fold (Segura *et al*., 2003).

Regarding the modification of regulatory systems controlling PHA synthesis to increase their production, some interesting examples are also found in *A. vinelandii*. Poly-(3-hydroxybutyrate) synthesis in this bacterium is regulated by the nitrogen-related phosphotransferase system (PTS^{Ntr}), where the non-phosphorylated form of the IIA^{Ntr} protein negatively regulates the expression of the P(3HB) biosynthetic operon (Segura and Espín, 1998; Noguez *et al*., 2008). Another system regulating P(3HB) synthesis in *A. vinelandii* is the post-transcriptional regulatory system RsmZ/Y-A, where the RsmA protein represses translation of the mRNAs of the *phbBAC* biosynthetic operon and of *phbR* that codes for its transcriptional activator (Hernández-Eligio *et al*., 2012). In each case, negative regulators IIA^{Ntr} and RsmA were identified (Fig. 4). In order to have P(3HB) overproducing strains of *A. vinelandii* OP, the gene coding for the IIA^{Ntr} (*ptsN*) was inactivated. This mutation increased 77% the specific production of P(3HB), equivalent to 4.1 g l^{-1} of PHB (3.5 g l^{-1} in the case of the wild type), with a 36% higher yield of product based on the consumed substrate (Peña *et al*., 2014). Later, a mutant where both negative regulators (IIA^{Ntr} and RsmA) were inactivated was constructed (Fig. 4), further increasing the P(3HB) accumulation capacity of *A. vinelandii*. This strategy, together with the implementation of a fermentation strategy allowed to produce 27 g l^{-1} of P(3HB) (García *et al*., 2014).

Another case illustrating production improvement by manipulation of regulatory systems is found in the cianobacterium *Synechocystis* sp. PCC 6803. In this bacterium, the overexpression of the sigma factor SigE, previously shown to activate the expression of many sugar catabolic genes and to enhance the levels of acetyl-CoA, increased the production of P(3HB) two or three times (Osanai *et al*., 2013).

Fast growth on simple media and the possibility to reach a high cell density in the culture with a high-content P(3HB) are important factors to consider for a successful P(3HB) production process. Because *E. coli* is an extensively studied bacterium with well-established technologies for genome manipulation, cultivation and downstream processing, many studies have focused on the use of *E. coli* to efficiently produce these polymers. This bacterium is a non-PHA producer; however, the genes of the P(3HB) producer *C. necator* H16 were cloned in *E. coli* for the first time by Slater *et al*. in 1988, enabling the production of P(3HB) in this organism. Since then, many different genetic modifications have been attempted, both to improve the accumulation of P(3HB) at low-cost with high productivity and to produce diverse copolymers using metabolic engineering and synthetic biology strategies. These strategies have been reviewed recently (Li *et al*., 2007; Wang *et al*., 2013).

Fermentation strategies to improve the production of P(3HB)

Effect of carbon source on P(3HB) production

The mayor expenses in the production of P(3HB) are determined by the cost of the fermentation substrate, the polymer extraction from the cells and the treatment of fermentation and extraction wastes (Chen, 2010). Of all these factors, the cost of the carbon source has the greatest influence on the price of P(3HB). Because of the

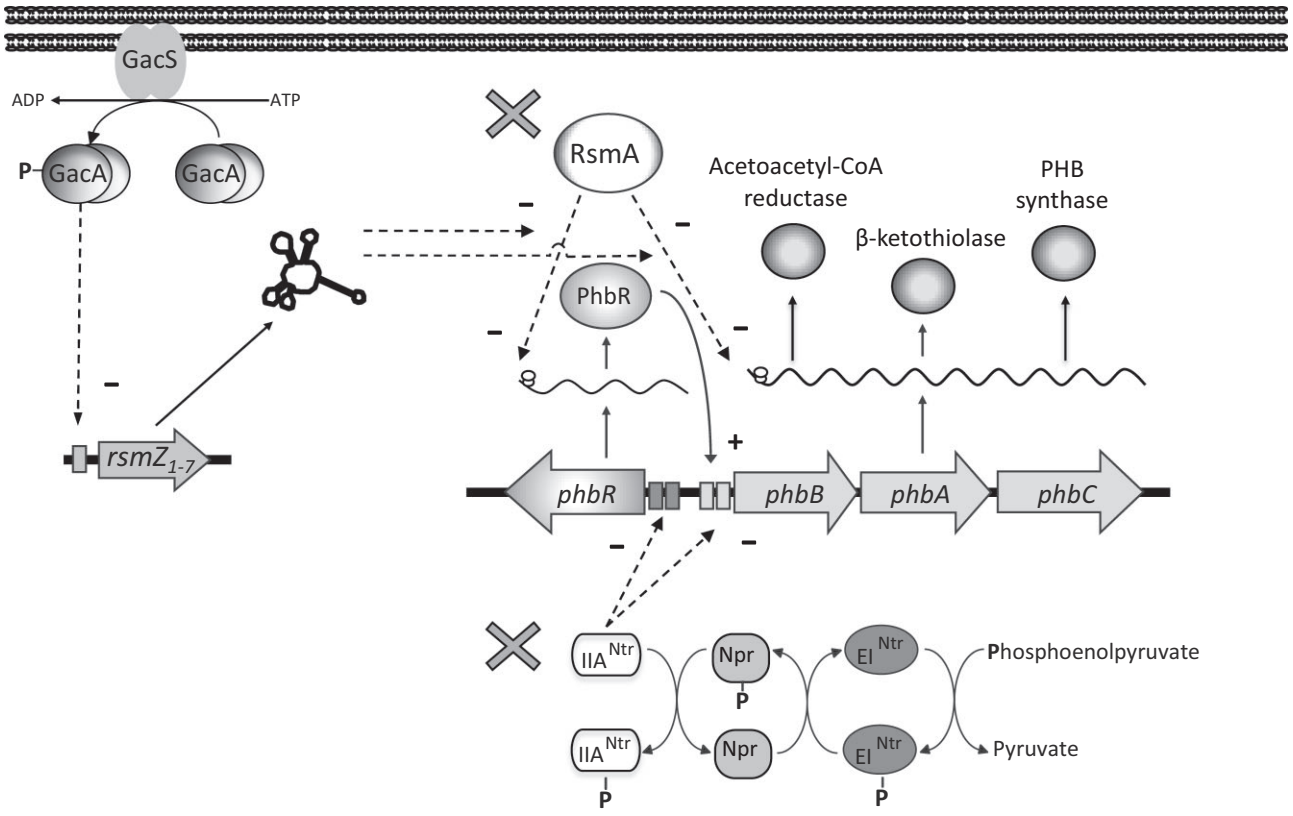


Fig. 4. Model of the regulatory systems controlling the expression of the *phb* genes in *A. vinelandii*. (+) indicate positive regulation; (–) indicate negative regulation. Promoters are indicated as rectangles. The regulators inactivated in the *A. vinelandii* improved strains OPN and OPNA are indicated by a grey cross.

above, new alternatives have been proposed to reduce the costs of raw materials. It is important to note that the selection of carbon sources should not focus only on the market prices but also on the availability and on global price (Chanprateep, 2010).

Table 3 summarizes different carbon sources used for the P(3HB) production. Fortunately, most P(3HB) producers can metabolize a wide range of raw materials. For example, it is known that several species of *Azotobacter* can use corn syrup, cane molasses, beet molasses or malt extract as carbon sources (Kim, 2000; Myshkina *et al*., 2008; Peña *et al*., 2011). For example, Kim (2000) reported the use of two inexpensive substrates, starch and whey, to produce P(3HB) in fed-batch cultures of *A. chroococcum* H23 and recombinant *E. coli*. These authors found that in fed-batch culture of *A. chroococcum* H23 a cell concentration of 54 g l^{-1} with 46% (w/w) P(3HB) was obtained with oxygen limitation, whereas 71 g l^{-1} of

Table 3. Comparison of P(3HB) volumetric production, content and yields using different carbon sources.

Organism	Carbon source	Quantity of carbon source (g) employed	DCW (g l^{-1})	P(3HB) concentration (g l^{-1})	P(3HB) content (%)	P(3HB) yield based on carbon source (g g^{-1})	Reference
A. lata	Sucrose	72.9	10.78	5.25	48	n.d.	Zafar *et al*., 2012a
C. necator DSM545	Glucose	523	164	125	76.2	0.22	Mozumder *et al*., 2014
	Waste glycerol	n.d	104.7	65.6	62.7	0.52	Mozumder *et al*., 2014
	Waste glycerol	170.8	76.2	38.1	50	0.34	Cavalheiro *et al*., 2009
	Pure glycerol	249	82.5	51.2	62	0.36	Cavalheiro *et al*., 2009
A. chrococcum H23	Alpechin/acetate	30/0.06	7.36	6.10	82.9	n.d	Pozo *et al*., 2002
	Starch	200	54	25	46	n.d	Kim, 2000
E. coli recombinant GCSC 6576	Whey	340	31	25	80	n.d.	Kim, 2000

n.d., data not described.

cells with 20% (w/w) P(3HB) was achieved without oxygen limitation. In the case of whey as carbon source, using recombinant *E. coli* 6576, Kim (2000) reported a P(3HB) content of 80%, with a cell concentration of 31 g l^{-1}.

On the other hand, P(3HB) and P[3HB-co-3HV]) copolymers were produced by *A. chroococcum* strain H23, when growing in culture media supplemented with wastewater from olive oil mills (alphechin), as the sole carbon source (Pozo *et al*., 2002). A maximal concentration of P(3HB) of 6.2 g l^{-1} was reached when the cells were cultured in shaken flasks at 250 r.p.m. for 48 h at 30°C in liquid medium supplemented with 60% (v/v) alpechin and 0.12% (v/v) ammonium acetate (Table 3). Production of PHAs by *A. chroococcum* strain H23 using alpechin looks promising, as the use of a cheap substrate for the production of these materials is essential if bioplastics are to become competitive products.

In this context, crude glycerol (a by-product of the large-scale production of diesel oil from rape) has been evaluated for its potential use as a cheap feedstock for P(3HB) production (Cavalheiro *et al*., 2009; Mozumder *et al*., 2014). Bacteria used has been *C. necator* DSM 545, which accumulated P(3HB) from pure glycerol up to a content of 62.5% (w/w) of cell dry mass, reaching a volumetric production of 51.2 g l^{-1}, with a yield on glycerol of 0.36 g $_{P(3HB)}$ g $_{gly}^{-1}$ (Cavalheiro *et al*., 2009). On the other hand, when this by-product was used by Mozumder and colleagues (2014), a maximal biomass concentration of 104.7 g l^{-1} was reached, with a P(3HB) concentration in the broth culture of 65.6 g l^{-1}. In addition, the molecular weight of P(3HB) produced with *C. necator* from glycerol varies between 7.86×10^2 kDa (with waste glycerol) and 9.57×10^2 kDa (with pure glycerol), which allows the processing by common techniques of the polymer industry (Cavalheiro *et al*., 2009).

