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Tese

Poli(3-hidroxibutirato) com propriedades térmicas diferenciadas produzido por *Pseudomonas* sp. CMM43 degradadora de agrotóxico

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Resumo

CROCHEMORE, Ane Gerber. **Poli(3-hidroxibutirato) com propriedades térmicas diferenciadas produzido por *Pseudomonas* sp. CMM43 degradadora de agrotóxico**. 2014. 119f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Os bioplásticos vêm ganhando espaço nos projetos de pesquisas e muitos já estão sendo produzidos em escala industrial e podem substituir polímeros de origem petroquímica, resultando em grande benefício ambiental. Também possuem grande potencial biotecnológico, podendo ser empregados como matéria-prima para diferentes produtos dependendo principalmente das propriedades físico-químicas e térmicas que apresentarem. Os polihidroxialcanoatos (PHAs), típicos bioplásticos totalmente biodegradáveis, são poliésteres acumulados, principalmente, por uma variedade de bactérias, como fonte de reserva de carbono e energia para a célula. O poli(3-hidroxibutirato) [P(3HB)], é o PHA mais estudado e mais comumente armazenado na forma de grânulos lipofílicos, podendo chegar a níveis de 90% em massa celular seca. O objetivo desta pesquisa foi, primeiramente, quantificar e avaliar as propriedades térmicas do polímero produzido pela *Pseudomonas* sp. CMM43, identificada como produtora de P(3HB), cultivada em meio nutritivo simples. Na sequência, o objetivo foi otimizar a produção de P(3HB) pela bactéria em incubador agitador orbital e biorreator bem como caracterizar os biopolímeros produzidos nas diferentes condições. Inicialmente, foi investigada a fase de multiplicação celular, alterando parâmetros de cultivo com intuito de aumentar a concentração de biomassa produzida e determinar se a produção está associada à multiplicação celular. O P(3HB) recuperado foi identificado e caracterizado. A *Pseudomonas* sp. CMM43 acumulou P(3HB) em 24 h, sem a necessidade de limitação de algum nutriente, sugerindo que a acumulação do polímero ocorra simultaneamente à multiplicação. O máximo conteúdo de P(3HB), 70% em relação à massa celular seca (MCS), foi obtido em meio com 1% de sacarose, pH 6,5; temperatura de 28 ºC, 250 rpm e menor aeração. O biopolímero apresentou propriedades térmicas e massa molecular reduzidas, o que permite aplicações específicas na área médica e farmacêutica. Em uma segunda fase, foram testados em agitador incubador orbital meios e concentrações de inóculo para a fase de produção do biopolímero na temperatura e aeração anteriormente selecionadas. Os P(3HB)s foram identificados e caracterizados. A máxima produção de polímero relativa à MCS foi de 83% em 48 h, no meio em que foi utilizado 10% (v/v) de inóculo e 1% de sacarose. Obtiveram-se polímeros de massa molecular reduzida e temperatura de fusão menor que a de degradação, indicando ser uma característica da bactéria e não influência do meio ou da concentração do inóculo. Por final foi avaliada a produção de P(3HB) em biorreator, aplicando o mesmo meio e condições de cultivo do estudo anterior, observando-se grande redução no acúmulo. Conclui-se que *Pseudomonas* sp. CMM43 possui propriedades promissoras para processos industriais de produção de P(3HB). Mesmo não tendo atingido altas concentrações celulares, a bactéria acumulou altos níveis de (P(3HB)/MCS) em 48 h, sem limitação de nutrientes e utilizando sacarose, substrato bastante econômico no Brasil. Adicionalmente, sintetiza polímeros com propriedades térmicas diferenciadas que permitem aplicabilidades específicas na área biomédica e farmacêutica.

Palavras-chave: P(3HB), *Pseudomonas*, baixa massa molecular, propriedades térmicas, sacarose, ausência de limitação de nutrientes

Abstract

CROCHEMORE, Ane Gerber. **Poly(3-hydroxybutyrate) with different thermal properties produced by *Pseudomonas* sp. CMM43 pesticide-degrading**. 2014. 119f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Bioplastics have been gaining espace in research projects and many are already being produced on an industrial scale and can replace petrochemical polymers of origin, resulting in greater environmental benefit. They also have great biotechnological potential and can be used as raw material for different products depending primarily on the physical, chemical and thermal properties that show. Polyhydroxyalkanoates (PHAs), typical bioplastics completely biodegradable, are polyesters accumulated mainly by a variety of bacteria, as a source of carbon and energy reserve for the cell. The poly(3-hydroxybutyrate) [P(3HB)], is the most studied and most commonly stored as lipophilic PHA granules, reaching levels of 90% of dry cell weight. The aim of this study, was first, to quantify and evaluate the thermal properties of the produced polymer by *Pseudomonas* sp. CMM43, identified as producing P(3HB), cultivated in simple nutrient medium. Following aimed to optimize the production of P(3HB) by the bacteria in orbital shaker and bioreactor, and to characterize produced biopolymers in different conditions. Initially, was investigated cellular growth phase, changing cultivation parameters in order to increase the concentration of biomass produced and to determine if production is growth-associated. The P(3HB) recovered was identified and characterized. The *Pseudomonas* sp. CMM43 accumulated, high levels of P(3HB) at 24 h, without any nutrient limitation, suggesting that accumulation of polymer occurs simultaneously on the growth. The maximum content, 70% in the dry cell weight (DCW) was obtained in medium with sucrose, pH 6.5, temperature of 28 º C, 250 rpm and less aeration. The biopolymer presented reduced thermal properties and molecular weight, which allows specific applications in the medical and pharmaceutical area. In a second phase, was tested still in an orbital shaker incubator, media and inoculum concentration for the biopolymer production phase, in the temperature and aeration previously selected. The P(3HB) are recovered were identified and characterized. The maximum polymer yield was 83% in DCW at 48 h, in the medium which 10% (v/v) of inoculum and1% of sucrose was used. Polymers with low molecular weight and with melting temperature less than degradation were obtained, indicating that a characteristic of bacteria, and no influence on the medium or the concentration of inoculum. By the end was evaluated P(3HB) production in bioreactor, using the same medium and culture conditions of the previous study, it was observed a large reduction in the accumulation. In conclusion*, Pseudomonas* sp. CMM43 has promising conditions for industrial P(3HB) production processes. Despite not having reached high cell concentrations, the strain acumulated high levels (P(3HB)/DCW) for 48 h, without nutrient limitation and using sucrose, quite economical substrate in Brazil. Additionally, synthesizes polymers with different thermal properties that allow specific applicability in the biomedical and pharmaceutical field.

Keywords: P(3HB), *Pseudomonas*, low molecular weigth, thermal properties, sucrose, absence nutrient limitation

Lista de Figuras

Revisão Bibliográfica

Produção de P(3HB) por *Pseudomonas* sp. CMM43 em biorreator

Lista de Tabelas

Produção de P(3HB) por *Pseudomonas* sp. CMM43 em biorreator

Lista de Abreviaturas

acetil-CoA – acetilcoenzima A
ATSM – American Society for Testing and Materials
DCW – dry cell weigth (massa celular seca)
DSC – *Differential scanning calorimetry* (calorimetria diferencial exploratória)
DNS - *dinitrosalicylic acid method*
FTIR – *Fourier transform infrared spectroscopy* (espectroscopia de infravermelho com transformada de fourier)
GC – *Gas chromatography* (Cromatografia gasosa)
GC/MS – *Gas chromatography/Mass spectrometry* (Cromatografia gasosa com espectro de massa)
GPC – *Gel permeation chromatography* (Cromatografia de permeação em gel)
MCS – massa celular seca
MET – microscopia eletrônica de transmissão
Mn - *number average molecular weight* (massa molecular numérica média)
Mw - *weight average molecular weight* (massa molecular ponderal média)
Mv - *Viscosity average molecular weight* (massa molecular viscosimétrica média)
Mw/Mn – *polydispersity* (polidispersão)
PA - polímeros de amido
PAA - poliésteres alifáticos aromáticos
PET – Politereftalato de etileno
PHA – Polihidroxialcanoatos
PHA_{SCL} - *short-side-chain*(cadeia lateral curta)
PHA_{MCL} - *midle-side-chain* (cadeia lateral média ou longa)
P(3HB) – Polihidroxibutirato
P(3HB) – Poli(3-hidroxibutirato)
P(3HB)V – poli(3-hidroxibutirato-co-3-hidroxivalerato)
PLA - polilactatos
PP – polipropileno
TEM – Transmission electron microscopy
TCA - ciclo do ácido tricarboxílico
TGA – análise termogravimétrica
Tg - temperatura de transição vítrea
Tm – *Melting temperature* (temperatura de fusão)
Tmax - temperatura de degradação
Tonset – temperatura inicial de degradação
RMN - ressonância magnética nuclear
Rpm – rotações por minuto
X_C – grau de cristalinidade

Sumário

1 INTRODUÇÃO GERAL

Os plásticos de origem petroquímica proporcionam muitos avanços e facilidades para a sociedade, devido a sua durabilidade, resistência e diversidade de usos. Sua produção e consumo aumentam a cada ano, tendo alcançado 288 milhões de toneladas em 2012 (PLASTICS EUROPE, 2013). Esses materiais são de lenta degradabilidade, pois não reagem quimicamente com a maioria das substâncias, provocando problemas nos aterros sanitários, dificultando a troca de gases e a decomposição de outros compostos (LUENGO et al., 2003). Prejudicam também os centros urbanos, com a obstrução parcial da rede de esgoto aumentando os danos causados por enchentes (BIOPLASTIC NEWS, 2009), e o campo, quando é feito o descarte inadequado de embalagens de agrotóxicos e fertilizantes (INPEV, 2013). Uma quantidade expressiva chega às águas dos mares e oceanos, afetando diretamente e enormemente a fauna aquática (CIÊNCIA HOJE, 2012). Frente a essa problemática, pesquisas vêm sendo desenvolvidas no intuito de minimizar ou solucionar essa questão, investigando novos materiais que possam substituir o plástico de origem petroquímica sem ou com reduzidas perdas na qualidade e funcionalidade.

Biopolímeros produzidos por bactérias cultivadas em biorreatores ganham importância na busca de substitutos para os plásticos derivados de petróleo, principalmente os biopolímeros do tipo polihidroxialcanoatos (PHAs). Esses polímeros são poliésteres armazenados por uma variedade de bactérias, com a finalidade de reserva de carbono e energia para a célula, podendo atingir níveis de até 90 % da massa celular seca (BRAUNEGG et al., 1998). Geralmente são acumulados em condições de limitação nutricional de N, P, O, ou Mg e em excesso de fonte de carbono (ANDERSON & DAWES, 1990), mas em algumas bactérias a acumulação de polímero ocorre durante o crescimento na ausência de limitação de nutrientes (LEE, 1996a). O grande atrativo desses biomateriais é que eles são totalmente biodegradáveis, biocompatíveis e podem ser produzidos a partir de fontes renováveis (CHANPRATEEP, 2010), resultando em uma substituição dos plásticos petroquímicos ambientalmente vantajosa.