A recent report on P(3HB) production using *A. lata* has been published (Zafar *et al*., 2012a). In this study, the optimization of P(3HB) production process using *A. lata* MTCC 2311 was carried out. By using a genetic algorithm on an artificial neural network, the predicted maximum P(3HB) production of 5.95 g l^{-1} was found, using 35.2 g l^{-1} of sucrose and 1.58 g l^{-1} of urea (Zafar *et al*., 2012a); however, the highest experimental P(3HB) concentration (5.25 g l^{-1}) was achieved using 36.48 g l^{-1} of sucrose. The same authors reported that the use of propionic acid together with cane molasses allowed the synthesis of the copolymer P(3HB-co-3HV) in maximal concentrations of 7.2 g l^{-1} in shaken flasks and of 6.7 g l^{-1} in 3-L bioreactor (Zafar *et al*., 2012b).

Fermentation strategies

Only a few species of bacteria producing P(3HB) have been used at industrial scale to produce the polymer. These include *C. necator*, *A. lata* and recombinant *E. coli* (Khanna and Srivastava, 2005). On the other hand, there are some bacteria, such as *A. vinelandii* and *A. chroococcum* which can accumulate a high P(3HB) content and therefore could be used for the synthesis of this polymer at large scale.

Several studies have been carried out which described the P(3HB) production by several microbial strains, either in batch, fed batch or continuous cultures. Batch fermentation for P(3HB) production is a popular process due to its flexibility and low operation costs. However, batch cultures have the disadvantage that, usually, the yields and productivities of P(3HB) are low. In this sense, the P(3HB) production in batch cultures of *C. necator* ATCC 17699 has been studied using acetic acid as a carbon source (Wang and Yu, 2001). In this study, the P(3HB) productivity was of only 0.046 g l^{-1} h^{-1} employing a carbon/nitrogen (C/N) weight ratio of 76, with a maximal accumulation of P(3HB) close to 50% (w/w).

The systems more often employed for P(3HB) production are those involving two or three stages. These fermentations have been widely used for the production of P(3HB) and other PHAs (Ruan *et al*., 2003; Rocha *et al*., 2008). The fed-batch cultures have been employed to achieve high cell densities and a high concentration of P(3HB) (Kulpreecha *et al*., 2009). Fed-batch cultivations are systems where one or more nutrients are supplied to the bioreactor and the products and other components are kept within the system until the end of fermentation. This means that there is an inflow but no outflow, and the volume changes with respect to time (Mejía *et al*., 2010). There are several ways to feed the cultures, and it is possible to add one or more components.

Currently there are reports in the literature about the use of exponentially fed-batch cultures for P(3HB) production with microorganisms as *A. lata* (Grothe and Chisti, 2000). These authors obtained a maximal biomass concentration of 36 g l^{-1} with a P(3HB) volumetric production of 20 g l^{-1} by varying the components in the culture. More recent studies have shown that a total concentration of 4.5 g l^{-1} of P(3HB) was obtained using limiting conditions of dissolved oxygen with processed cheese whey supplemented with ammonium sulfate in fed-batch culture *of Methylobacterium sp. ZP24* (Nath *et al*., 2008). This investigation reflects the possibility of developing a cheap biological route for production of green thermoplastics.

Recently, an integrated model was used for the optimization of the production of P(3HB) with tailor-made molecular properties in *A. lata*. A single-shot feeding strategy with fresh medium free of nitrogen was designed and experimentally tested. Using this strategy, a maximal concentration of P(3HB) of 11.84 g l^{-1} was obtained, equiva-

Table 4. Comparison of P(3HB) production using different microorganism and fed-batch strategies.

Organism	Feeding strategy	DWC (g l^{-1})	P(3HB) (g l^{-1})	P(3HB) productivity (g l^{1} h^{-1})	P(3HB) content (% wt)	Reference
B. megabacterium BA-019	pH stat	72.6	30.5	1.27	42	Kulpreecha *et al*., 2009
	Intermittent	90.7	41.6	1.73	46	Kanjanachumpol *et al*., 2013
C. necator	Pulses	75	53	0.92	71	Tanadchangsaeng and Yu, 2012
	Pulses	83	67.2	2.5	81	Pradella *et al*., 2012
	Pulses	82.5	51.2	1.52	62	Cavalheiro *et al*., 2009
	Exponential + coupled to alkali addition monitoring + constant with N2 limitation	164	125	2.03	76.2	Mozumder *et al*., 2014
E. coli	pH stat	119.5	96.2	2.57	80	Ahn *et al*., 2000
A. vinelandii	Exponential + pulses	37.2	27.3	0.5	73.3	García *et al*., 2014

lent to polymer content equal to 95% (w/w) of dry cell weight (Penloglou *et al*., 2012a).

Table 4 shows the more recent results reported about of maximal concentration and productivity of P(3HB) reached using different microorganism and fed-batch systems. From these studies, the cases for P(3HB) production using *Bacillus megaterium, C. necator*, recombinant *E. coli* and *Azotobacter* are highlighted. For example, Kulpreecha and colleagues (2009) reported a high P(3HB) production (30.5 g l^{-1}) and P(3HB) productivity (1.27 g l^{-1} h^{-1}) in a fed-batch culture of *B. megaterium* BA-019 using sugarcane molasses as a carbon source. More recently, Kanjanachumpol and colleagues (2013) found that in cultures of *B. megaterium* BA-019 with intermittent feeding of the sugarcane molasses and an increase of the C/N ratio at 12.5 improved the biomass and volumetric productivity of P(3HB), reaching a maximal biomass concentration of 90.7 g l^{-1} with 45.84% (w/w) of P(3HB) content and a productivity of 1.73 g l^{-1} h^{-1} P(3HB).

In the case of *C. necator*, Tanadchangsaeng and Yu (2012) reported a significant increase in P(3HB) volumetric production and productivity (53 g l^{-1} and 0.92 g l^{-1} h^{-1} respectively) in a fed batch using glycerol as a carbon source. Considering this, they suggested that glycerol is an ideal feedstock for producing bioplastics via bacterial fermentation due to its ubiquity, low price and high degree of reduction. However, the productivities reported using glycerol as carbon source (Cavalheiro *et al*., 2009) are still relatively low compared to other reports. An example is the high P(3HB) productivity reached by *C. necator*, using soybean oil in fed-batch culture (Pradella *et al*., 2012). In this study, the authors reported a maximal P(3HB) concentration of 67.2 g l^{-1} with a volumetric productivity of 2.5 g l^{-1} h^{-1}. On the other hand, Mozumder and colleagues (2014) using *C. necator*, developed a three-stage feeding strategy using glucose as the sole carbon source that resulted in a P(3HB) concentration of 125 g l^{-1}, with a P(3HB) content of 76% achieving a productivity of 2.03 g l^{-1} h^{-1}.

Another successful case is that reported by Ahn and colleagues (2000), who developed fermentation strategies for P(3HB) production from whey by recombinant *E. coli* strain CGSC 4401. Using a pH stat fed-batch cultures, adding a concentrated whey solution containing 280 g l^{-1} was possible to reach final cell and P(3HB) concentrations of 119 and 96 g l^{-1} respectively, at 37.5 h, with a maximal productivity of 2.57 g l^{-1} h^{-1} (Table 4). The strategies developed in this study provide an attractive solution to whey disposal and utilization of this raw material for the P(3HB) production at large scale.

For several decades the synthesis of P(3HB) by *Azotobacter* strains has been the subject of studies, either in batch (Page and Knosp, 1989; Page *et al*., 2001; Myshkina *et al*., 2008), continuous (Senior *et al*., 1972; Senior and Dawes, 1973) or fed-batch cultures (Page and Cornish, 1993; Chen and Page, 1997; Kim and Chang, 1998; García *et al*., 2014). However, the information related with the fermentation systems has been scarce in recent years. On the other hand, to our knowledge, none of these processes has yet been adopted for the industrial production of P(3HB).

Recently, our group reported (García *et al*., 2014) a mixed fermentation strategy based on exponentially fed-batch cultures (EFBC) and nutrient pulses with sucrose and yeast extract to achieve a high concentration of P(3HB) by *A. vinelandii* OPNA, which carries a mutation on the genes encoding IIA^{Ntr} (*ptsN*) and RsmA (*rsmA*) that negatively regulate the synthesis of P(3HB). Using a strategy of exponential feeding coupled with nutrient pulses (with sucrose and yeast extract), the production of P(3HB) increased sevenfold (with respect to the values obtained in batch cultures) to reach a maximal P(3HB) concentration of 27.5 ± 3.2 g l^{-1} at 60 h of fermentation (Table 4). Overall, the use of the OPNA mutant of *A. vinelandii*, impaired in the P(3HB) regulatory systems, in combination with a mixed fermentation strategy, could be a feasible strategy to optimize the P(3HB) production at industrial level (García *et al*., 2014).

Table 5. Influence of culture conditions on the molecular mass of PHB.

Organism	Carbon Source	Condition	MMW (kDa)	PHB content (%)	Reference
C. necator	Acetic Acid	Low C/N ratio = 4	820	50	Wang and Yu. 2001
		High C/N ratio = 72	520		
A. lata	Sucrose	C/N ratio = 20	2576	15	Penloglou *et al*., 2012b
		C/N ratio = 8	596	35	
		C/P ratio = 8	2076	27	
A. vinelandii UWD	Beet molasses	5% (w/v)	4100	N.S.	Chen and Page, 1994
	Beet molasses	10% (w/v)	3500		
	Sucrose	5% (w/v)	1600		
A. chroccoccum 7B	Sucrose	2% (w/v)	1200–1600	74–79	Myshkina *et al*., 2008
	Sucrose+Molasses		590	60	
E. coli XL-1	Glucose Xylose	pH = 6.0–7.0	2000–2500	32–35	Bocanegra *et al*., 2013
A. chroccoccum 6B	Glucose	0.5 VVM	1100	63.5	Quagliano and Miyazaki, 1997
		2.5 VVM	100	7.6	
A. vinelandii OPN	Sucrose	Low aeration	2020	67	Peña *et al*., 2014
		High aeration	1010	62	

N.S., not specified.

Influence of the culture conditions on the P(3HB) molecular mass

The molecular mass (MM) of P(3HB) determines the elastic behaviour of the material and its mechanical resistance (Iwata, 2005). Fibres of P(3HB) with a MM of about 3.0×10^2 kDa have a tensile strength of 190 MPa and an elongation at break of 5%. In contrast, the tensile strength of fibres of P(3HB)-UHMW with a MM of 5.3×10^3 kDa could be manipulated to increase up to sevenfold (1320 MPa) with an elongation at break of 57% (Iwata, 2005). Therefore, for P(3HB) commercial production, it is desirable to obtain polymers with a suitable molecular mass for their final application, especially in the medical field.

It has been described by several authors how the P(3HB) molecular mass depends on the culture conditions such as: medium composition, pH and oxygen availability. In the next section, the influence of these parameters on the molecular weight of the P(3HB) will be discussed.

Medium composition. The effect of the medium composition on the P(3HB) MM has been reported for *Azotobacter* species, *C. necator, A. lata* and for methane-utilizing mixed cultures (Chen and Page, 1994; Wang and Yu, 2001; Helm *et al*., 2008; Myshkina *et al*., 2008; Penloglou *et al*., 2012b).

Wang and Yu (2001) reported that the mean molecular mass (MMM) of P(3HB) produced by *C. necator* could be altered by the medium composition, under chemically defined conditions and using acetic acid as carbon source. These authors evaluated the effect of C/N ratio on the MMM. The MMM of the polymer was higher (8.2×10^2 kDa) in cultures developed under low C/N ratio, with respect to those obtained under high C/N ratio ($MM = 5.2 \times 10^2$ kDa) (Table 5). However, the amount of P(3HB) per residual biomass increased from 0.5 to 1.2 $g_{P(3HB)}\ g_{biomass}^{-1}$ increasing the C/N ratio.

On the other hand, in *A. lata*, Penloglou and colleagues (2012b) evaluated in 2-L shaken flasks cultures the effect of the initial C/N ratio and carbon/phosphates (C/P) weight ratio on the MM of P(3HB). These authors reported that the polymer reached highest MMM values (2.5×10^3 kDa) for a C/N ratio of 20 and an MM of 2.0×10^3 kDa when C/P ratio was 8; however, under such growth conditions, the P(3HB) accumulation was lower than 30%. Also, these authors observed that the MM diminished up to 20 and 3 times-fold as the C/N or C/P ratios decreased to 6 and 0.8, respectively.

The role of the potassium, iron and sulfur deficiency on the MM of the P(3HB) has been studied in methane-utilizing mixed cultures by Helm and colleagues (2008). In two-stages cultures (with a continuous-growth phase and a discontinuous P(3HB)-accumulation phase), P(3HB) accumulation was higher in those cultures under potassium deficiency (33.6% w/w) than the accumulation obtained under iron and sulfur-deficiency conditions. With respect to the MM of the P(3HB), the highest value (3.1×10^3 kDa) was obtained in the cultures developed under potassium deficiency, and the lowest value (1.7×10^3 kDa) was achieved in those cultures lacking iron. It must be emphasized that the MM of 3.1×10^3 kDa is up to now the highest value reported for methanotrophic bacteria.

In the case of *A. vinelandii*, Chen and Page (1994) reported that strain UWD produced a polymer with a high-molecular weight (4.1×10^3 kDa), when this bacterium was grown in 5% w/v beet molasses medium. The polymer MM decreased when the beet molasses concentration was increased. Similar results were obtained in equivalent concentrations of sucrose (as raw sugar), but

the polymer MM was not greater than 1.6×10^3 kDa (Table 5).

For the producer strain *A. chroococcum* 7B, it has been shown that the MM of P(3HB) depends on changes in the medium composition, specially carbon source (Myshkina *et al*., 2008). These authors described that the MM of P(3HB) obtained using glucose, sucrose or starch as carbon sources, oscillated around 1.2×10^3 and 1.6×10^3 kDa (Table 5). However, the P(3HB) MM decreased to 5.9×10^2 kDa when *A. chroococcum* 7B was cultured using sucrose complemented with molasses at 2% (w/v). The negative effect of the introduction of molasses suggested that presence of organic acids in this kind of raw material affected P(3HB) biosynthesis. To confirm this behaviour, the MM of P(3HB) was evaluated in cultures of *A. chroococcum* 7B using sucrose (2% w/w) supplemented with sodium acetate at different concentrations (from 2 to 5 g l^{-1}). Under such conditions, the MM of P(3HB) decreased as the acetate concentration increased. These results, provided an original method for production of P(3HB) with predetermined MM within a wide range, from 2.7×10^2 kDa (using 2% sucrose w/v and acetate 5 g l^{-1}) to 1.5×10^3 kDa (with sucrose as a sole carbon source).

Influence of pH. The pH of the broth culture is a critical parameter for the optimal production of P(3HB). Reports have been published about the influence of this parameter on the concentration and molecular weight of this polymer (Kusaka *et al*., 1998; Myshkina *et al*., 2008; Bocanegra *et al*., 2013).

In this line, Myshkina and colleagues (2008) reported in shake flask cultures using *A. chroococcum* strain 7B that the mean molecular weight (MMW) of P(3HB) was influenced by the pH of the broth culture, finding that the MMW was maximum (1485 kDa) when the bacterium was grown at neutral pH (7.0). A variation of pH in the interval of 6.0 to 8.0 allowed the synthesis of PHB of predetermined MMW in a wide range from 354 to 1485 kDa, determined by capillary viscometry.

On the other hand, Kusaka and colleagues (1998) reported that in cultures of recombinant *E. coli* XL-1 Blue (pSYL105), harbouring *C. necator* P(3HB) biosynthesis *phbCAB* genes, the MM of P(3HB) could be manipulated by changes in pH, reaching one of the highest values of MM reported for P(3HB) (11×10^3 kDa) when *E. coli* cultures were grown at pH 6.5, and this value dropped up to 10-fold times (1.1×10^3 kDa) when pH increased to 7.0.

More recently, Bocanegra and colleagues (2013) evaluated P(3HB) production by recombinant *E. coli* XL-1 Blue harbouring plasmid pSK::*phbCAB* at three different pHs (6.0, 6.5 and 7.0). Cultures in bioreactor using glucose as the sole carbon source at variable pH values (6.0, 6.5, or 7.0) allowed the production of P(3HB) with MMW varying between 2.0 and 2.5×10^3 kDa. These values were significantly higher than those obtained by natural bacterial strains (0.5–1.0 MDa). However, in contrast to that reported by Kusaka *et al*., 1998, no influence of pH was observed on the MMW of the polymer produced (Table 5).

Influence of aeration conditions. There are reports in the literature where the influence of the aeration conditions on the MMW of P(3HB) has been evaluated. Quagliano and Miyazaki (1997) evaluated different levels of aeration in a stirred bioreactor for *A. chroococcum* 6B. These authors reported that at lower aeration (0.5 vvm), the MM of P(3HB) (determined by the intrinsic viscosity) was of 1.1×10^3 kDa. In contrast, at higher aeration (2.5 vvm), the molecular weight significantly decreased at values of 1.0×10^2 kDa. In addition, Myshkina and colleagues (2008) found that by culturing *A. chroococcum* 7B in shake flasks, the molecular mass of P(3HB) increased from 1.48×10^3 to 1.67×10^3 kDa when the agitation rate decreased from 250 to 190 r.p.m. (Table 5). However, the yield of P(3HB) on biomass was very similar in both conditions evaluated.

Previous studies in our group revealed that the MM of P(3HB) is strongly influenced by both the aeration condition and the strain tested (Peña *et al*., 2014). In that study, a maximal MM of 2.02×10^3 kDa was observed for the P(3HB) isolated from the cultures of OPN mutant under low aeration conditions at 60 h of cultivation. A similar behaviour was observed in the polymer produced by the OP strain, obtaining a P(3HB) with a MW of 1.65×10^3 kDa at the same time. In contrast, in the cultures at high aeration, the molecular weight of P(3HB) decreased to 1.01×10^3 kDa and 5.51×10^2 kDa for the OPN and parental strain (OP) respectively (Table 5).

Finally, it is important to point out that the MM can be controlled to some extent by genetic manipulation. An interesting example was reported by Hiroe and colleagues (2012). They showed that the concentration of active PHA synthase, relative to that of the enzymes supplying the monomer has a negative correlation with the P(3HB) molecular weight. They were able to construct strains producing a high molecular weight polymer by changing the order of the *phaA, phaB* and *phaC* genes within the operon, which in turn determines their relative expression level. Another example illustrating the effect of genetic changes on P(3HB) MM control was reported by Zheng and colleagues (2006). A deletion of 78 amino acid residues from the highly variable N-terminal fragment of the P(3HB) synthase of *C. necator*, resulted in a 60-fold increase in the average molecular weight, reaching a size of 2.84×10^3 kDa. An α-helix structure was predicted in this region, and mutations disrupting this structure at amino acids 75 and 81 were shown to also increase

50-fold the size of the polymer, allowing simultaneously a higher production of the P(3HB).

Conclusions and future prospects

In this article, several aspects about P(3HB) polymer production using different microorganisms and fermentation strategies have been reviewed. It is clear that the commercial applications of P(3HB) depend on the characteristics of the polymer. In this sense, it has been shown that the strain and culture conditions employed determine the molecular mass of the P(3HB) produced, and that this characteristic can also be further modified by genetic alteration of the producer strain. The understanding of the regulatory mechanisms controlling the synthesis of P(3HB) has also helped in some cases to construct mutants improved for P(3HB) production. In addition, some recombinant strains have shown to produce sufficient P(3HB) for large-scale production. The development of fermentation strategies has also shown promising results in terms of improving the productivity. Undoubtedly, the fed-batch fermentation and the multistage systems seem to be the more suitable strategies for improving the P(3HB) production. By using this kind of systems, it has been possible to reach a very high yields and productivities of P(3HB). Overall, the use of recombinant strains, in combination with a multistage fermentation process and raw materials for low cost could be a feasible strategy to optimize the P(3HB) production at the industrial level. However, the cost of the substrates for P(3HB) production and extraction of these materials is still the bottleneck, which limits the possibility to market them at larger scale. For this reason, the implementation of systems of production by mixed microbial cultures and wastes as substrates seems to have many advantages in the close future. In addition, the use of Archaeabacteria could be a feasible strategy to the PHA production, because they can utilize cheap carbon sources and are able to grow under extreme conditions, in which other microorganisms do not survive.

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Conflict of interest

None declared.

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Approaches for the Synthesis of Tailor-Made Polyhydroxyalkanoates

2

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Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics. These have been proposed for a wide range of biotechnological applications, especially in the field of the medicine and chemistry. PHAs are produced by more than 300 bacterial species, the most efficient being *Cupriavidus necator* (*formerly Ralstonia eutropha*), *Alcaligenes latus*, and recombinant strains of *Escherichia coli*. PHAs are produced by fermentation using different culture systems, from batch culture to exponentially fed-batch cultures, and it is known that culture conditions, such as pH, aeration, and nutritional conditions, influence the chemical characteristic PHAs synthesized by microorganisms; because of that, it has been proposed that by manipulating the microbial metabolism and culture conditions, it is possible to design biopolymers with specific chemical properties. This paper describes four cases of PHAs production: the copolymers of poly-3-hydroxybutyrate-co-poly-3-hydroxyvalerate [P(3HB-co-3HV)] and poly-3-hydroxybutyrate-co-poly-3-hydroxyhexanoate [P(3HB-co-3HHx)], the medium-chain-length PHAs, the P3HB of ultrahigh molecular mass, and finally, the production of other short-chain-length PHAs, with a special emphasis on the species that have been reported for their production as well as the molecular and fermentation strategies evaluated in order to modify the chemical composition of PHAs.

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2.1 Introduction

Commercial interest in bioplastics has increased due to the possibility of replacing synthetic materials, which have disadvantages from the environmental perspective. In this regard, polyhydroxyalkanoates (PHAs) are a suitable option to substitute the plastics derived from petroleum.

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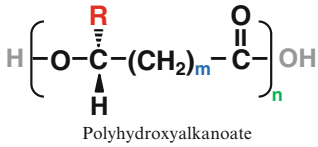

Polyhydroxyalkanoate

PHA	R	m	Name of the monomer
SCL	H	1	3-hydroxypropionate (3HP)
	CH_3	1	3-hydroxybutyrate (3HB)
	H	2	4-hydroxybutyrate (4HB)
	C_2H_5	1	3-hydroxyvalerate (3HV)
	H	3	5-hydroxyvalerate (5HV)
MCL	C_3H_7	1	3-hydroxyhexanoate (3HHx)
	C_5H_{11}	1	3-hydroxyoctanoate (3HO)
	C_7H_{15}	1	3-hydroxydecanoate (3HD)
	C_9H_{19}	1	3-hydroxydodecanoate (3HDD)
	C_9H_{18}	1	3-hydroxydodecenoate (3HDDe)
	$C_{11}H_{23}$	1	3-hydroxytetradecanoate (3HTD)

n = 1 - 230,000 monomers.