Poli(3-hidroxibutirato) [P(3HB)] é o PHA mais estudado, pois apresenta propriedades que o tornam altamente competitivo com o polipropileno (PP) (REDDY

et al., 2003), termoplástico com a maior taxa de crescimento anual no mundo, devido às suas excepcionais propriedades e versatilidade de aplicação e uso (ABREU et al, 2006).

Assim como o PP, o P(3HB) é cristalino e com ponto de fusão em torno de 180 ºC (HOLMES, 1985). Também é resistente à água, estável à radiação ultravioleta e constitui barreira à permeabilidade de gases (LINDSAY,1992; OJUMU e SOLOMON, 2004). Possui aplicações em diferentes áreas, como na medicina, sendo utilizados em fios de sutura e próteses ósseas (HAYATI et al., 2012), na farmacêutica para cápsulas medicamentosas (BAZZO, 2008), na agricultura para encapsulamento de inoculantes e pesticidas (SAVENKOVA et al., 2001) e em embalagens e itens descartáveis de uso geral (KULKARNI et al., 2011).

A obtenção de PHAs ainda têm um alto custo comparado a plásticos convencionais, sendo o tipo de substrato utilizado, a etapa de extração (JACQUEL et al., 2008) e o rendimento do polímero os fatores determinantes (KHANNA & SRIVASTAVA, 2005).

Na tentativa de diminuir o custo total do processo, novos microrganismos produtores têm sido testados (ARUN et al., 2009), resíduos são utilizados como substrato, alcançando a conversão em polímero (KHARDENAVIS et al., 2006; FERNÁNDEZ et al., 2005; TRIPATHI et al., 2012) e novas metodologias de extração são desenvolvidas (DALCANTON, 2006; KAPRITCHKOFF et al., 2006).

Os organismos mais estudados para produção de PHA são dos gêneros *Ralstonia*, *Azotobacter*, *Bacillus* e *Pseudomonas*. Muitos acumulam P(3HB), porém poucos produzem em quantidades suficientes para serem utilizados em processo industriais (LEE, 1996b). Dessa forma, buscam-se microrganismos, nativos ou modificados geneticamente, com capacidade de acumular elevados níveis do polímero.

A maioria das *Pseudomonas* são capazes de sintetizar PHAs de cadeia média (MCL) (HABA et al., 2007). Algumas vezes produzem copolímeros, consistindo de P(3HB) (cadeia curta - SCL) e PHAs MCL (HANG et al, 2002), e raramente acumulam apenas P(3HB) (JIANG et al., 2008;. MOHAN et al., 2010). Entretanto, *Pseudomonas* sp. CMM43 tem esta capacidade, além de possuir as vantagens de acumular polímero em curto período de tempo utilizando sacarose como substrato e sem limitação de nutrientes (CROCHEMORE et al., 2012).

O objetivo desta pesquisa foi otimizar a produção de Poli(3-hidroxibutirato) sintetizado pela bactéria *Pseudomonas* sp. CMM43 em diferentes escalas de crescimento e parâmetros fermentativos, caracterizando o biopolímero produzido.

2 REVISÃO BIBLIOGRÁFICA

2.1 Bioplásticos

Atualmente, duas categorias de polímeros recebem a denominação "bioplásticos": plásticos produzidos a partir de matérias-primas renováveis, transformados em produtos biodegradáveis e não biodegradáveis, e plásticos biodegradáveis produzidos a partir de matérias-primas renováveis ou fósseis, chamados também de polímeros biodegradáveis (BPD). Para que um bioplástico seja biodegradável sua decomposição deve ser resultante da ação de bactérias e fungos (SECOM, 2007).

Os principais bioplásticos produzidos são: polímeros de amido (PA), polilactatos (PLA), polihidroxialcanoatos (PHAs) e poliésteres alifáticos aromáticos (PAA). São utilizados diferentes tipos de matéria-prima renováveis para produção dos bioplásticos como: milho, batata, beterraba, cana de açúcar (BRITO et al, 2011).

Com o aumento do preço do petróleo e um cenário de poluição cada vez maior devido ao consumo exagerado de plásticos petroquímicos e seu descarte e destino inadequados, as pesquisas no setor de bioplásticos ganham espaço e incentivos financeiros. A grande vantagem que esses materiais apresentam é o reduzido tempo de degradação em relação aos petroquímicos, que levam cerca de 450 anos para degradarem-se (SECOM, 2007). Como muitas indústrias e consumidores buscam por produtos ecologicamente corretos, o mercado de bioplásticos deverá crescer até 32%, considerando o período 2010-2014 (UNICA, 2011).

Destacam-se entre os bioplásticos os PHAs, produzidos por bactérias a partir de diversas fontes renováveis, que são biocompatíveis e totalmente biodegradáveis, sendo decompostos por microrganismos em poucos meses, apresentando um biociclo fechado, gerando ao final CO_2 e água (FORMOLO et al*.,* 2003). Esses poliésteres possuem propriedades semelhantes a dos plásticos petroquímicos podendo ser vistos como substitutos desses, com a vantagem de serem totalmente biodegradáveis (COUTINHO et al., 2004).

2.2 Polihidroxialcanoatos

Os polihidroxialcanoatos (PHAs) são homo ou heteropoliésteres bastante simples, sintetizados e intracelularmente armazenados por numerosos procariotos como substâncias naturais de reserva de carbono e de energia. São acumulados pela célula microbiana em forma de grânulos, podendo chegar até 90% de seu peso seco (SCHLEGEL; KALTWASSER & GOTSCHALK, 1961; MADISON & HUISMAN, 1999). São conhecidos mais de 300 microrganismos diferentes que sintetizam PHAs, mas somente poucas bactérias têm sido utilizadas na produção desses polímeros, entre elas *Ralstonia eutropha*, *Alcaligenes latus*, *Azotobacter* v*inelandii*, espécies de metilotroficas, espécies de *Pseudomonas* e *Escherichia coli* (LEE, CHOI & WONG, 1999).

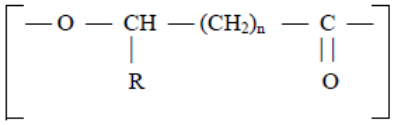
Os PHAs são termoplásticos atóxicos, biocompatíveis e biodegradáveis que podem ser produzidos a partir de produtos de fontes renováveis. Os PHAs têm alto grau de polimerização, são altamente cristalinos, opticamente ativos e insolúveis em água. Essas características os tornam similares aos plásticos convencionais e altamente competitivos com o polipropileno, plástico derivado do petróleo (HOCKING & MARCHESSAULT, 1994; REDDY et al., 2003).

As propriedades termoplásticas mais importantes dos plásticos são: elevado ponto de fusão, baixa rigidez, alta resistência à pressão, resistência ao alongamento antes da ruptura, forte resistência ao impacto, temperatura de transição vítrea (Tg) cristalinidade e tempo de cristalização (CANEVAROLO, 2003). A busca de PHAs que apresentem essas propriedades é bastante ampla, pois cada bactéria, seja naturalmente produtora ou geneticamente modificada, tem condições de originar PHAs com diferentes propriedades termoplásticas que conferem características diferentes aos produtos obtidos.

PHAs podem ser produzidos em grandes quantidades a partir de fontes renováveis por meio de processos conhecidos de fermentação, sob imposição de condições particulares de cultivo, em regime de batelada, semibatelada ou contínuo (RIESENBERG & GUTHKE, 1999). Vários métodos químicos e físicos são conhecidos para extraí-los da biomassa produzida (FULLER & LENZ, 1990 *apud* BRAUNEGG et al., 1998).

As unidades monoméricas que compõem os PHAs dependem da fonte de

carbono utilizada pelas bactérias e diferem entre si pela composição da cadeia lateral ou do radical R e pelo valor de n (LEE, 1996a). A figura 1 apresenta a estrutura geral da unidade monomérica destes polímeros.



n=1	R= hidrogênio	Poli (3-hidroxipropionato)
	R= metil	Poli (3-hidroxibutirato)
	R= etil	Poli (3-hidroxivalerato)
	R= propil	Poli (3-hidroxihexanoato)
	R= pentil	Poli (3-hidroxioctanoato)
	R= nonil	Poli (3-hidroxidodecanoato)
n=2	R= hidrogênio	Poli (4-hidroxibutirato)
	R= metil	Poli (4-hidroxivalerato)
n=3	R= hidrogênio	Poli (5-hidroxivalerato)
	R= metil	Poli (5-hidroxihexanoato)
n=4	R= hexil	Poli (6-hidroxidodecanoato)

Figura 1 - Estrutura geral dos polihidroxialcanoatos. Fonte: LEE, 1996a.

PHAs podem ser classificados em dois grupos: de cadeia lateral curta e de cadeia lateral média ou longa. Os polímeros constituídos de unidades monoméricas de cinco carbonos ou menos, são denominados de cadeia lateral curta – (*short-side-chain*) (PHA_{SCL}). Os polímeros com unidades monoméricas com mais de seis carbonos são os de cadeia lateral média ou cadeia lateral longa (*midle-side-chain*) (PHA_{MCL}) (RAMSAY, 1994).

Os PHAs de cadeia média apresentam-se como materiais flexíveis e emborrachados, com baixa cristalinidade e tem vasta aplicação onde o P(3HB), seu co-polímero P(3HB-3HV) e outros PHAs de cadeia curta e alta cristalinidade não podem ser utilizados (GAGNON *apud* LEE, CHOI & WONG, 1999), como por exemplo em filmes de recobrimento para papel e papelão (DE KONING et al., 1997), componentes de tintas à base de água (VAN DER WALLE et al.,1999) e suportes para engenharia de tecidos (WILLIAMS et al., 1999).

No citoplasma celular os PHAs apresentam-se na forma de inclusões que podem ser visualizadas por microscopia óptica utilizando amostras coradas com Negro Sudão (ANDERSON & DAWES, 1990; ROMEIRO, 2001) ou por microscopia óptica com fase de contraste. As células bacterianas contendo PHA podem ser

observadas também por microscópio eletrônico de transmissão, e as inclusões aparecem, de maneira mais nítida, como corpos eletrônicos densos (LAGEVEEN et al., 1988; SUDESH, ABE & DOI, 2000).