Fig. 2.1 Chemical structure of polyhydroxyalkanoates. *R* alkyl group, *m* length of carbon chains, *n* number of monomers

PHAs are polyesters composed of 3-hydroxy fatty acid monomers (Fig. 2.1) (Chen 2010; Peña et al. 2014a). The main advantages of these biopolymers are their biodegradability and biocompatibility, making them suitable for a wide range of applications, from the traditional plastic industry to their use as materials in the biomedical field, with emphasis on chemical composition and purity of the product (Peña et al. 2014a; Leong et al. 2014). PHAs are synthesized by many microorganisms as energy reserve material. In general, it has been well documented that these polymers are produced under nutrient limitation, mainly nitrogen, phosphorus, or oxygen (Anderson and Dawes 1990; Peña et al. 2011, 2014a; Ienczak et al. 2013). There is a wide variability in PHA composition that includes homopolymers, heteropolymers, and up to 150 different types of monomers (Steinbüchel and Lütke-Eversloh 2003). The thermomechanical properties of PHAs and therefore their specific applications will depend on their chemical structure, specifically monomer composition (type, ratio, and distribution) and, in the case of homopolymers, their mean molecular mass (MMM). In this line, several attempts, that include genetic manipulation of microorganisms as well as changes on the culture conditions in which the cells are grown, have been evaluated in order to obtain materials of specific characteristics (Reddy et al. 2003; Peña et al. 2014a; Leong et al. 2014). The subjects covered by this chapter include properties of PHAs and their applications, bacterial sources and PHAs biosynthesis, as well as the influence of culture conditions (i.e., medium composition, temperature, pH, etc.), which determine the composition of PHAs, including specific examples regarding the production of PHAs with different chemical compositions.

2.2 Chemical Structure, Physicochemical Properties, and Applications of PHAs

Polyhydroxyalkanoates (PHAs) are polyesters produced and accumulated by several bacteria as a carbon and energy reservoir. These polymers protect organisms against starvation and may enable them to survive under adverse conditions. The PHA accumulation occurs mainly under conditions of excess of carbon and limitation of other nutrients (Anderson and Dawes 1990; Peña et al. 2011, 2014a). These polymers are water-insoluble and they are stored in the cytoplasm as granules (Legat et al. 2010). The monomeric composition of PHAs depends primarily on the microbe and the type of the carbon used for growth. Based on their monomeric chemical

structure, three PHA groups can be defined: the short-chain-length PHAs (SCL-PHAs), with monomers from 3 to 5 carbon atoms, the medium-chain-length PHAs (MCL-PHAs) composed of units from 6 to 15 carbon atoms, and, finally, the long-chain-length PHAs (LCL-PHAs) with monomers of more than 15 carbon atoms (Peña et al.2014a; Leong et al. 2014; Fig. 2.1). On the other hand, PHAs could also be classified as homopolymers, such as P3HB, or copolymers that could be found as SCL copolymers, MCL copolymers, and SCL-MCL-PHA copolymers. The thermoelastic properties of the PHAs will be influenced by the type, ratio, and distribution of the monomer units (Leong et al. 2014; Table 2.1); homopolymers of SCL-PHAs such as the poly-3-hydroxybutyrate P3HB are brittle and stiff materials, while copolymers of MCL-PHAs have improved elastomeric properties (Reddy et al. 2003). It must be emphasized that thermoprocessibility, biodegradability, and biocompatibility of PHAs make them of great interest for biomedical applications such as the emerging field of tissue engineering (Hazer et al. 2012; Peña et al. 2014a; Leong et al. 2014).

2.3 Bacterial Sources of Polyhydroxyalkanoates

PHAs are produced by several bacterial and archaea species (Olivera et al. 2001; Chanprateep 2010; Peña et al. 2014a). It is noteworthy that species able to produce and accumulate these biopolymers could be found in diverse environments, from marine sediments with genera such as *Vibrio*, *Beneckea*, and *Paracoccus* (López-Cortés et al. 2010) to soil environments, where species such as *Azotobacter vinelandii, Bacillus* spp., *Cupriavidus necator*, and *Pseudomonas* spp. are natural producers of PHAs, or even species which can be found on extreme hypersaline environments, in which the *haloarchaeal* genera *Haloferax, Halococcus, Halobacterium, Halorubrum*, and *Haloarcula* are an interesting group for PHA production (Legat et al. 2010; Poli et al. 2011; Kumar et al. 2013). Until now only two species have been successfully used for PHA production at a commercial scale: *C. necator* and *Azohydromonas lata* (Chen 2009; Ienczak et al. 2013); however, some of the genera and species listed above could bring advantages for the tailor-made production of these biopolymers, such as *Haloferax mediterranei*, which produces the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer in high-cell-density cultures, reaching PHA concentrations of up to 77.8 g L^{-1} (Huang et al. 2006). Furthermore, production of PHAs using this archaea has additional advantages. For example, some archaea have the ability to grow on hypersaline conditions, using inexpensive carbon sources, and the feasibility to lyse cells using distilled water, which could be of great impact for PHA recovery costs (Hezayen et al. 2000; Huang et al. 2006; Poli et al. 2011). In contrast, *Pseudomonas* species are able to produce a wide range of MCL-PHAs from cheap and renewable substrates, such as plant oils. In addition, various *Bacillus* spp. are able to accumulate homopolymer P3HB and copolymer P(3HB-co-3HV) using different carbon sources as glycerol, carbohydrates, and biowaste (pea shells) and also produce MCL-PHAs, when various carbon sources are co-fed or when this bacterium was employed as host for overexpression of the biosynthetic operon *phaCAB* from *P. aeruginosa* and *C. necator* (Singh et al. 2009; Kumar et al. 2009, 2013, 2015). Another interesting case is *E. coli*, a nonnatural PHA producer; however, recombinant strains of this bacterium harboring PHA biosynthetic genes from *C. necator, A. lata*, *A. vinelandii*, or *Pseudomonas oleovorans* are important alternatives for the production of a wide range of PHAs, which *E. coli* can synthesize using a wide range of substrates. *E. coli* can grow in high-cell-density cultures and does not have PHA depolymerases, unlike natural PHA producers (Lee 1996; Olivera et al. 2001; Reddy et al. 2003; Chen 2009; Centeno-Leíja et al. 2014; Leong et al. 2014). In addition to the production of PHA by bacteria and archaea in pure cultures, the use of microbial mixed cultures for the production of these polymers is an attractive alternative. The mixed culture of several microbial species in one single process allows the use of very low-cost complex substrates, or mixtures of substrates, such as those present in waste materials (derived from agro-industry or other waste sources), with no sterilization requirements and with the possibility of a continuous process (Kleerebezem and Loosdrecht

Table 2.1 Mechanical properties and applications of PHAs

PHA	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (GPa)	Crystallinity (%)	Applications	References
P3HB (1400 kDa)	161	45	2.8	71	P3HB with Mw: 1143 for scaffolds for nerve cells	Domínguez-Díaz et al. (2015); Chan et al. (2014)
P3HB (230 kDa)	43	5	–	46	Construction of cast film for the growth of human embryonic cells (HEK293) without any cytotoxic effect	Domínguez-Díaz et al. (2015)
P(3HB-4HB) (62–38 mol%)	–	48	0.65	–	Biomaterials for human dermal fibroblasts and orthopedic support. Furthermore, P(3HB-4HB) is a potential temporary substrate that can be used in transplantation to replace damaged bone or skin	Chanprateep et al. (2010)
P(3HB/3HB-co-3HHx) (100/70–30 mol%)	2.0	10	0.19	53.7	Construction of matrices as cell growth supporting materials for applications in skin engineering and in nerve regeneration	Li et al. (2008)
P(3HB/3HB-co-3HHx) (100/60–40 mol%)	3.5	17	0.32	50.8	–	–
P(3HO-co-3HHx) (88–12 mol %)	9.0	380	0.008	–	Scaffolds for tissue engineering of cardiac valves; suitable for engineering both soft and hard tissues	Jana et al. (2014)
P(3HB-co-3HV) (80–20 mol %)	32	50	1.2	–		

2007). In this type of cultures, it is very difficult to determine the species composition; however, some studies have started to identify some of the PHA-producing species present. Particularly interesting are those works reporting *Alcaligenes, Azoarcus, Amaricoccus, Comamonas, Achromobacter, Pseudomonas, Kluyvera, Acine-tobacter, Paracoccus, Xanthobacter, Curto-bacterium, Flavobacterium*, and *Thauera* (Dionisi et al. 2005, 2006, 2007; Serafim et al. 2006; Lemos et al. 2008) as the dominant genera present in mixed cultures promoting high PHA accumulation.

2.4 PHA Biosynthesis Pathways

PHAs biosynthesis and its regulation has been well documented (Anderson and Dawes 1990; Slater et al. 1992; Steinbuchel and Schlegel, 1991; Peña et al. 2011, 2014a). The synthesis of P3HB, the simplest SCL-PHA, involves three enzymatic reactions; the first reaction involved the condensation of two molecules of acetyl-CoA, mainly from the tricarboxylic acid (TCA) cycle, into acetoacetyl-CoA by the β-ketothiolase (encoded by *phaA*). Then, acetoacetyl-CoA gets reduced to 3-hydroxybutyryl-CoA (3HB-CoA) with the help of the enzyme acetoacetyl-CoA reductase (encoded by *phaB*). Finally, the PHA synthase (encoded by *phaC*) polymerizes the 3-hydroxybutyryl-CoA monomers to P3HB, with the subsequent liberation of CoA (Stubbe et al. 2005; Peña et al. 2011, 2014a). However, biosynthesis of PHAs with different monomeric compositions involved biosynthetic pathways of hydroxyacyl-CoA thioester precursors (Fig. 2.2). In the case of the biosynthesis of SCL-copolymers such as P(3HB-co-3HV), two pathways are involved, leading to C4 monomer (3-hydroxybutyryl-CoA) or to C5 monomer

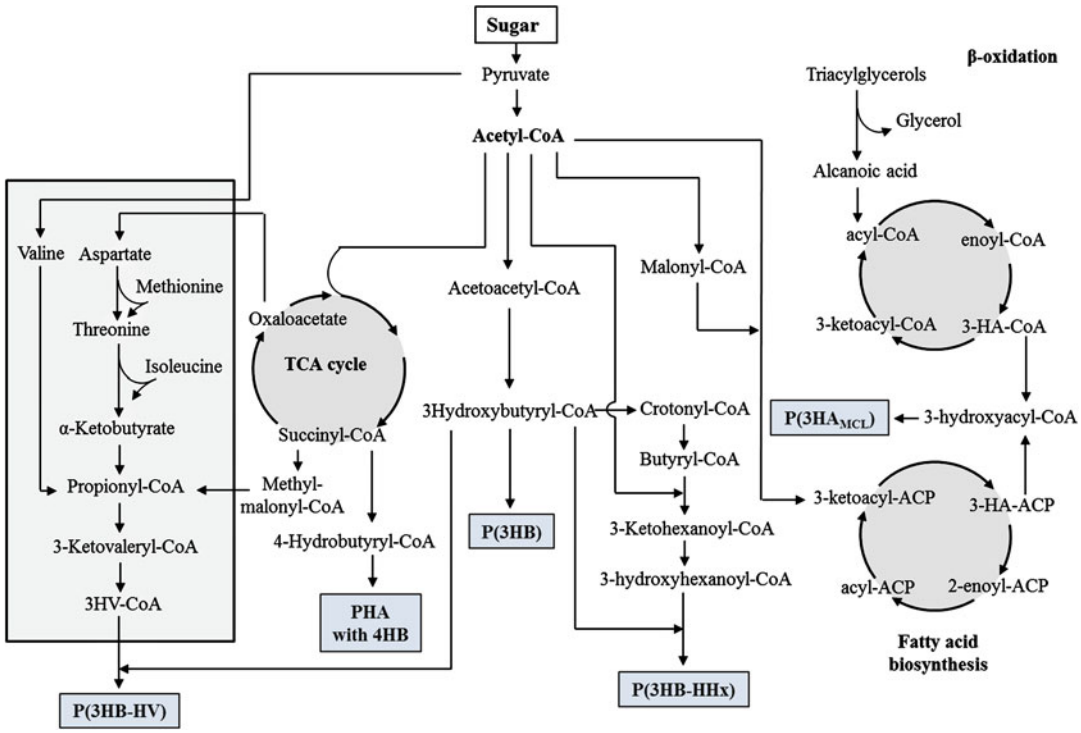

Fig. 2.2 Pathways involved in the biosynthesis of polyhydroxyalkanoates. Amino acid metabolic pathways, the tricarboxylic acids cycle, butyrate metabolism, fatty acid biosynthesis, and β-oxidation pathways (from left to right) are shown. Abbreviations: *ACP* acyl-carrier protein, *3HB* 3-hydroxybutyric acid, *3HA* 3-hydroxyalkanoic acid, *HV* hydroxyvaleric acid, *4HB* 4-hydroxybutyric acid, *HHx* hydroxyhexanoic acid, *MCL* medium chain length

(3-hydroxyvaleryl-CoA) (Steinbuchel and Schlegel 1991). As it has been previously described, the synthesis of the monomer of 3-hydroxybutyryl-CoA involves the condensation of two molecules of acetyl-CoA and its further reduction to 3-hydroxybutyryl-CoA which will be available for its incorporation into the copolymer by the PHA synthase (*phaC*). On the other hand, formation of the 3-hydroxyvaleryl-CoA involves the condensation of acetyl-CoA and propionyl-CoA into 3-ketovaleryl-CoA, a reaction catalyzed by the β-ketothiolase (*phaA).* Afterward 3-ketovaleryl-CoA is reduced by the acetoacetyl-CoA reductase (*phaB*) into the monomer 3-hydroxyvaleryl-CoA which could be incorporated into the growing polymer chain by the PHA synthase or polymerase (*phaC*). In this case, the propionyl-CoA, precursor of the 3-ketovaleryl-CoA, could be the result of the amino acid metabolism, from threonine, which could be converted in α-ketobutyrate and then reduced to propionyl-CoA with the help of the enzyme pyruvate dehydrogenase (Slater et al. 1998; Fig. 2.2), or it could be synthesized through β-oxidation during the growth of bacteria on fatty acids, amino acids, and other substrates that can first be converted into fatty acids (Steinbuchel and Schlegel 1991).

For the biosynthesis of the MCL-PHAs, which are composed of C_6 to C_{15}, two pathways are involved: one of these is the biosynthesis and degradation of fatty acids (*β*-oxidation pathway), wherein a wide variety of substrates are available for the polymer production (Lageveen et al. 1988; Timm and Steinbüchel 1990). From the fatty acid metabolism, precursors such as enoyl-CoA, hydroxyacyl-CoA, and ketoacyl-CoA could be used as substrates for the PHA polymerase for their further conversion into MCL-PHAs (Kraak et al. 1997; Lageveen et al. 1988; Fig. 2.2). Fatty acid biosynthesis is built by adding two carbons through intermediates linked to acyl-carrier protein (ACP), whereas in the *β*-oxidation pathway, two carbons are reduced from the fatty acyl substrates, the whole process liberates a molecule of acetyl-CoA in each cycle and their intermediates are linked to CoA (Fig. 2.2). Although, both fatty acid metabolic pathways are present in all organisms, carbon sources can vary and affect MCL-PHA production. The capability of incorporating different hydroxyacyl-CoA units will be dependent on the PHA synthase (*phaC*). There are two types of these enzymes: the Type I which is harbored by organisms such as *C. necator* and synthesizes SCL-PHAs and the Type II which is present mainly in *Pseudomonas* and is able to polymerize MCL-PHAs.

2.5 Effect of the Culture Conditions on the PHAs Synthesized by Native and Recombinant Bacteria

It is known that the culture conditions affect the chemical characteristics of PHAs synthesized by microorganisms, and those chemical properties have an important effect on the mechanical properties and therefore the final applications of PHAs. In this section, some cases regarding the manipulation of the chemical composition of PHAs by the manipulation of strains and the culture conditions will be discussed (Tables 2.2 and 2.3).

Case 1: Production of the Heteropolymers P(3HB-co-3HV) and P(3HB-co-3HHx)

The copolymers poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] are conformed by monomers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) or 3-hydroxyhexanoate (3HHx), respectively. Both copolymers are interesting candidates as alternative materials for replacement of petrochemical plastics. In the case of P(3HB-co-3HV), this was manufactured and commercialized by ICI (Biopol), Zeneca BioProducts, Biomer Inc. (Biomer), and Tianan Biologic (Enmat) (Braunegg et al. 1998; Chanprateep 2010).

Due to the presence of 3HV or 3HHx residues, polymer crystallinity is reduced, and these residues contribute to an increase of flexibility, elasticity, and melting temperature as compared with the homopolymer P3HB (Feng et al. 2002; Zhuang et al. 2014). In this line, when the molar ratio of 3HV is of only 20 mol %, the copolymer

Table 2.2 Production of polyhydroxyalkanoates under different culture conditions

PHA	Organism	Production scale	Culture conditions	Biomass (g L^{-1})	PHA content (%)	References
P3HP	Recombinant *E. coli*	Fed-batch fermentation 2 L	Crude glycerol Pure glycerol For both: 300 mM, 37°C and 400 rpm for 92 h	5.2 12.0	5.2 11.8	Andreeβen et al. (2010)
P3HB, P3HHx, P3HO, P3HD	Recombinant *E. coli*	Shake flasks	Decanoate Glucose and decanoate Cultures kept at 37 °C and 200 rpm	2.5–3.0 4.3–5.1	1.8–9.3 5.9–36.4	Li et al. (2011)
P4HB	Recombinant *E. coli*	Bioreactor 1 L	Glycerol (20 g L^{-1}) with K-4HB (4 g L^{-1}), 32 °C, pH 7, 800 rpm, 30 % DO_2, 1 L air/min, for 48 h Glucose (20 g L^{-1}) with K-4HB (4 g L^{-1}), propionic acid, and NZ-amines (1 g L^{-1}), 32 °C, pH 7, 800 rpm, 30 % DO_2, 1 L air/min. for 48 h	N.S. 6.5–6.7	2.0–61.0 63.0–65.0	Kämpf et al. (2014)
P(3HB-co-3HHx) (90–10 mol %)	*E. coli* LS5218 (pBBJPC)	Shake flasks	M9 medium supplied with glucose (20 g L^{-1}), 37 °C, 200 rpm, for 48 h	7.8	14.1	Wang et al. (2015)
P3HP	*E. coli* Q1911 (harboring both pHP302 and pHP513)	Baffled shake flasks 500 mL	100 mL of minimal medium with glycerol (20 g L^{-1}) and glucose (3 g L^{-1})	4.9	10.2	Wang et al. (2014)
P(3HB-3HHx-3HO-3HD-3HDD-3HTD) (21.2, 6.1, 45.8, 11.0, 9.2, 6.8 mol %)	*E. coli* LS5218	Shake flasks batch 300 mL	50 mLof medium supplemented with glucose (30 g L^{-1}), 30 °C, 250 rpm, for 72 h	6.5	12.1	Zhuang et al. (2014)
P(3HB-co-3HV) (85–15 mol %)	*E. coli* XL10	Fed-batch (first stage, glucose; second stage, propionate)	Continuous feeding of glucose (20 g L^{-1}) and propionic acid (2 g L^{-1})	39.8	60.5	Liu et al. (2009)
P(3HB-co-3HHx) (60–40 mol %)	*C. necator Re2133/pCB81*	Shake flasks 250 mL	Butyrate (0.5 %)	0.6	65	Jeon et al. (2014)

N.S. not specified

has an excellent strength and flexibility (Luzier 1992). Besides, in some cases, these copolymers have better biocompatibility compared to either P3HB or polylactic acid, which makes them promising materials for medical fields, for example, in cardiovascular problems, wound-healing process, orthopedic issues, drug delivery, and tissue engineering (Yang et al. 2002; Chen and Wu 2005; Table 2.1).

The ability to produce these copolymers is directly attributed to the specificity of polymerase synthase, which has been characterized only at preliminary level in *Bacillus* sp. (Lee et al. 2008). The P(3HB-co-3HV) is synthesized by several

Table 2.3 Culture conditions for the P3HB production with different molecular masses using recombinant *E. coli* strains and *Azotobacter* species

Organism	Mean molecular mass Mw or Mn* (kDa)	Production scale	Culture conditions	Biomass (g L^{-1})	PHB content (%)	References
E. coli XL-1 Blue (pSYL105)	20,000*	Bioreactor 2.6 L	LB medium with glucose 20 g L^{-1}, pH 6, 37 °C	7.4	48	Kusaka et al. (1997)
E. coli JM109 (pGEM-phaC$_{Re}$AB)	1800*	Shake flasks 500 mL with 100 mL medium	LB medium with glucose 20 g L^{-1}, 37 °C, 14 h culture time	3.2	33	Agus et al. (2006)
E. coli JM109 (pGEM-phaC$_{Da}$AB)	4000*			5.8	51	
E. coli JM109 (pGEM-phaC$_{Ac}$AB)	380*			2.7	24	
E. coli JM109 (pGEM-phaRCBspAB)	170–48*	Shake flasks	LB medium with glucose 20 g L^{-1}, 37 °C at 14 and 60 h of culture time	7.9–9.2	54–61	Agus et al. (2010)
E. coli JM109 (pGEM-phaRCBspAB)	1800*	Shake flasks	LB medium with glucose 20 g L^{-1}, 25 °C	5	24	Agus et al. (2010)
E. coli DH5α (pGETS109-pha) with order gene phaABC, phaACB, phaBAC, phaBCA, phaCAB, and *phaCBA*	2000–6200	Shake flasks	LB medium with glucose 20 g L^{-1}, 30 °C, 130 rpm at 72 h	8.0–11.2	31–57	Hiroe et al. (2012)
A. vinelandii UWD	4100	Shake flasks	5 % (w/v) beet molasses at 24 h	N.S.	N.S.	Chen and Page, (1994)
A. vinelandii OPN (ptsIIA^{Ntr}-)	3670	Shake flasks	Low aeration conditions (200 mL PY medium)	N.S.	62	Peña et al. (2014b)
A. chroococcum 7B	2215	Shake flasks	Microaerophilic conditions	2.87	61.3	Myshkina et al. (2008)

N.S. not specified

bacteria such as *C. necator*, some species of *Bacillus*, *Azotobacter*, recombinant strains of *E. coli*, and *Haloferax mediterranei*. This last is a natural P(3HB-co-3HV) producer (Don et al. 2006). The composition of P(3HB-co-3HV) produced by bacterial sources could be manipulated by the kind of carbon sources employed. Several studies have shown that the supply of propionyl-CoA in cells is the key factor for the production of the 3HV fraction during the synthesis of P(3HB-co-3HV) (Aldor et al. 2002). Therefore, most attempts aimed to produce P(3HB-co-3HV) copolymer or increasing the 3HV fraction are based on the strategies to improve propionate utilization. On the other hand, the P(3HB-co-3HHx) is generally produced from plant oil and fatty acids by several wild-type and recombinant bacteria. Previous reports have shown that it is possible to produce P(3HB-co-3HHx), containing greater than 20 % content of 3HHx monomer, using plant oils as carbon source (Kahara et al. 2004; Budde et al. 2011; Riedel et al. 2012). More recently, Jeon et al. (2014) demonstrated that engineered

C. necator can produce P(3HB-co-3HHx), when this strain was grown on mixed acids or on butyrate as carbon source. This strain produced a polymer containing up to 40 wt % of 3HHx monomer. It is important to point that this was the first report for the production of P(3HB-co-3HHx) copolymer in *C. necator* using butyrate. In this section the more recent attempts to improve composition and production of the copolymers P(3HB-co-3HV) and P(3HB-co-3HHx) will be discussed.