A biodegradabilidade do bioplástico só interessa que ocorra após a vida útil e esta só ocorrerá em ambiente propício pela ação de bactérias, fungos e algas, sendo os dois primeiros os principais participantes nos processos de biodegradação do mundo natural, que lhes fornece precursores para os componentes celulares e energia (HOLMES, 1985; BRAUNEGG et al., 1998). Os microrganismos degradadores secretam enzimas que quebram o polímero em seus blocos moleculares, chamados de hidroxiácidos, que são utilizados como uma fonte de carbono para crescimento (FLEMMING, 1998).

As taxas de biodegradação dependem de uma variedade de fatores, principalmente os relacionados com a facilidade de colonização microbiana (HOLMES, 1985), incluindo área de superfície, hidrofobicidade, atividade microbiológica, umidade, pH, temperatura e a presença de nutrientes para que ocorra a adesão de microrganismos, colonização e formação de biofilme sobre o material (FLEMMING, 1998). Os PHAs degradam-se em diversos ambientes, aeróbios e anaeróbios, ocorrendo degradação mais rápida em sistemas anaeróbios e mais lenta no mar (LUZIER, 1992, MUKAI et al., 1993).

As inúmeras condições de biodegradabilidade já seriam suficientes para tornar os PHAs tão fascinantes, principalmente na importante contribuição destes na manutenção do equilíbrio ambiental. No entanto a produção dos PHAs ainda apresenta outras importantes contribuições que vem desde o seu processo, pois a sua síntese é a partir de fontes renováveis de carbono baseadas na agricultura ou até mesmo em resíduos industriais, permitindo um processo de ciclo fechado sustentável para a produção e uso destes poliésteres em vez de tecnologias ligadas à produção e o uso dos plásticos clássicos (DOI, 1990; BRANDL et al., 1995; BRAUNEGG et al., 1998).

A figura 2 mostra o ciclo fechado de produção e degradação dos polihidroxialcanoatos. As plantas, ao realizarem fotossíntese, utilizam a luz solar, CO_2 e H_2O para síntese de carboidratos. Estes carboidratos podem ser utilizados como fonte de carbono em um processo fermentativo com microrganismos acumuladores de PHAs. Os PHAs são extraídos, purificados e transformados nos

mais diversos produtos plásticos, exemplificado em uma garrafa. Após seu uso, os produtos são descartados em ambiente microbiano ativo ocasionando a total degradação destes com formação de CO_2 e H_2O e o ciclo recomeça.

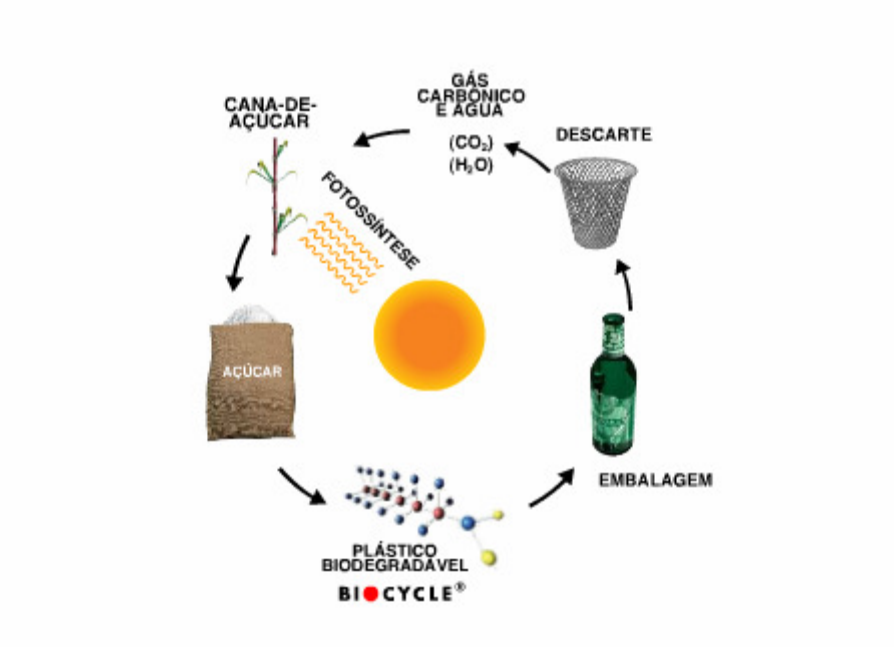


Figura 2 – Biociclo de PHAs. Fonte: http://www.biocycle.com.br/.

A preocupação com os rejeitos não degradáveis ou de difícil degradação, como os plásticos a base de polietileno ou polipropileno, no meio ambiente vem crescendo a cada dia pelos problemas de poluição que estes causam. É de fundamental importância conhecer como sintetizar e caracterizar os polímeros biodegradáveis, e como usá-los.

Os países desenvolvidos possuem uma preocupação ecológica, incentivando a substituição dos materiais poliméricos sintéticos não biodegradáveis por biodegradáveis, e leis em relação ao uso destes materiais. No Brasil, só recentemente foram redigidos projetos de lei relacionados a este assunto. Um exemplo é o projeto de lei nº 631 de 2009 da Câmara do Estado de São Paulo, e ainda em tramitação, que "proíbe o uso de embalagens plásticas à base de polietileno, polipropileno e o PET à base de polipropileno para acondicionamento de gêneros alimentícios, bebidas e cosméticos. A substituição das embalagens plásticas deverá ser feita por plásticos biodegradáveis e os estabelecimentos industriais têm um prazo de 6 anos para se adequarem" (SÃO PAULO, 2009).

Os polímeros biodegradáveis recebem diferentes definições. Uma muito

difundida é a da ASTM (proposta D20. 96), segundo a qual plásticos degradáveis são materiais plásticos que sofrem cisão nas ligações da estrutura do polímero por forças químicas, físicas e/ou biológicas no ambiente levando a fragmentação ou desintegração do plástico.

2.2.1 Poli(3-hidroxibutirato)

O polihidroxibutirato (P(3HB)) foi isolado pela primeira vez de *Bacillus megaterium* e caracterizado em 1925 por Lemoigne, no Instituto Pasteur, em Paris, porém sem nenhuma intenção de usá-lo na produção de plástico, pois suas características de termoplasticidade ainda não haviam sido descobertas. Em 1958, Macre e Wilkinson, também utilizando *Bacillus megaterium,* estudaram condições operacionais para obtenção de P(3HB) e concluíram ser este uma fonte de reserva de carbono e energia a ser utilizada pelo microrganismo em condições desfavoráveis. As bactérias armazenam P(3HB) como uma reserva de energia, da mesma forma que mamíferos acumulam gordura (HOLMES, 1985). Geralmente a produção do P(3HB) ocorre em duas etapas distintas: no primeiro estágio são oferecidas condições de multiplicação celular, fase de crescimento e o segundo estágio compreende o acúmulo do polímero com o cessar da multiplicação celular (BYROM, 1987; KIM et al., 1997). O P(3HB) é sintetizado na maioria dos microrganismos a partir da acetil-CoA, em condições de excesso de fonte de carbono e limitação de algum nutriente essencial, por uma sequência de três reações catalisadas pelas enzimas beta-cetotiolase, acetil-CoA redutase e PHA sintase. Em condições de crescimento balanceado a acetil-CoA entra no ciclo do ácido tricarboxílico (TCA) para geração de energia e material celular (SUDESH et al., 2000).

Entretanto algumas bactérias são capazes de acumular P(3HB) associado ao crescimento celular sem necessidade de limitação de nutrientes (LEE, 1996a). A produção de P(3HB) associada ao crescimento celular contribui para dois processos metabólicos: o fornecimento de energia para sintetizar os constituintes celulares e acumular o material de armazenamento, cuja síntese não é desencadeada pela taxa de crescimento diminuindo (BORMAN, 2000).

Dentro da célula o P(3HB) é um fluído e se apresenta em estado amorfo. Ao

ser extraído da célula, utilizando-se solventes orgânicos, ele se torna altamente cristalino e rígido, porém quebradiço (MADISON & HUISMAN, 1999). É também termoplástico podendo ser comparado ao polipropileno pelas suas propriedades físicas; possui semelhante ponto de fusão (próximo de 180 ℃), grau de cristalinidade e temperatura de transição vítrea. Esta comparação é útil para que se saiba o tipo de produtos que podem ser desenvolvidos a partir de P(3HB) (HOLMES, 1985).

O P(3HB) é insolúvel em água e solúvel em alguns solventes apolares como o clorofórmio, caracterizando-se por ser um material com alta cristalinidade, o que dificulta seu processamento e o torna extremamente quebradiço, limitando seu uso como plástico comercial (HONG & CHEN, 2006).

2.3 Microrganismos acumuladores de PHAs

Os PHAs são sintetizados por um grande número de bactérias Gram-negativas e Gram-positivas pertencentes a pelo menos 75 gêneros diferentes (DOI, 1990). Alguns exemplos de cultura puras usadas industrialmente para produzir PHAs incluem a *Ralstonia eutropha*, *Alcaligenes latus*, *Azotobacter vinelandii* e diversas espécies de *Pseudomonas* (SERAFIM, LEMOS & REIS, 2003).

Microrganismos capazes de acumular P(3HB) foram isolados de ecossistemas de plantações de cana de açúcar do nordeste do Brasil, sendo isoladas 82 bactérias P(3HB) positivas com grande incidência de *Pseudomonas* (SILVA & GOMEZ, 2007).

A produção de PHAs ocorre geralmente em duas etapas, sendo primeiramente produzida grande quantidade de biomassa celular para, após, oferecer-se as condições necessárias à acumulação do polímero (KIM et al., 1997).

Segundo LEE (1996a), os microrganismos usados para produção de PHAs podem ser divididos em dois grupos, baseados nas condições de culturas requeridas para síntese de polímero. O primeiro grupo de bactérias requer a limitação de um nutriente essencial como N, P, Mg, K, O ou S para uma síntese eficiente de PHAs, com excesso de fonte de carbono. Nesse grupo, fazem parte *Alcaligenes eutrophus, Protomonas extorquens, Pseudomonas oleovorans* e muitas outras bactérias. O segundo grupo não requer limitação de nutriente para síntese de PHA, e podem

acumular o polímero durante o crescimento. As bactérias *Alcaligenes latus, Alcaligenes vinilandii* recombinante e *Escherichia coli* recombinante são representantes desse grupo.

2.3.1 Acúmulo de Polihidroxialcanoatos por *Pseudomonas*

A versatilidade bioquímica observada em espécies do gênero *Pseudomonas* é ampliada pela presença de plasmídeos degradadores, os quais são elementos extracromossômicos que codificam enzimas necessárias ao catabolismo desses compostos. Bactérias do gênero *Pseudomonas* têm a capacidade de utilizar um grande número de compostos orgânicos complexos e raros como fonte de carbono e energia. Além disso, *Pseudomonas* são capazes de rapidamente desenvolver novas atividades metabólicas em resposta a mudanças nas condições ambientais (BARBIERI, 1990).