Choi and Lee (1999) described a strategy for production of copolymer P(3HB-co-3HV) at high concentration using a recombinant strain of *E. coli* with different feeding solutions containing propionic acid and glucose. In that study, a maximal copolymer concentration of 141.9 g L^{-1} with a P(3HB-co-3HV) up to 62.1 wt % and a 3HV component of 15.3 mol % was reached. It has been reported that the copolymer composition can be manipulated by adding propionate in the feed (Fidler and Dennis 1992; Slater et al. 1992, 1998; Yim et al. 1996; Choi and Lee 1999). However, industrial production of propionate is more expensive than glucose (Poirier et al. 1995; Aldor et al. 2002) making difficult the scale-up process for P(3HB-co-3HV) production. In addition, propionate being toxic must be fed at relatively low concentrations (Steinbüchel and Lütke-Eversloh 2003). An alternative strategy has been to design genetically modified strains in which it is possible to induce the expression of a critical gene in the polymer-producing pathway (Aldor and Keasling 2001). Some examples of genetic modifications that increased P(3HB-co-3HV) synthesis have been reported in different bacteria. For example, Yang et al. (2012), by introducing the genes of propionyl-CoA transferase (*pct*), β-ketothiolase *(bktB)*, acetoacetyl-CoA reductase *(phaB)* and PHA synthase *(phaC)* from *C. necator* into *E. coli* strain YH090, were able to produce P(3HB-co-3HV) with an ultra-high 3HV monomer composition reaching over 80 wt %. More recently, Yang et al. (2014) reported the *E. coli* strain (XL-1) harboring *E. coli poxB L253F V380A* gene along *C. necator prpE* (propionyl-CoA synthase) and *phaCAB* genes, which was able to produce propionyl-CoA via citramalate pathway. When this strain was cultured in a defined medium having 20 g L^{-1} of glucose as carbon source, P(3HB-co-3HV) was produced up to polymer content of 61.7 % based on dry weight. Furthermore, the 3HV monomer fraction in P(3HB-co-3HV) increased up to 5.5 mol % by additional deletion of the genes responsible for the metabolism of propionyl-CoA (*prpC* and *scpC*). Another interesting case is that of *Salmonella enterica* serovar *Typhimurium* that by expression of the *E. coli* (2R)-methylmalonyl-CoA mutase (YliK) and (2R)-methylmalonyl-CoA decarboxylase (YgfG) was able to biosynthesize P(3HB-co-3HV) from a single carbon source through the generation of propionyl-CoA from succinyl-CoA (Aldor et al. 2002).

It is important to point out that the production cost of these polymers can be significantly reduced by using activated sludge instead of pure substrates. This step enables easy operation, since this does not require sterile conditions and uses renewable substrates as carbon sources (Bosco and Chiampo 2010). Others have focused on the use of dairy waste (Pandian et al. 2010), sewage water (Hu et al. 1997; Wong et al. 2000), waste from food processing industry (Wong et al. 2000), as well as agricultural feed stocks (Solaiman et al. 2006). More recently, Narayanan et al. (2014) reported that the culture of *B. mycoides* DFC1 in rice husk hydrolyzate in combination with gluten hydrolyzate resulted in maximum synthesis of P(3HB-co-3HV) in the presence of valeric acid as co-substrate at different induction intervals and concentrations.

On the other hand, focus has been on the production of P(3HB-co-3HHx), which take advantage of the microbial fatty acid degradation pathways (Khanna and Srivastava 2005; Budde et al. 2011). A recent report of P(3HB-co-3HHx) production by *C. necator* has showed that the engineered *C. necator* accumulated P(3HB-co-3HHx) from fructose via the inverted β-oxidation pathway (Insomphun et al. 2015). Since the metabolic flux from acetyl-CoA to 3HB-CoA was too high in the natural PHA producer *C. necator*, a cellular content of 48 wt % P(3HB-co-3HHx) composed of 22 mol % 3HHx was obtained. In this context, Wang et al. (2015) with the purpose

to produce P(3HB-co-3HHx) from glucose as carbon source designed an *E. coli* recombinant strain, where they combined the BktB-dependent condensation pathway with the inverted β-oxidation cycle pathway, by cloning five exogenous genes (*bktB, phaB1, phaJ, ter*, and *phaC*). The resultant recombinant strain was able to produce a copolymer with a 3HHx fraction of 10 mol %. On the other hand, the biosynthesis of P(3HB-co-3HHx) from sugars involves an artificial pathway that allowed to build up the C6-monomer from three acetyl-CoA molecules, which is a challenge from metabolic engineering point of view. Based on this strategy, the recombinant *E. coli* strain PHB4 designed by Fukui et al. (2002) was able to accumulate P(3HB-co-3HHx) up to 48 wt % of dry weight from fructose, although the (3HHx) monomer composition in the copolymer was lower than 1.5 mol %.

Case 2: Production of Medium-Chain-Length PHAs (MCL-PHAs)

The MCL-PHAs may be used in diverse applications due to their better physical and mechanical properties as compared with the SCL-PHAs (Table 2.1). The MCL-PHAs are characterized due to their low degree of crystallinity, low melting point, and glass-transition temperatures combined with their improved flexibility, elasticity, and sticky properties that are required for applications in certain biomedical areas (Abe et al. 2012; Chen et al. 2014; Table 2.1). *Pseudomonas* spp. are able to produce MCL-PHAs, and their composition is directly related to the carbon source used as growth substrate. This is because the former monomers, as it was previously described (Fig. 2.2), are derived from intermediates of fatty acid biosynthesis or β-oxidation pathways; therefore, the nature of the PHA monomers produced by *Pseudomonas* species will depend on the metabolism of the specific carbon sources. When the carbon sources are carbohydrates, *P. aeruginosa* accumulates C10 (3-hydroxydecanoate; 3HD) from the biosynthetic fatty acid pathway as the predominant monomer. However, when the carbon source used are fatty acids, the precursors for the PHA synthesis are produced by β-oxidation pathway, and the predominant monomers are C8 (3-hydroxyoctanoate; 3HO), C10, and C12 (3-hydroxydodecanoate; 3HDD) (Madison and Huisman 1999; Nitschke et al. 2011). In addition, *Pseudomonas* spp. produce MCL-PHAs due to their PHA synthases (type II), which are able to polymerize hydroxy acids of short and medium chain length ($3HA_{SCL}$ and $3HA_{MCL}$), covalently linked within the same polyester molecules (Steinbüchel and Lütke-Eversloh 2003; Chen et al. 2014). MCL-PHAs such as poly-3-hydroxydodecanoate (P3HDD) and poly-3-hydroxyoctanoate (P3HO) are of commercial interest, because these polymers exhibit a considerable interval of thermomechanical properties and elastomeric behavior.

Simon-Colin et al. (2008) showed that *P. guezennei* was able to produce MCL-PHA copolymers with a great diversity in their structures and properties. The carbon sources include saturated and unsaturated monomers, from C4 to C14, but preferably C8 and C10 monomers. Furthermore, this strain was able to use a broad range of carbon sources as carbohydrates or fatty acids. For example, when the strain was grown in glucose, cells accumulated 3-hydroxybutyrate (3HB: 1.3 mol %), 3-hydroxyhexanoate (3HHx: 0.9 mol %), 3-hydroxyoctanoate (3HO: 22 mol %), 3-hydroxydecanoate (3HD: 62.8 mol %), 3-hydroxydodecanoate (3HDD: 6.2 mol %), 3-hydroxydodecenoate (3HDDE: 5.6 mol %), and 3-hydroxytetradecanoate (3HTD: 1.2 mol %). In contrast, when the bacterium was cultivated using oleic acid as carbon source, the MCL-PHAs included 3HTD (13.8 mol %) with less fraction mol of monomer 3HD (35.6 mol %). In another study, Simon-Colin et al. (2012) using sodium octanoate as sole carbon source observed that *P. guezennei* synthesized MCL-PHAs mainly composed of 3HO accounting for up to 94 mol % and lower amounts of 3HHx and 3HD. Recently, in cultures of the *P. fulva* strain TY16 grown on petrochemical wastes as carbon source, the production of MCL-PHAs was reported (Ni et al. 2010). Interestingly, when this strain was grown in glucose, toluene, benzene, ethylbenzene, gluconic acid, and acetic acid, it was able to synthesize MCL-PHA copolyesters, containing saturated

and unsaturated units of 3HDD, 3HHx, 3HO, and 3HD. On the other hand, copolyesters – MCL-PHAs synthesized by *P. fulva* strain TY16 from octanoic and decanoic acids – were composed of repeating units of 3HHx, 3HO, and 3HD with a mean molecular mass (MMM) between 42 and 43 kDa (Ni et al. 2010).

Another interesting case was reported using the strain of *P. putida* KT2440, grown with nonanoic acid and glucose as carbon sources at a 1:1–1.5 (w/w) ratio for the PHA production. Under such conditions, this strain accumulated a biopolyester with the following composition: 3-hydroxynonanoate (66 mol %), 3-hydroxyheptanoate (32 mol %), and 3HV (1 mol %). The terpolymer produced exhibited a MMM of 11 kDa, with a polydispersion index of 1.8 (Sun et al. 2009). Chan et al. (2014) evaluated the PHA production employing the strain of *P. mosselii* TO7 through utilization of plant oils such as soybean and palm kernel oil as carbon sources. These authors demonstrated that this strain accumulated up to 50 % (cell dry weight) of poly-3-hydroxyoctanoate (P3HO) achieving a productivity of 2.05 $g_{PHA}\ L^{-1}\ h^{-1}$.