Esperava-se que a via metabólica de síntese de PHAs das *Pseudomonas* fosse exclusivamente baseada em ácidos graxos, porém estudos mostram que cepas de *P. putida* são capazes de acumular estes biopolímeros a partir de glicose e outros açúcares. Timm & Steinbuchel (1990) e Haywood et al. (1990) demonstraram que diversas cepas de *Pseudomonas* quando cresciam em outros substratos como carboidratos acumulam PHAs que consistem predominantemente de unidades monoméricas de 3-hidroxidecanoato. *P. putida* KT2442 é capaz de produzir PHAs a partir de substratos tais como a glicose e o glicerol. A acumulação de PHA foi observada quando o meio de cultura continha um excesso de fonte de carbono (> 10 g/L) e uma quantidade limitante de nitrogênio (HUIJBERTS et al., 1992). Outros estudos mais recentes confirmam a possibilidade de utilização de carboidratos para produção de PHAs (MADISON & HUISMAN, 1999; HABA et al., 2007).

A limitação de um nutriente é condição essencial para o acúmulo de PHAs para grande maioria das *Pseudomonas*. A limitação de amônio no cultivo foi necessária para promover o acúmulo de PHAMCL em *P. putida* (80% da massa celular seca) (KIM et al., 1997), em *P. oleovorans* (33% da massa celular seca) (HAZENBERG & WITHOLT, 1997) e *Pseudomonas* sp. K (57% da massa celular seca) (SUZUKI, YAMANE & SHIMIZU, 1986). Annuar et al. (2006) relatam que as porcentagens obtidas por esses autores são 2 a 7 vezes maiores que as obtidas a

partir da limitação de outros nutrientes como sulfato, magnésio, ferro, etc. Daniel et al. (1992), utilizando *Pseudomonas* 135, metilotrófica facultativa, produziram P(3HB) a partir de metanol como única fonte de carbono e em deficiência de nutrientes como nitrogênio, magnésio e fósforo. Em meio complexo suplementado com ácido butírico ocorreu a incorporação de 3-OH ácido butírico em polímeros de cadeia média produzido por *Pseudomonas,* resultando na produção de blendas ou numa mistura de copolímeros com quantidade significativa de 3-OH ácido butírico (SCHROLL et al., 1996).

A utilização de sacarose como fonte de carbono para produção de P(3HB) é uma característica almejada, pois apresenta um baixo custo em relação a outros substratos por ser proveniente de fontes renováveis amplamente cultivadas, como cana-de-açucar e beterraba, ou até mesmo de resíduos industriais que apresentem na sua composição sacarose como carboidrato predominante. Porém não são todas as bactérias que conseguem convertê-la em polímero, sendo essa uma característica incomum para este gênero (BELAL, 2013; BONATTO et al., 2004), que preferencialmente utiliza glicose e ácidos graxos (BRANDL et al, 1988; ANDERSEN et al., 2000; ASHBY, SOLAIMAN & FOGLIA, 2002; DAS, CHOWDHURY & ANWAR, 2005; LI et al, 2013).

Também a acumulação de P(3HB) por espécies de *Pseudomonas* é uma característica rara ou incomum (KESSLER; PALLERONI, 2000), pois normalmente armazenam PHAs compostos de monômeros de cadeia média MCL (HUISMAN et al., 1989). Algumas cepas de *Pseudomonas* são capazes de acumular simultaneamente polímeros de cadeia curta, como o P(3HB), e polímeros de cadeia média, a partir de várias fontes de carbono. As análises de criofraturas indicaram que os polímeros são armazenados em inclusões diferentes na célula (FUKUI et al., 1998).

Porém alguns poucos autores relatam o acúmulo de P(3HB) por esse gênero. Daniel et al. (1992) é, talvez, a citação mais antiga. Após um hiato de vários anos sobre o tema, Jiang et al. (2008) relataram o isolamento de uma cepa de *Pseudomonas fluorescens*, de solo do Alaska, com capacidade de acumular elevada quantidade do polímero (22 g/L) a partir de caldo de cana. Koller et al. (2008) demonstraram em seu trabalho que uma cepa de *Pseudomonas hydrogenovora* acumulou P(3HB) utilizando glicose e galactose da lactose hidrolisada do soro de

leite. *Pseudomonas* sp. 14-3, uma cepa exposta a condições extremas que prevalecem no seu habitat natural da Antártida, apresenta um alto nível de resistência ao estresse quando acumula P(3HB) a partir de octanoato. A síntese de P(3HB) inicia na fase de crescimento exponencial, sugerindo que a associação entre a alta resistência ao estresse e alta acumulação de P(3HB) é uma característica geral de bactérias que habitam áreas com rápida mudança e condições extremas, que geram um ambiente estressante (AYUB et al., 2004). Em 2006, Ayub e colaboradores relataram em seu trabalho que essa *Pseudomonas*, classificada como mutante natural, acumula P(3HB) por meio de uma deleção no gene que codifica para enzima β-cetotiolase, usando uma rota alternativa para a produção de P(3HB) que permite a síntese do polímero a partir de ácidos graxos, mas não a partir da glicose ou gluconato. Posteriormente (AYUB et al., 2007) foi constatado que os genes envolvidos na biossíntese do P(3HB) pela *Pseudomonas* sp. 14-3 foram encontrados dentro de uma ilha genômica chamada pha-GI, indicando que os genes phaB e phaC foram adquiridos por transferência horizontal, provavelmente derivados de espécies de *Burkholderia*, sugerindo que a transferência horizontal dos genes pha é um mecanismo de adaptabilidade às condições de estresse.

Em estudo realizado por Crochemore et al. (2012), foram identificadas três bactérias do gênero *Pseudomonas* degradadoras de agrotóxico, isoladas de solo de arroz irrigado, como produtoras de P(3HB) a partir da utilização direta de sacarose e sem limitação de nutriente, uma característica diferenciada para o gênero. Essas bactérias são provenientes de solo que recebeu aplicação de um inseticida, sendo expostas a um ambiente de estresse. A capacidade de degradação de agrotóxico e a sobrevivência da *Pseudomonas* sp. CMM43 em condição de estresse ambiental podem estar relacionadas com o acúmulo de P(3HB). Esta bactéria pode apresentar ilha genômica, já que ilhas genômicas (GIs) e vários plasmídeos são frequentemente encontrados associados à adaptação e degradação de xenobióticos em cepas de *Pseudomonas* (HE et al., 2004; PITMAN et al., 2005; GAILLARD et al., 2006; MA, WANG & SHAO, 2006;). Assim, os genes envolvidos na síntese de P(3HB) podem ter sido adquiridos por transferência horizontal, sugerindo tratar-se de um mecanismo de adaptabilidade a ambientes em mudança (AYUB et al., 2007).

2.4 Aplicabilidade dos biopolímeros

PHAs são poliésteres naturais termoplásticos, cujas propriedades físicas permitem potenciais aplicações biotecnológicas em muitas áreas, como em embalagem biodegradáveis, na medicina e na agricultura. A diferença é que para embalagens o volume buscado é de grande produção e baixo custo, enquanto que para área médica o volume pode ser pequeno e com maior valor agregado. O destino da aplicação dos PHAs vai depender principalmente das suas características térmicas e da massa molecular.

Filmes de P(3HB) podem ser utilizados para matrizes biodegradáveis para imobilização de agrotóxicos, prolongando e regulando sua liberação, e assim as plantas utilizam-no com mais eficácia (SAVENKOVA et al., 2001; MARTINS et al., 2008). Estudos feitos por Bucci et al. (2007) demonstraram que o P(3HB) utilizado como material para embalagem de alimentos tem um grande potencial, pois apresenta fácil degradação em diferentes ambientes e atua como uma boa barreira para incidência de luz. A biomassa rica em P(3HB), não purificada, pode ser utilizada na manufatura de fibra natural reforçada com compostos termoplásticos (NFRTCs), com utilização na construção civil, resultando, assim, um produto totalmente biológico e biodegradável (COATS et al., 2008).

Esses compostos também são extremamente atrativos na preparação de matrizes biodegradáveis para liberação controlada de drogas de depósito (medicamentos, hormônios), inseticidas e herbicidas. Também são usados como materiais osteossintéticos, na estimulação de crescimento de ossos devido a suas propriedades piezoelétricas, em suturas cirúrgicas e como vasos sanguíneos substitutos (REDDY et al., 2003). Contudo, se os PHAs podem ser considerados polímeros com alto potencial para aplicações biomédicas, o P(3HB), com suas normalmente elevadas massa molecular e cristalinidade, temperatura de fusão alta e próxima à temperatura de degradação, tem essas aplicações restringidas.

A massa molecular (Mw) é um fator de extrema importância, pois afeta diretamente a resistência mecânica do polímero, bem como a capacidade de intumescimento e de hidrólise e, consequentemente, a taxa de biodegradação, a qual está relacionada com a sua cristalinidade. Com isso, para o desenvolvimento de sistemas de liberação controlada utilizando-se os PHAs, há a necessidade de

que o polímero tenha a massa molecular reduzida. Em adição, o aumento da taxa de degradação promoverá um acréscimo na velocidade de liberação do princípio ativo e, consequentemente, a matriz polimérica será mais facilmente absorvida pelo organismo (MONTORO, 2005). Além disso, PHAs de baixa massa molecular podem ser usados como componentes para construir diversas arquiteturas, tais como, copolímeros em blocos (ARSLAN, HAZER & KOWALCZUK, 2002; ZHANG et al., 2005) e enxertados (NGUYEN & MARCHESSAULT, 2004; LAO et al., 2007).

A massa molecular dos PHAs varia em função do microrganismo, do substrato oferecido e das condições de cultivo (OLIVEIRA et al*.,* 2007; VAN DER WALLE et al., 2001). A massa molecular (Mw) do P(3HB) produzido por bactérias nativas é usualmente na faixa de $1x10^4$ a $3x10^6$ Da, com polidispersão (Mw/Mn) em torno de 2,0 (SUDESH et al., 2000). Poucas bactérias produzem P(3HB) com baixa massa molecular, que geralmente fica em torno de 10^6 Da (CHEN & PAGE, 1994; MYSHKINA et al., 2008; OLIVEIRA et al., 2007). Os métodos de extração também podem reduzir a massa molecular, causando sérios danos nos grânulos (DAWES & SENIOR, 1973; HOLMES, 1988). E a massa molecular ainda pode ser reduzida por vias de modificação química como, por exemplo, hidrólise ácida (YU & MARCHESSAULT, 2000) ou via reação de transesterificação (DENG & HAO, 2001).

A associação de polifosfato e íons de cálcio (REUSCH & SADOFF, 1988; REUSCH, 1999) ou a incorporação de 3-hidroxivalerato (3HV) na produção de copolímero de poli(3-hidroxibutirato-co-3-hidroxivalerato) – P(3HB)V – também resultam na formação de um polímero com características mais interessantes do ponto de vista industrial (ARAUJO, 2005; MARANGONI, 2000). Sendo esse copolímero menos rígido e quebradiço do que o P(3HB), podendo ser aplicado no preparo de filmes que impossibilitem ou reduzam a permeabilidade à água e gases, semelhantemente ao polipropileno (MADISON & HUISMAN, 1999).

Enquanto o homopolímero P(3HB) tem uma temperatura de fusão (Tm) em torno de 173 a 180 °C e uma temperatura de transição vítrea (Tg) de cerca de -5 a 5 °C, a adição de 3HV reduz a temperatura de fusão dos cristais (variando de 75 a 170 °C), a resistência à tração e módulo elasticidade e, ao mesmo tempo, aumenta a flexibilidade, resistência ao impacto e ductilidade. Isto é particularmente benéfico, pois com a diminuição da temperatura de fusão, o copolímero pode ser processado

em temperaturas mais baixas, reduzindo significativamente a degradação térmica (ERCEG, KOVAI & KLARI, 2005). A cristalinidade do P(3HBV) é normalmente de 39-69%, dependendo da percentagem dos monômeros correspondentes. A alta porcentagem de 3HV diminui a cristalinidade, tornando o polímero mais flexível (GARCIA, 2006).

Um biopolímero apresentar temperatura de fusão (Tm) menor que a temperatura de degradação (Tmax) é uma característica que lhe permite aplicações específicas, podendo ser facilmente transformado em películas e revestimentos, apresentando resistência sem ser quebradiço e boa estabilidade térmica no processamento, porque têm relativamente baixa Tm e alta Tmax (NODA, SATKOWSKI & SATKOWSKI, 2002). A maioria dos P(3HB)s sintetizados apresenta baixa estabilidade à fusão devido à temperatura de degradação ser próxima à temperatura de fusão (LEE, 1996b).

Embora existam inúmeras possibilidades de aplicação, os plásticos biodegradáveis ainda são pouco presentes em nossa vida cotidiana, contribuindo com uma pequena participação no mercado atual. Essa situação mercadológica é devida ao seu alto custo em relação aos plásticos petroquímicos (KHANNA & SRIVASTAVA, 2005). O quilo do P(3HB) tem saído da empresa P(3HB) Industrial a US$ 5,00, enquanto os plásticos à base de petróleo custam, em média, US$ 2,00 (RAC, 2012).

O consumidor, normalmente, só está disposto a pagar, pelos plásticos biodegradáveis, preço menor ou igual aos dos plásticos derivados petroquímicos. Segundo Kessler et al. (2001), o consumidor, de modo geral, por mais consciente que seja, ainda não esta disposto a pagar pela sua contribuição com o equilíbrio do meio ambiente. Enquanto o custo de produção não puder ser significativamente reduzido (ou a utilização dos derivados petroquímicos fortemente reprimida), a única forma de convencimento será a criação de uma estratégia onde sejam exaltadas suas propriedade incomparáveis, como a biodegrabilidade e a biocompatibilidade, e não apenas apresentá-los como substitutos dos plásticos convencionais (HÄNGGI, 1995; LEE, 1996b; DALCANTON 2006).

2.5 Aspectos da produção de PHAs

Mundialmente, poucas são as indústrias que se lançaram nesta produção porque as pesquisas são muito abrangentes e interdisciplinares. Para chegar-se a uma produção economicamente viável de PHA, além de a bactéria ser capaz de sintetizar é fundamental que se encontrem as condições operacionais satisfatórias para que a mesma possa produzir e armazenar esse constituinte de forma estável. Também devem ser estabelecidas as condições de recuperação e purificação do polímero. A ocorrência do consumo do P(3HB) por ação do metabolismo celular ou, ainda, qualquer tipo de degradação durante o processo de obtenção é indesejável.

Entre a descoberta do P(3HB), em pesquisas básicas, e a possibilidade de sua obtenção em escala industrial decorreram vários anos e foram feitos muitos investimentos que possibilitaram o avanço necessário na área de engenharia de fermentadores. O desenvolvimento de biorreatores ou fermentadores com controles precisos de oxigênio ou mistura de gases, pH, agitação e temperatura foram fundamentais para possibilitarem a transposição da descoberta de PHAs em escala de laboratório e a produção em grandes volumes com condições de manipulação do processo, incluindo as características de termoplasticidade e biodegradabilidade (ANDERSON & DAWES, 1990; STEINBÜCHEL, 1991; FÜCHTENBUSCH & STEINBÜCHEL, 1999).

A tão desejada redução dos custos desse processo, no entanto, não é simples de se obter, pois depende de bactérias acumuladoras e do desenvolvimento de condições operacionais que permitam a exploração do potencial máximo da bactéria na obtenção do polímero (KHANNA & SRIVASTAVA, 2005).

Para que um processo de produção de PHAs seja economicamente viável a cepa produtora deve conseguir acumular no mínimo 60% de sua massa celular em polímero. Já se conhecem bactérias capazes de acumular até 93%, como *Alcaligenes latus* DSM 1122 e *Ralstonia eutropha* com capacidade de 80 a 90%. Outras, com capacidade entre 60 e 80%, são as *Pseudomonas* (67%), *Alcaligenes latus*, *Azotobacter vinelandii* (73%), *R. eutropha* recombinante (80%), *Escherichia coli* e *Klebsiella aerogenes* recombinantes e *Rhodobacter,* entre outras (RAMSAY et al., 1994; LEE, 1996b).

A fonte de carbono deve ser pouco dispendiosa, pois é o maior contribuinte para o custo total do meio de cultivo. Assim, o fato de utilizar fontes renováveis para produção de PHAs é uma característica tão importante quanto à biodegradabilidade.

No mesmo contexto, o rendimento do PHA com relação ao substrato de carbono (definido como gramas de PHA produzido por gramas de substrato consumido) precisa ser elevado, o que demonstra não ter sido desperdiçado para produzir materiais que não sejam PHA. O conteúdo de PHA pode ser mensurado como a habilidade da célula de acumular PHA em uma determinada condição. Elevados conteúdos de PHA geralmente resultam em elevados rendimentos do polímero, sendo benéfico para o processo de recuperação (LEE, 1996b).

2.6 Produção de polímeros biodegradáveis

A primeira produção comercial de P(3HB) foi em 1960, pela empresa W. R. Grace Co. Em 1976 a Imperial Chemical Industrie iniciou pesquisas de produção e aplicação do P(3HB), porém somente em 1990 foi lançada na Alemanha a primeira embalagem produzida exclusivamente com plástico biodegradável.

Embora a descoberta do P(3HB) tenha quase um século, somente nos últimos 20 anos a pesquisa com propósito de obtenção de polímero biodegradável foi realmente impulsionada no Brasil. Em meados da década de 90, deu-se início ao desenvolvimento de tecnologia para a produção de plásticos biodegradáveis e biocompatíveis empregando matéria-prima renovável pela agricultura, em especial derivados da cana-de-açúcar. Após um levantamento de oportunidades, selecionou-se um grupo de polímeros que compreendem a família dos polihidroxialcanoatos (PHA) que podem ser produzidos por bactérias em biorreatores a partir de carboidratos (COUTINHO et al., 2004).

O desenvolvimento de pesquisas para produção de bioplásticos no país ocorreu pelo consórcio feito por grandes instituições de pesquisa e empresas do setor sucroalcooleiro, que são: o Instituto de Pesquisas Tecnológicas de São Paulo (IPT), o Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB/USP), a cooperativa Copersucar e a Usina da Pedra (VELHO & VELHO, 2008).

O IPT realizou estudos de fermentação para obtenção do polímero biodegradável. Concomitantemente, o Centro de Tecnologia Copersucar - CTC desenvolveu uma tecnologia de extração e purificação do polímero com a utilização de solvente menos tóxico e mais seguro, um álcool superior. O objetivo da tecnologia de extração era a utilização de insumos produzidos pela própria Usina.

Tendo sido atingido esse objetivo, em 2000 foi criada a P(3HB) Industrial S/A, na localidade de Serrana, em São Paulo, na planta piloto da Usina da Pedra, a qual foi modificada em função das pesquisas da nova rota de produção, através da qual foi possível obter um produto economicamente viável. No mesmo ano a indústria começou a produzir P(3HB), sendo a produção inicial de 10 ton/ano. Em 2008, a produção de P(3HB), agora com a marca Biocycle, foi de 30 toneladas; em 2009 foi de 35 ton e o produto foi exportado para os Estados Unidos, Japão e Alemanha (BIOPLASTIC NEWS, 2010). Em 2012 a produção atingiu 50 ton/ano e pela primeira vez a P(3HB) Industrial entregou um produto ao mercado final, um kit de escova e pente com cabos à base de cana. A produção de P(3HB) ainda tem caráter laboratorial e utiliza equipamentos semi-industriais. Para migrar ao nível industrial, a capacidade de produção deveria subir para 10 mil toneladas, ou seja, 200 vezes mais (RAC, 2012).

Os principais produtores de PHAs são: a Mitsubishi (Japão), que produz o P(3HB) de marca Biogreen®, a partir de metanol; a Biofan (P(3HB)/P(3HB)V), também no Japão; a Biomer (P(3HB)), na Alemanha; a Monsanto, com a marca Biopol® (P(3HB)/PHV/PHA), na Itália; co Metabolix® (UK); a Procter & Gamble, nos EUA, com o Nodax®, copolímero P(3HB)/HHx produzido a partir de glicose e óleos vegetais (óleo de palma) (MCKEEN, 2012); a Bi-on, na Itália, que produz o Minerv-PHA, a partir do açúcar da beterraba, que é totalmente degradado em 10 dias em água normal dos rios e considerado substituto para PET, PE, PP, HDPE, LDPE (BIO-ON, 2014). A indústria brasileira P(3HB) Industrial S/A, a única que utiliza sacarose extraída de cana-de-açúcar como substrato, e que tem a marca Biocycle®, é hoje reconhecida mundialmente pelo seu produto inovador que tem uma ampla aplicabilidade (PRADELLA, 2006).

2.7 Identificação e caracterização

A caracterização do PHA produzido é de suma importância, pois trata-se de uma grande família de diferentes poliésteres, com mais de 100 unidades monoméricas já identificadas. Logo, suas propriedades irão depender principalmente da composição monomérica, que definirá a aplicação do bioplástico conforme suas características específicas (STEINBÜCHEL & VALENTIN, 1995).