Hori et al. (2011) evaluated the effect of temperature (within a range from 15 to 30 °C), in the biosynthesis of the PHAs produced by *P. aeruginosa* IFO3924. The results indicated that the MCL-PHA composition was closely dependent on the temperature and the culture time in which the biopolymer accumulation is carried out. At the beginning of the culture, 3HD and 3-hydroxydodecenoate (C12:1) units were found in the PHA samples at all temperatures evaluated (15, 20, and 25 °C). In contrast, the 3HO was detected only at 30 °C. On the other hand, when the maximum cellular content of PHA was achieved, 3HO and 3HD were the major monomer units present at all the temperatures tested. Haba et al. (2007) studied the effect of temperature (in the range of 18–42 °C) on PHA composition in cultures of *P. aeruginosa* 47 T2. In this study, *P. aeruginosa* was grown in mineral medium supplemented with urea as nitrogen source and 2 % of waste cooking oil. The results obtained indicated that the most abundant monomer was 3HD, except for PHAs produced at 42 °C in which 3HO was the monomer present in greater proportion (43.2 %). At elevated temperatures, long chain monomers such as 3HD, 3HDD, and C14:1 decreased, whereas at 37 °C the content of unsaturated monomers (C12:1, C14:2, C14:1) increased. Later on a significant decrease was observed at 42 °C (Haba et al. 2007). Another interesting example is the production of MCL-PHAs by cultures of *P. mediterranea* using reagent-grade or partially refined glycerol (Pappalardo et al. 2014). The gas chromatography analysis indicated that the biopolymer structure was composed by six monomers: 3HHx, 3HO, 3HD, 3HDD, *cis* 3-hydroxydodec-5-enoate ($C12{:}1\Delta^5$), and *cis* 3-hydroxydodec-6-enoate ($C12{:}1\Delta^6$).

Besides *Pseudomonas*, recombinant strains of *E. coli* are also an alternative for production of MCL-PHAs. For example, Li et al. (2011) reported an *E. coli* recombinant strain harboring *phaA* and *phaB* genes from *C. necator* and the *phaC2* gene from *P. stutzeri* (pCJY02). When this strain was cultivated in medium with decanoate, the bacterium accumulated SCL-MCL-PHAs with a monomer composition of 3HB, 3HHx, 3HO, and 3HD in mol ratios of 43.2:12.8:10.3:33.6. However, when this strain was grown on decanoate and glucose, the recombinant strain synthesized the same biopolymer, but the mol ratios were 3HB (83.4), 3HHx (4.0), 3HO (5.6), and 3HD (7.0). These results indicated that it is possible to modulate the monomer content and type of the PHA accumulated by adding different carbon sources and manipulating metabolic pathways of the host. In the same line, Zhuang et al. (2014) designed in its *E. coli* host metabolic pathways to synthesize MCL-PHAs directly from glucose. Engineering the reversed fatty acid β-oxidation cycle, Zhuang et al. (2014) employed this route to generate the key intermediates for the production of MCL-PHAs in *E. coli*. By using a PHA synthase with broad substrate specificity and using glucose as carbon source, recombinant *E. coli* was able to produce MCL-PHA copolymers with monomer composition ranging from 4 to 14 carbons. The PHA compositions in mol %

were 3HB:21.2, 3HHx:6.1, 3HO:45.8, 3HD:11.0, 3HDD:9.2, and 3HTD:6.8 (Table 2.2).

Case 3: Production of Poly-3-hydroxybutyrate of High Molecular Mass (HMM-P3HB)

The third case of microbial PHAs is the poly-3-hydroxybutyrate (P3HB), the common homopolymer of SCL; it is composed by monomers of 3-hydroxybutyrate (3HB), which are linked by ester bonds between the hydroxyl group and the carbonyl groups of the two adjacent monomers (Fig. 2.1). This polymer has similar thermomechanical properties to those found in conventional petrochemical plastics (Chanprateep et al. 2010), and these properties of P3HB are highly dependent on the mean molecular mass (MMM) of the polymer (Peña et al. 2014a; Domínguez-Díaz et al. 2015). Previous reviews have pointed out two interesting bacterial sources for the production of P3HB of high molecular mass (HMM-P3HB): one of them belongs to the genus *Azotobacter*, which is able to accumulate P3HB that exhibits a high molecular mass (>1000 kDa), and the other are recombinant strains of *E. coli* (Peña et al. 2014a; Leong et al. 2014). Some of the most relevant cases are discussed here.

Several authors have studied the culture parameters that could affect the MMM of the P3HB synthesized by *Azotobacter*, finding that the composition of the culture medium, the oxygen availability, and temperature are some of the factors that could have an important effect on the MMM. For example, Chen and Page (1994) observed that the UWD strain of *A. vinelandii* accumulated P3HB with a high MMM (4100 kDa), when it was cultivated using beet molasses of 5 % (w/v) in contrast with cultures without this substrate. These authors suggested that the nitrogen compounds of the beet molasses such as organic acids and salts stimulate the synthesis of P3HB of very high molecular mass (Chen and Page 1994). More recently, Peña et al. (2014b), in shaken flask cultivations of mutant strain OPN, reported a polymer with an MMM of 3670 ± 270 kDa in cultures conducted under low aeration conditions (conventional shaken flasks) as compared with cultures under high aeration. They proposed that by manipulating the aeration conditions of the culture and therefore the oxygen availability, it is possible to modify the MMM of the P3HB. Similar results were reported by Myshkina et al. (2008); these authors evaluated the P3HB production by *A. chroococcum* 7B, under microaerophilic conditions. Under low aeration condition, this strain was able to synthesize P3HB with a MMM of 2215 kDa and an increase on aeration negatively affected the MMM of P3HB. They also found that the optimal temperature for P3HB production with a high molecular mass was 30 ° C; in contrast, at low (20 °C) or high (37 °C) temperatures, the MMM decreased.

On the other hand, an interesting example for production of P3HB with a high molecular mass is that reported for *E. coli* recombinant strains harboring the *C. necator* biosynthesis *phbCAB* genes (Kusaka et al. 1997). These authors reported, for first time, the production of a polymer with ultrahigh molecular mass (20,000 kDa) during the stationary phase of growth by culturing *E. coli* XL-1 Blue (pSYL105), in a bioreactor of 2.6 L, under controlled pH conditions at 6.0 with LB medium supplemented with glucose (20 g L^{-1}). Interestingly, when this *E. coli* strain was grown at pH within a range of 7.0–8.0, the molecular mass of P3HB decreased to values below 5000 kDa after 12 h of culture.

Another interesting case was reported by Agus et al. (2006), who demonstrated that MMM of the P3HB accumulated by recombinant strains of *E. coli* depends on the specific PHA synthase (type and organism of origin) employed. Their results indicated that P3HB with high number-average molecular mass (Mn: 1500-4000 kDa) were synthesized by PHA synthases from *C. necator* (type I), *Delftia acidovorans* (type I), and *Allochromatium vinosum* (type III). P3HB with the lowest Mn (170–790 kDa) were accumulated by PHA synthases from *Aeromonas caviae* (type I), *Pseudomonas* sp. (type III), and *Bacillus* sp. (type IV). On the contrary, these authors found out that the highest MMM were obtained using the PHA synthase from *D. acidovorans* (4000 kDa). They also observed that for the strain harboring the PHA synthase from *D. acidovorans*, an acid pH (4.8) favored the P3HB production with high Mn (2100 kDa) as compared with the

biopolymer produced under basic conditions (pH 7.4–7.8), where the Mn was 1500 kDa, and these results were similar to those observed by Kusaka et al. 1997. When they investigated the effect of temperature on the Mn of P3HB using *E. coli* recombinant strains with PHA synthase *D. acidovorans*, they found that at 37 °C the biopolymer exhibited a higher Mn (4300 kDa) than that accumulated in the condition of 30 °C (580 kDa). In contrast, when Agus et al. (2006) used a recombinant *E. coli* strain with PHA synthase from *Bacillus* sp., they found a different behavior to that observed with the strain containing PHA synthase from *D. acidovorans*. They found that the Mn of P3HB produced by the strain with PHA synthase from *Bacillus* sp. in the condition of 37 °C was lower (440 kDa) than the Mn of the P3HB synthesized at 25 °C (nearly 1900 kDa).

On the other hand, through rearrangement of gene order of *phbCAB* operon biosynthesis (*phaABC*, *phaACB*, *phaBAC*, *phaBCA*, *phaCAB*, and *phaCBA)* in recombinant *E. coli* DH5α (pGES109-pha), Hiroe et al. (2012) found that it was possible to produce P3HB with different MMM in the range between 2000 and 6200 kDa. The results indicate that the MMM of P3HB accumulated by the six strains was higher during the exponential growth phase (12 h of cultivation) as compared with the biopolymer produced at the stationary phase (72 h). They also found an inverse correlation between MMM and P3HB synthase activity, in contrast to the accumulation percentage (quantified as dry weight), which increased as the synthase activity increased (Hiroe et al. 2012; Table 2.3).

Case 4: Production of Homopolymers of Short Chain Length: Poly-4-hydroxybutyrate (P4HB) and Poly-3-hydroxypropionate (P3HP)

Another example of SCL-homopolymers is the poly-4-hydroxybutyrate (P4HB; Fig. 2.1), which is one of the most promising PHA for biomedical applications, because of its unique properties, which include biodegradability, biocompatibility, nontoxicity, and superior mechanical properties. It must be emphasized that synthesis of P4HB requires precursor like 4-hydroxybutyric acid, 1.4-butanediol, or γ-butyrolactone (Valappil et al. 2007). Recently, Kämpf et al. (2014) investigated the production of P4HB using recombinant *E. coli* JM109 that harbors a 4-hydroxybutyric acid CoA transferase gene (*orfZ*) from *Clostridium kluyveri*, using glycerol and propionic acid. They found that biopolymer accumulation in the cells was of 80 % (dry weight) achieving 3.7 gL^{-1} (Table 2.2). On the other hand, Le Meur et al. (2013) reported that recombinant *E. coli* JM109 was able to produce P4HB using xylose as carbon source and sodium-4-hydroxybutyrate (Na-4HB) as biopolymer precursor. The highest P4HB concentration achieved was 4.33 g L^{-1} with a yield ($Y_{P4HB/Na\text{-}P4HB}$) of 92 % g g^{-1}. Also, Le Meur et al. (2014) using fed-batch high-density bacterial mass using glycerol as the sole carbon source along with precursor 4HB for biopolymer synthesis achieved a concentration of 15 g L^{-1} of P4HB.

The last example is the poly-3-hydroxypropionate (P3HP), which combines the properties of P3HB and poly-2-hydroxypropionate (known as polylactic acid). Andreeβen et al. (2010) reported the conversion of glycerol to P3HP in an *E. coli* recombinant strain, harboring genes encoding for glycerol dehydratase (*dhaB1*) of *Clostridium butyricum*, the propionaldehyde dehydrogenase *(pduP*) of *Salmonella enterica*, and the PHA polymerase (*phaC1*) of *C. necator.* After 92 h of incubation at 37 °C with 300 mM of pure glycerol, 1.42 gL^{-1} of P3HP were achieved with a yield of 17.5 mmol $_{P3HP}$ mol $_{glycerol}^{-1}$ consumed, with the drawback production of ethanol (8.04 gL^{-1}), succinate (48.92 gL^{-1}), and acetate (0.26 gL^{-1}) as by-products. Another case was reported by Wang et al. (2014). They built a recombinant strain of *E. coli* (with *panM, panD, pp0596, ydfG, prpE*, and *phaC1*) for the P3HP production. This strain was able to produce 0.5 g L^{-1} of biopolymer when it was cultivated in shaken flasks, using glycerol and glucose as carbon sources and without any addition of precursors. In cultures of the same strain in stirred bioreactors in fed-batch aerobic cultures, they obtained up to 10.1 g L^{-1} of P3HP (Wang et al. 2013). In the same line, Gao et al. (2014) designed a recombinant stable *E. coli* strain harboring seven exogenous genes of P3HP synthesis pathway. This strain in aerobic fed-batch cultures

was able to produce 25.7 g L^{-1} of biopolymer from glycerol.