Uma grande quantidade de técnicas analíticas tem sido usada para identificação e caracterização de polímeros. Pode-se citar ressonância magnética nuclear (RMN), espectroscopia no infravermelho com transformada de Fourier (FT-IR), e difração de raios-X (CANEVAROLO, 2006). Técnicas de análise térmica, como calorimetria exploratória diferencial (DSC) e termogravimetria (TGA) diferenciam os polímeros em termos de propriedades e auxiliam na observação das alterações nas propriedades do material antes e depois da degradação. Para determinação da distribuição da massa molecular, a técnica mais difundida é a cromatografia de permeação em gel (GPC).

Também são utilizadas as análises de cromatografia gasosa (GC), espectrometria de massas (MS) ou técnicas conjugadas como GC/MS, SFC/MS e HPLC/MS. Algumas vezes é feita a derivatização do composto antes da análise, sendo que esta e o método analítico a ser empregado dependem do problema específico em estudo (MOLDOVAN; JOVER; BAYONA, 2002).

Outros métodos têm sido desenvolvidos para detecção de P(3HB), como microextração da fase sólida acoplado à cromatografia gasosa, resultando em menor prejuízo para o meio ambiente e consumindo menor tempo de análise (RIVERA et al., 2007). A utilização de eletroforese capilar também mostra-se como método alternativo e a detecção é feita com quantidades de amostras duas ou três vezes menor do que as utilizadas em cromatografia gasosa (HE, CHEN & YU, 2002).

O espectro de absorção no infravermelho (IV) é hoje um dos métodos mais rápidos para caracterizar os grupamentos funcionais de um composto químico (COSTA NETO, 2005). A espectroscopia na região do infravermelho é considerada uma das mais importantes técnicas experimentais para a caracterização de polímeros, em termos de identificação e/ou determinação de características estruturais de polímeros (SPERLING, 1992). O espectro de IV apresenta muitas bandas de absorção, a possibilidade de dois compostos terem o mesmo espectro é praticamente inexistente. Por isso, o espectro de IV é a "impressão digital" da molécula. Por meio dele é possível reconhecer a presença de bandas de absorção provenientes de vibrações de grupos funcionais característicos (NAKANISH & SOLOMON, 1977).

As análises térmicas DSC e TGA são amplamente utilizadas, pelo fato de os

polímeros serem compostos orgânicos, sendo suas características físicas e químicas fortemente influenciadas pela temperatura (SILVERSTEIN et al., 2007; CANEVAROLO, 2006).

DSC é uma técnica na qual é medida a absorção ou liberação de calor em função da temperatura em que ocorrem as mudanças químicas ou físicas no polímero. São medidas as temperaturas de fusão (Tm) e de transição vítrea (Tg) (GALEGO et al., 2000). Já a termogravimetria é uma técnica muito utilizada na caracterização do perfil de degradação de polímeros e outros materiais. A exposição à temperatura elevada pode, algumas vezes, alterar a estrutura química e, por conseqüência, as propriedades físicas dos materiais. Portanto, a curva de degradação térmica, em condições não isotérmicas, mostra o perfil da resistência ou estabilidade térmica que o material apresenta quando submetido a uma varredura de temperatura (SPIER, 2005).

Os materiais poliméricos diferenciam-se dos demais por apresentarem uma cadeia longa, identificada por sua massa molecular. Esta característica exerce grande influência sobre suas propriedades físico-químicas de tal modo que seu conhecimento e controle é de fundamental importância (CANEVAROLO, 2003). São apresentados três tipos de massa molecular: massa molecular numérica média (*Mn*), que depende do número de moléculas de polímero presentes na solução, massa molecular ponderal média (*Mw*), que depende do número e da massa das moléculas em solução e massa molecular viscosimétrica e média (*Mv*), que depende do número, da massa e também da forma das moléculas presentes na solução. O valor ponderal médio é mais elevado que o valor numérico médio, e que é mais expressivo no campo dos polímeros, pois valoriza a característica dominante dos polímeros que é o tamanho da molécula. Assim, a polidispersão, que é o quociente, *Mw*/*Mn*, será tanto maior quanto mais heterogêneas forem as massas moleculares, ou seja, quanto maior for a distribuição de massa molecular (MANO & MENDES, 1999).

Assim, a análise conjunta das propriedades que identificam e caracterizam o biopolímero, permitem a previsão de sua aplicabilidade.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

Pseudomonas sp. CMM43, acumula P(3HB) com propriedades térmicas e massa molecular reduzidas e em quantidade desejável para sua utilização em bioprocessos industriais.

3.2 Objetivo Geral

O objetivo desta pesquisa foi otimizar a produção de Poli(3-hidroxibutirato) sintetizado pela bactéria degradadora de agrotóxico *Pseudomonas* sp. CMM43 em incubador agitador orbital e biorreator, modificando parâmetros fermentativos e caracterizar o biopolímero produzido.

3.3 Objetivos Específicos

● Quantificar e analisar as propriedades térmicas do P(3HB) produzido preliminarmente em estudo de bioprospecção;
● Aumentar a concentração celular da fase de crescimento (inóculo) modificando parâmetros como temperatura, carboidrato, pH, agitação e aeração, e juntamente verificar se o acúmulo de P(3HB) ocorre associado ao crescimento;
● Estabelecer condições ótimas de produção de P(3HB) em Incubador agitador orbital, avaliando meios, temperatura, agitação, aeração e concentração de inóculo;
● Produzir P(3HB) em biorreator de 5 L;
● Quantificar o polímero acumulado pela *Pseudomonas* sp. CMM43 degradadora de agrotóxico;
● Caracterizar o biopolimero produzido quanto suas propriedades térmicas e sua massa molecular.

4 CAPÍTULOS

4.1 Artigo 1 – Identification of pesticide-degrading *Pseudomonas* strains as poly-β-hydroxybutyrate producers

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Identification of Pesticide-degrading *Pseudomonas* strains as poly-β-hydroxybutyrate producers

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Abstract

Polyhydroxybutyrate (P(3HB)) is a ecological promising substitute for polypropylene because is biocompatible, biodegradable and can be produced by renewable sources. This study investigate P(3HB) accumulation on *Pseudomonas* pesticide-degrading. Fourteen isolated from subtropical lowland soil in southern Brazil were analyzed using optical microscopy. Lipid inclusions were identified in four strains, and three of them, which degrade the pesticide carbofuran, had extensive granules accumulation detected by transmission electron microscopy. These strains were cultivated in shaker at 28 ^{o}C and the polymer was characterized by FTIR spectroscopy. Strain CMM43 had the best accumulation after 48 h. The biopolymer was identified as poly-β-hydroxybutyrate.

Keywords: Polyhydroxybutyrate. P(3HB). Pesticide-degrading *Pseudomonas* sp. Microscopy. Subtropical lowland soil

1 Introduction

The increasing search for polyhydroxyalkanoate (PHA)-producing bacteria is due to its potential applications in packaging (Kulkarni et al., 2011), as matrices for pesticides (Savenkova et al., 2001), and in suture lines (Volova et al., 2003). These polymers have properties that make them highly competitive with polypropylene (Reddy et al., 2003); and their replacement of petrochemical plastics is environmentally advantageous because they are biodegradable (Sudesh et al., 2000). Polyhydroxybutyrate (P(3HB)) is the most studied PHA and is the one that is most frequently accumulated by bacteria. These polyesters work as carbon and energy reserves for the cell and can be produced from a large variety of substrates, including renewable resources (Madson and Huisman, 1999).

P(3HB) are accumulated as granules by bacterial cell at up to 80% of the cell dry weight (Braunegg et al., 1998). Usually the granules formation occurs under conditions of nutritional limitation of N, P, O, or Mg and in excess carbon source (Anderson and Dawes, 1990) and in some bacteria the polymer accumulation occur during growth in the absence of nutrient limitation (Lee, 1996).

Most *Pseudomonas* bacteria are capable of biosynthesizing medium chain

length (MCL) PHAs (Haba et al., 2006). They seldom produce blends of PHAs consisting of P(3HB) (short chain) and MCL PHAs (Hang et al., 2002), and they rarely accumulate P(3HB) (Jiang et al., 2008; Mohan et al., 2010).

In this study, three strains of pesticide-degrading *Pseudomonas* from a culture collection were identified as P(3HB) producers. An initial classification of the P(3HB)-accumulating strains was performed using optical microscopy and transmission electron microscopy to determine the presence of granules in bacterial cells. The selected strains were cultivated in a sucrose medium for later polymer extraction and characterization by Fourier transform infrared spectroscopy (FTIR).

2 Materials and methods

2.1 Microorganisms

14 strains of pesticide-degrading *Pseudomonas* were used to identify lipid bodies. The strains were isolated from subtropical lowland soil in Rio Grande do Sul and conserved at the Culture Collection of Multifunctional Microorganisms at Embrapa Temperate Agriculture (CCMMETA). Six of the strains are known to degrade pyrazosulfuron-ethyl (CMM16, CMM17, CMM18, CMM19, CMM20 and CMM21), two to degrade glyphosate (CMM38 and CMM39), one to degrade clomazone (CMM1), and five to degrade carbofuran (CMM41, CMM42, CMM43, CMM44 and CMM45). *Bacillus megaterium* (CMM105) was used as a positive control for P(3HB) production (Gouda et al., 2001).

2.2 Detection of lipophilic inclusions by Sudan Black

To detect lipid bodies by optical microscopy, the strains were cultivated on plates with nutrient agar and 5% sucrose in a bacteriological incubator at 28 °C for 48 h. A preliminary screening for lipid bodies in the fourteen strains was performed using Sudan Black B staining according to Schaad, Jones and Chun (2001). Strains with positive Sudan Black B results were further assessed by transmission electron microscopy.

2.3 Detection of lipophilic inclusions by Transmission electron microscopy

The quantities and sizes of lipid inclusions were characterized using

transmission electron microscopy (TEM) for the strains previously selected by optical microscopy. For TEM analysis, cells were incubated on nutrient agar with sucrose for 48 h. The cells were washed with saline solution, bathed in two fixers, and then dehydrated in increasing concentrations of alcohol, 30%, 50%, 70%, 90%, and 95%(v/v), and P.A. acetone. Finally, the samples were embedded in Epon AB-DinP30 resin and cured for 5 days at 60 ºC. Ultra-thin sections were observed using TEM (ZEISS EM900).

2.4 P(3HB) production in shaker flasks

Because strain CMM43 was identified by TEM as a good accumulator of lipid granules, it was used as a model for analyzing growth versus polymer accumulation. The inoculum was prepared by inoculating YM medium (Jeanes, 1974) with a loop of a pure culture of the organism grown for 24 h. The YM culture was grown in an orbital shaker for 18 h at 28 ºC and 150 $rev.min^{-1}$. Ten percent (v/v) of inoculum was added to nutrient broth with $12g.L^{-1}$ of sucrose (NB+SUC) and incubated in a shaker at 28 ºC and 150 $rev.min^{-1}$. Four time points (12, 24, 48 and 72 h) were assessed by TEM for the accumulation of inclusions. In 24, 48 and 72 h cell dry weights (CDW) were also determined by gravimetry, and the amounts of P(3HB) were measure in 10 mg of CDW, wich were subjected to acid methanolysis and analyzed by gas chromatography (Brandl et al., 1988). Residual sucrose was measured using dinitrosalicylic acid method (DNS), after the hydrolysis of samples (Miller, 1959).