2.6 Perspectives

PHAs are biomaterials of great importance not only due to their biodegradability and thermomechanical capabilities similar to those of the plastics derived from the petrochemical industry but also due to their biocompatibility, which is a characteristic required in medical and biomedical fields. In addition, the success of application of these biopolymers will depend on their chemical nature, mainly the monomer composition and mean molecular mass, and other properties which influence the mechanical properties, biodegradability, and biocompatibility of PHAs. Current advances in fermentation, purification technology, as well as the design of mutant strains by recombinant DNA technology would allow the tailor-made production of new PHAs. These tailor-made PHAs can be used as materials for biomedical uses, such as tissue engineering. From the economic viewpoint, the efforts are now focusing on the design of new strains, which can use complex substrates of very low cost, such as those present in waste materials, and having the versatility to produce PHAs with a wide chemical variety and molecular mass.

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ANEXO B. Establecimiento de la metodología de extracción del P3HB y análisis del peso molecular por GPC

Existen reportadas en la literatura diferentes técnicas de recuperación y purificación del biopolímero de los restos celulares. La extracción con solventes, principalmente empleando hidrocarburos clorados es el método más utilizado debido a su simplicidad y rapidez. Además, permite obtener elevados porcentajes de recuperación del material; sin embargo se requieren grandes cantidades de solventes que resultan dañinos al medio ambiente (Jacquel *et al*., 2008; Kunasundari y Sudesh, 2011). Para disminuir la cantidad de solvente se emplea hipoclorito de sodio el cual digiere la membrana celular y facilita la recuperación del material, obteniéndose un biopolímero de mayor pureza. Sin embargo, existen evidencias experimentales que indican que la concentración de la solución de hipoclorito de sodio, así como el tiempo de contacto con el paquete celular, causa ruptura de las cadenas de P3HB, afectado su peso molecular (Hahn *et al.*, 1994).

La metodología empleada en nuestro grupo de investigación para la recuperación del P3HB ha sido la descrita por Hahn *et al.*, (1994), con algunas modificaciones, la cual emplea hipoclorito de sodio para la ruptura celular. Por lo tanto, para el desarrollo de este proyecto, fue necesario establecer una técnica de extracción que garantizara el menor daño posible al biopolímero. Para ello, se evaluaron tres métodos de ruptura celular: 1) hipoclorito de sodio al 2 % (como control), 2) sonicado y 3) acetona. A continuación se detalla la metodología establecida.

Las muestras de cultivo fueron conservadas en congelación a -20 °C hasta su análisis. Para la extracción del P3HB, a cada muestra se le realizó el siguiente procedimiento: se recuperó la biomasa por centrifugación a 11,000 rpm x 10 minutos. Para eliminar componentes del medio de cultivo, el paquete celular se resuspendió en agua destilada y nuevamente se centrifugó a 11,000 rpm por 10 minutos. Posteriormente, la biomasa fue sometida a los diferentes tratamientos de ruptura celular.

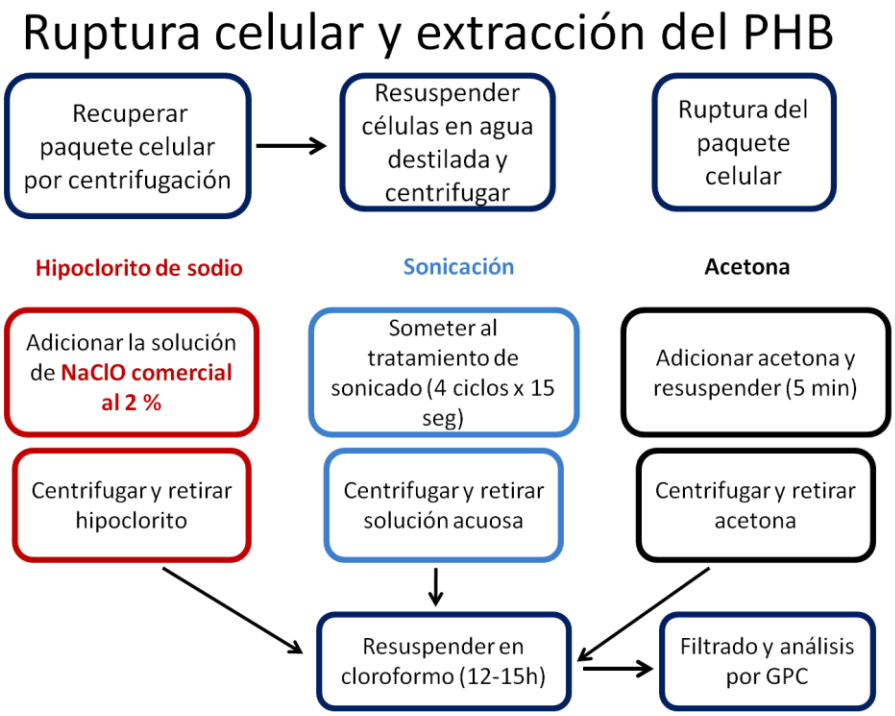
1) En las muestras que fueron sometidas a sonicación, la biomasa fue resuspendida en agua y se aplicaron cuatro ciclos de sonicado de 15 segundos cada uno. Después, se centrifugaron las muestras a 11,000 rpm x 5 minutos, se desechó el sobrenadante y se adicionó el cloroformo para la extracción del P3HB, quedando en contacto por 12-15 h.

2) Para la ruptura celular con hipoclorito de sodio al 2 %, la biomasa fue resuspendida en la solución y se dejó en contacto por 2 minutos. Posteriormente, se adicionó cloroformo y se dejó en contacto y después de 12 h se recuperó la fase inferior que contenía el P3HB.

3) En la ruptura celular con acetona, el paquete celular se resuspendió en acetona y se dejó en contacto por 10 minutos. Después se centrifugó la muestra a 11,000 rpm por 5 minutos, se desechó la acetona y se adicionó cloroformo para solubilizar el P3HB, dejando en contacto durante 12-15 h.

La solución de cloroformo que contenía el P3HB fue filtrada en membranas de 0.45 µm y se colocó en viales para realizar el análisis del peso molecular del biopolímero. Un

esquema de la metodología empleada en la recuperación del P3HB se muestra a continuación.



La distribución de pesos moleculares se determinó por cromatografía de permeación en gel (GPC), usando dos columnas (Styragel HR 6 y Styragel HR 5E) conectadas en serie. Dichas columnas nos permiten analizar muestras de P3HB con pesos moleculares desde 2,000 hasta 10 x 10^6 de Dalton. Dicho arreglo en serie se acopló a un equipo de HPLC (Waters Alliance 2695) con un detector de índice de refracción (Waters, 2414). Las condiciones de operación fueron: 30 °C y 0.7 mL min^{-1} de cloroformo como fase móvil. El volumen de inyección fue de 50 µL y se utilizaron estándares de poliestireno para la construcción de la curva de calibración con pesos moleculares entre 1.2 x 10^3 - 2 .78 x 10^6 Da.

La figura B1 presenta la distribución de pesos moleculares de muestras de P3HB recuperado empleando las tres condiciones de ruptura celular. Se encontró que de los métodos de ruptura celular evaluados, la acetona resultó ser el método de ruptura celular que causa el menor daño al P3HB. Cuando las muestras fueron tratadas con hipoclorito de sodio o sonicado, el 4 y 6 % de la muestra presentó pesos moleculares menores a 100 kDa; mientras que en las muestras tratadas con acetona, prácticamente no se observó dicha fracción de moléculas de bajo peso molecular (Tabla B1).

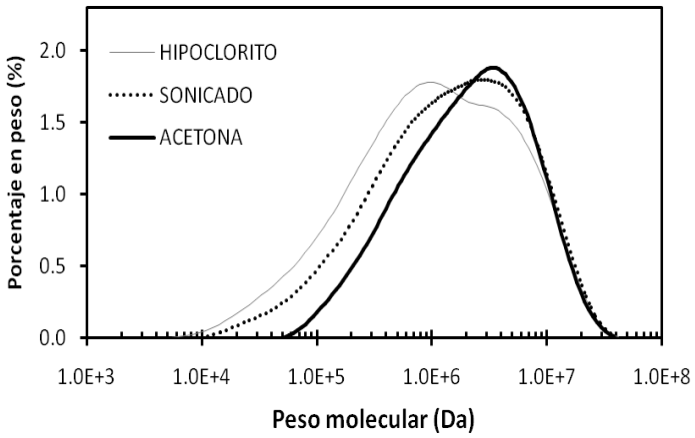


Figura B1. Distribución característica de pesos moleculares de muestras de P3HB sometidas a diferentes tratamientos de ruptura celular.

En general, las muestras de P3HB que se extrajeron usando acetona en la ruptura celular presentaron un mayor porcentaje de moléculas de peso molecular entre 1,000 a 10,000 kDa, comparado con el porcentaje presente en las muestras donde se utilizó hipoclorito de sodio y sonicado. El porcentaje de cadenas poliméricas con pesos moleculares en el intervalo de 100 a 1,000 kDa fue mayor para la ruptura realizada con hipoclorito de sodio y disminuyó en las muestras tratadas con acetona.

Tabla B1. Porcentajes de la distribución de pesos moleculares y peso molecular promedio de las muestras de P3HB sometidas a diferentes tratamientos de ruptura celular.

	Porcentaje que reprensenta en la muestra				**Peso molecular promedio (kDa)**		**Indice de**
Método de ruptura	$< 1x10^5$	$1x10^5$-$1x10^6$	$1x10^6$-$1x10^7$	$> 1x10^7$	Mw	Mn	**polidispersión**
Hipoclorito de Sodio	6.6	39.4	48.1	5.9	3,252 ± 407	375 ± 73	8.8 ± 1.4
Sonicado	4.1	33	55.3	7.6	3,191 ± 138	469 ± 95	7.0 ± 1.2
Acetona	0.5	28.6	63.2	7.7	3,442 ± 118	997 ± 58	3.5 ± 0.3

No se observaron cambios en el peso molecular promedio en peso (Mw) del P3HB mayores a 200 kDa entre los diferentes tratamientos; sin embargo, al emplear acetona, el peso molecular promedio en número (Mn) fue 600 kDa mayor comparado con el obtenido en las muestras sometidas a ruptura con hipoclorito de sodio. El índice de polidispersión (IP) nos permite conocer la heterogeneidad de la muestra, y cuando este parámetro es cercano a 1 indica que el polímero es monodisperso. La muestra de P3HB tratada con hipoclorito de sodio presentó un mayor grado de heterogeneidad, mientras que con acetona, el IP de la muestra disminuyó prácticamente a la mitad. Con base en estos resultados, para la extracción del P3HB de las muestras de cultivo, se empleó acetona como solvente para la ruptura celular.