The strains CMM41, CMM43 and CMM44, wich were previously identified as accumulators of lipid inclusions, were grown in NB+SUC for 48 h, using the same inoculation, temperature and shaking methods previously described, for later extraction and polymer identification and characterization.

2.5 Polymer extraction

For P(3HB) extraction, cells were centrifuged at 6300g for 20 min, and pellets of harvested cells were bathed in 100 ml of chloroform. Samples were placed in closed tubes and heated at 58-59 ºC with magnetic shaking for 2 h. They were then centrifuged at 1100 g for 15 min, and the bottom organic phase in each tube was removed with a Pasteur pipette and transferred to a Petri plate. The plates were then covered by glass cubes for slow chloroform evaporation inside a gas exhaust

chamber.

KBr tablets with ground polymer films and the P(3HB) standard (Sigma) were used to identify the biopolymers produced by CMM41, CMM43 and CMM44 strains. A Fourier transform infrared spectrometer Shimadzu® model IR Prestige 21 was used to obtain the Fourier transform infrared (FTIR) spectra. Sixty-four images were acquired over a wavelength range of 4500 to 500 cm^{-1} with a resolution of 4 cm^{-1}.

The thermal properties of P(3HB) samples and standard P(3HB) were investigated by differential scanning calorimetry (DSC). DSC was performed with a Perkin Elmer, model Pyris 6. It was used a nitrogen atmosphere at a rate of 20 $ml.min^{-1}$. The samples were subjected to the following test conditions: isotherm 20°C for 1 min, first heating cycle of 20°C to 200°C at a rate of 10°C min^{-1}, isotherm 200°C for 5 min; cycle cooling 200°C to 20°C at a rate of 10°C min^{-1}; isotherm of 20°C for 5 min, second heating cycle of 20°C to 200°C at a rate of 10°C min^{-1}. To assess the effect of thermal history in obtaining the samples were observed values of melting temperature (Tm) in the first heating cycle. However, in order to eliminate the thermal history of the material the Tm was taken in the second heating cycle. The crystallinity degree (X_c) was determined from the melting enthalpy of the sample (ΔHm) and the melting enthalpy of pure cristalline P(3HB) (Δ 0mH = 146 J/g) (Jianchun et a.l, 2003; Gogolewski et al., 1993).

3 Results and discussion

3.1 Identification of lipophilic bodies

Among the 14 strains pesticide degrading assessed by optical microscopy for the presence of lipophilic bodies, four were positive: CMM41, CMM42, CMM43 and CMM44. All four belong to a group of carbofuran-degrading *Pseudomonas* spp.. These strains were visualized using TEM.

TEM assessment of the bacterial cells showed that strains CMM41, CMM43 and CMM44 accumulated a high amount of intracytoplasmic granules (Figures 1a, c and d), which suggested that they were promising PHA biopolymer producers; hence, they were selected for further study. The general appearance of inclusions accumulated for these strains (Figures 1a, c and d) are very similar to granules stored for the positive control *Bacillus megaterium* (Figure 1e), though the

micrographs suggested that the *Pseudomonas* spp. accumulated a higher numbers of granules. The granules presented as dense electron bodies, very similar to PHA inclusions found in other bacteria (Loo and Sudesh, 2007). Granule diameters in strain CMM41 were between 0.37 and 0.75 µm; in strain CMM42, between 0.19 and 0.31 µm; in strain CMM43, between 0.6 and 1.1 µm; and in strain CMM44, between 0.4 and 0.8 µm. Granule diameters in *B. megaterium* were between 0.2 and 0.75 µm. According to Yoo et al. (1997), typical P(3HB) granules have diameters between 0.2 and 0.7 µm.

3.2 Cell growth and accumulation of lipophilic bodies in strain CMM43

In the Figure 1 can be seen also the accumulation of lipophilic inclusions as a function of cultivation time of strain CMM43. The highest accumulations and largest inclusions were found after 48 h (c3), when the maximum CDW of 5,8 $g.L^{-1}$ with a P(3HB) concentration of 29% CDW. The highest P(3HB) accumulation in a short time is a result very relevant because generally P(3HB) accumulation is a slowly process (Jiang et al., 2008). And was observed that the carbon source was readily consumed during P(3HB) accumulation registering a consumption of 8,6 $g.L^{-1}$ making 70 % of initial sugar concentration. Most cells had not divided because in conditions of excess carbon, growth becomes unbalanced, and substrates are converted to intracellular polymer, which disfavors cellular synthesis (Beccari et al., 1998). At 12 h of incubation (c1), there was a substantial quantity of accumulated granules, but the cells were also in a process of dividing; the amounts of CDW, P(3HB) and residual sugar were not analyzed. At 24 h (c2) there were evident reductions in the amount of inclusions and the extent of cell division, the CDW content of 2,14 $g.L^{-1}$ was found when strain CMM43 growth in this time and P(3HB) content in the CDW was 9%. The sugar concentration obtained was 4,4 $g.L^{-1}$ represents a consumption of 32% . At 72 h (c4), there were fewer inclusions than at other times, which could be due to the reserves being used by the cells (Sudesh et al., 2000). The quantitative P(3HB) results demonstrated that the amounts of biopolymer in this time decreased to almost 4% of P(3HB) content verified after 48 h. The results of CDW in this time were 6,3$g.L^{-1}$. The sugar consumption was almost the same that in 48 h; it was 72% (8,8$g.L^{-1}$) showing the use of the polymer for the cell and not the carbon source.

3.3 Identification of accumulated biopolymer

Infrared spectra showed that the three strains had similar biopolymer compared to the P(3HB) standard (Sigma) (Figure 2). All three samples analyzed displayed P(3HB) characteristics. The band at 1454-1459 cm^{-1} corresponds to C-H asymmetrical stretching for CH_2 groups, while the band at 1378 cm^{-1} is equivalent to CH_3 group deformation. These bands are comparable to those found by Oliveira et al. (2007). The bands near 3000 cm^{-1} signify the asymmetrical stretching of C-H in aliphatic CH_3 and CH_2 groups (Khardenavis et al., 2007). The presence of strong absorption bands at 1724-1727 cm^{-1} and 1281 cm^{-1} is a consequence of the ester C-O bonds stretching, representing carboxylic (C=O) and carbonylic (C-O) bonds, respectively. The series of intense bands from 1250 to 950 cm^{-1} also correspond to the C-O enlargement for the ester group (Oliveira et al., 2007; Arun et al., 2009). The wide band located near 3440 cm^{-1} corresponds to the OH group (Oliveira et al., 2007; Arun et al., 2009). Some of the P(3HB) bands produced by strain CMM41 were smaller compared to the standard spectrum pattern, which may be a result of differences in molecular weight or crystallinity (Lugg et al., 2008). In other study we verified that these strains can accumulate mainfold PHAs. The polymer spectrum produced by strain CMM44 is almost identical to the standard P(3HB), possibly indicating that in this sample the P(3HB) proportion is higher than in another samples.

The results of thermal analysis are expressed in Table 1. The melting temperature of the different samples obtained from P(3HB) synthesized by the strains and the standard P(3HB) was a little lower to the values found in the literature such as 177,3°C described by Hong et al. (2008) and 177,2°C according to Chaijamrus and Udpuay (2008). This values may possibly be related with external contaminants like the residual biomass and inorganic elements from fermentation processes that could affected the thermal property of P(3HB) (Kim et al., 2006) or with the technique of extration, Valappil et al. (2007) reported the T_m of P(3HB) extracted from the *Bacillus cereus* of 160,83°C using chloroform for the extration.

The X_C obtained in the P(3HB) produced by strains and the standard P(3HB) are lower too than the usual range of crystallinity value. El-Hadi et al. (2002) verified a X_C of 60% for the P(3HB) from Copersucar/Brazil produced by the fermentation of molasses using *Alcaligens euthrophus*. The crystallinity degree obtained by the

P(3HB) produced by strain CMM43 is the closest comparing to values found by Reddy et al. (2009) that varying according to the substrate used between 42 and 50%. These results are desirable because high levels of cristallinity becomes the polymer brittle and stiff resulting in very poor mechanical properities (Savenkova et al., 2000).

4 Conclusions

This study showed that pesticide-degrading *Pseudomonas*, specially the carbofuran-degrading strains constitutes a promising group to investigate polyhydroxybutyrate (P(3HB)) accumulation capability.

Pseudomonas sp. CMM41, CMM43 and CMM44, all of which degrade carbofuran pesticide, have the capacity to accumulate P(3HB) from sucrose, an inexpensive substrate in Brazil, a trait that is not common in *Pseudomona s*p. that, normally, accumulate MCL-PHA. The P(3HB) accumulated by these strains was characterized as having low crystallinity degree, desirable feature for making the material less brittle.

Strain CMM43 had the best accumulation and largest inclusions just after shaking for 48 h compared to other cultivation times. Currently the strain CMM43 is being investigated to increase the production of P(3HB) by optimization of fermentative parameters.

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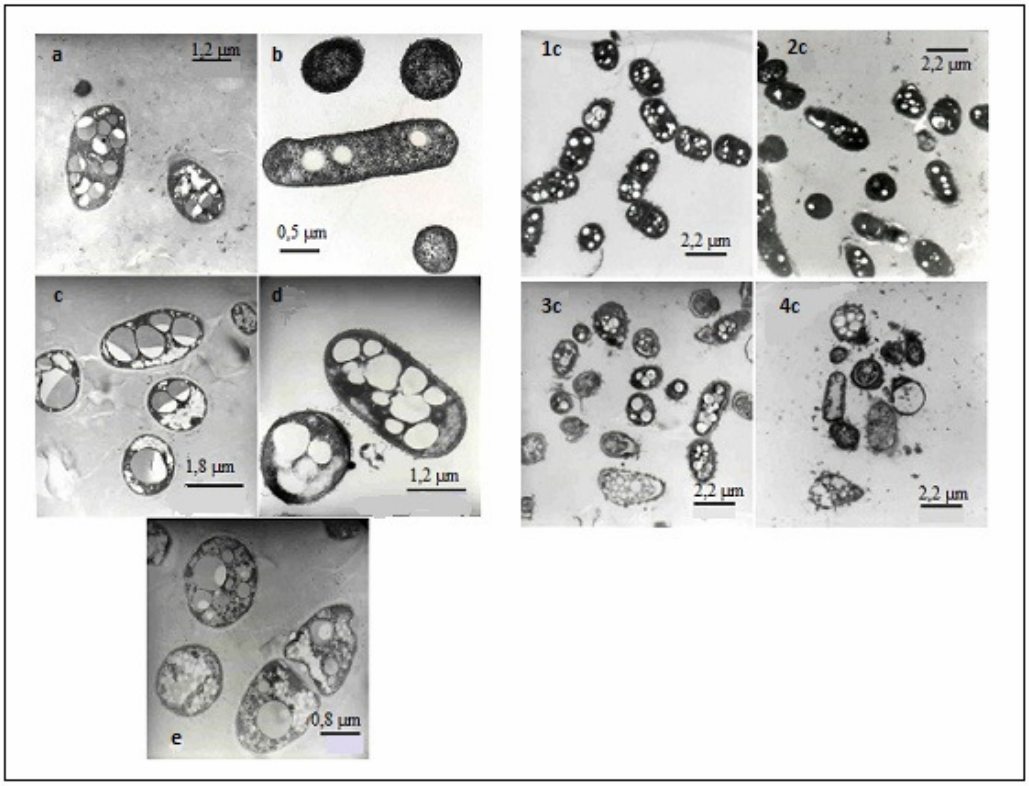

Fig. 1 Transmission electron micrographs showing P(3HB) inclusions in bacterial cells of *Pseudomonas* strains (a) CMM41, (b) CMM42, (c) CMM43, and (d) CMM44 and in cells of (e) the control bacteria *B. megaterium* cultivated in NB+SUC for 48 h and accumulation of inclusions as a function of cultivation time in strain CMM43 after (c1) 12 h, (c2) 24 h, (c3) 48 h and (c4) 72 h.

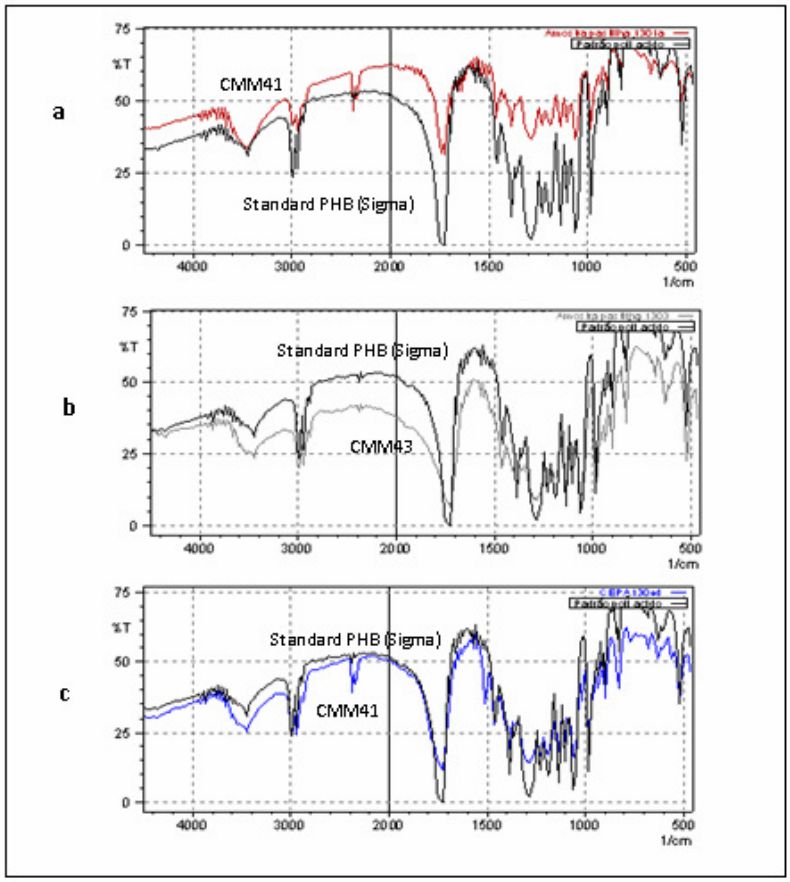

Fig. 2 FTIR spectra of the polymers from strains (a) CMM41, (b) CMM43 and (c) CMM44 compared to the P(3HB) standard pattern.

Table 1. Thermal properties of P(3HB).

Samples of P(3HB)	T_m (℃) (1)	T_m (℃) (2)	(X_C) %
Standard	170	148	41
CMM41	168	131	27
CMM43	164	155	45
CMM44	166	156	37

4.2 Manuscrito 1 – Poly(3-hidroxybutyrate) accumulation in cell growth phase by *Pseudomonas* sp. CMM43

Manuscrito formatado de acordo com as normas da revista *New Biotechnology*

Poly(3-hidroxybutyrate) accumulation in cell growth phase by *Pseudomonas* sp. CMM43

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Abstract

The P(3HB) production generally occurs in two distinct phases: the first phase is called growth phase, that gives conditions to cell growth, and in the second one the polymer production occurs with the stop or reduction of cellular multiplication process. But in some bacteria this production polymer process happens in a single phase, it is a growth-associated production of P(3HB). Due to the importance of the growth phase in order to achieve high cell concentration, conditions like aeration, agitation speed, carbohydrate source, pH and temperature was investigated. At the same time we evaluated the production of biopolymer in the growth phase to determine whether the biosynthesis is growth-associated. The recovered biopolymers were identified as P(3HB) by FTIR. The *Pseudomonas* sp. CMM43 accumulated high amounts of P(3HB) during growth phase without limiting nutrient and in a short time, suggesting that the accumulation occurs simultaneously to the growth. The use of high agitation combined with high medium volume, that result in less aeration, provided the maximum P(3HB) content. The GPC and DSC analysis showed that recovered biopolymer presents reduced thermal properties and low molecular mass. Thus enabling more specific applications in the biomedical area, such as microspheres, scaffolds for cartilage engineering and sutures.

Key words: P(3HB), growth phase, growth associated production, absence nutrient limitation, *Pseudomonas*

1 Introduction

Polyhydroxyalkanoates (PHAs) comprising important class of polyesters, called biodegradable plastic, having polyhydroxybutyrate P(3HB) as its main representative (Reddy et al., 2008). P(3HB) presents a wide application range that depends on their primary properties like melting temperature, crystallinity degree and molecular weigth. Most times P(3HB) production process are constituted of two main phases: a cell growth or inoculum phase and polymer production, that use different cultivation conditions. In the first phase the cells are grown in a medium which contain the essential nutrients, carbon and nitrogen source as carbohydrate and proteins, supporting cell growth to a certain biomass concentration and only minor polymer accumulation occurs (Byrom, 1987). In the second phase, the process blocks cell

growth and the cells start to accumulate the maximum amount of product. However some wild-type strains are capable of growth-associated production of PHA in the absence of nutrient limitation (Lee, 1996). The growth-associated P(3HB) production contributes to two metabolic processes: energy supply, to synthesize the non-P(3HB) cell constituents, and polymer accumulation, whose synthesis is not triggered by the decreasing growth rate (Borman, 2000). The growth phase of P(3HB) production is of utmost significance mainly because it is an intracellular polymer, therefore high-cell-density is necessary to achieve a high production of polymer. Some parameters need to be evaluate to increase cell concentration thus strategies are required like selecting the initial cellular concentration, temperature, agitation speed, pH, aeration, culture medium. Normally, when enough carbon and nitrogen are available, a small amount of P(3HB) is synthesized (Macrae and Wilkinson, 1958 *apud* Jacob et al, 1986). It is not common a high accumulation of polymer in the growth phase (Piccoli, 1995; Tay et al., 2010). The purpose in this study was to elevate the cellular concentration in growth phase, evaluating culture conditions, i.e. aeration, agitation speed, carbohydrate source, pH and temperature, in the meantime to determine whether P(3HB) production is growth-associated and characterize the synthesized polymer by TEM, GPC, DSC and FTIR.

2. Material and methods

2.1 Microorganism

The *Pseudomonas* strain CMM43 was from the Embrapa Temperate Agriculture Multifunctional Microorganism Culture Collection. Originally the bacterium was isolated from soil irrigated rice in Rio Grande do Sul, Brazil.

2.2 Culture medium

A classical medium, YM medium (Jeanes, 1974), containing in g/L: glucose, 10; malt extract, 3, yeast extract, 3, and peptone, 5 and variations of carbon source were used. After, the following combinations between carbohydrate source and pH - glucose and initial values of pH 5.5 (Gluc 5.5) and pH 6.5 (Gluc 6,5), and sucrose pH 5.5 (Suc 5,5) and pH 6.5 (Suc 6.5) - were tested.

2.3 Culture conditions

To establish the optimal conditions to cell growth in the growth phase for *Pseudomonas* sp. CMM43, we investigated the influence of different cultivation parameters such as incubation temperature, aeration, agitation speed, pH, and carbohydrate source. In all experiments, in order to investigate the capability of growth-associated polymer production, the amounts of P(3HB) accumulated was determined. The pre-inoculum were prepared from bacterial cell suspensions with 24 h of incubation time in YM agar, containing about $1.05x10^8$ CFU/mL, which were transferred to Erlenmeyer flasks containing YM broth variations and incubated in an orbital shaker for 24 h. In preliminary assays were evaluated the temperatures of 25 and 28 ℃, the media were Gluc 5.5, Gluc 6.5, Suc 5.5 and Suc 6.5, and agitation speed of 150 rpm. The volume of 50 mL (20% of total flask volume) was dispensed into 250 mL Erlenmeyer flasks. To determine the aeration and agitation influence volumes of 20 (8% of total flask volume) and 50 mL (20% of total flask volume) were used in the same volume Erlenmeyer flasks, the media Suc 6.5 and agitation speed of 150 and 200 rpm. Tests to verify the influence of aeration, agitation speed and temperature increasing the production volume were performed; the volumes of 100 (20% of total flask volume) and 250 mL (50% of total flask volume) were dispensed into 500 mL Erlenmeyer flasks. Using the media Suc 6.5, agitation speeds of 150 and 250 rpm and the following temperatures, 28, 30 and 32 ℃, were evaluated. A final experiment, to evaluated the dry cell weigth, P(3HB) accumulation and carbohydrate consumption at 24, 48 and 72 h of incubation time, was realized. The operational conditions were 28℃, 150 and 250 rpm, with volume of 250 mL into 500 mL (50% of total flask volume) Erlenmeyer flasks. The evaluation of aeration was performed indirectly by modifying the useful volume.

2.4 Determination of dry cell weight and P(3HB) quantification

Dry cell weight (DCW) was determined gravimetrically and the amounts of P(3HB) in 20 mg DCW were determined by acid methanolysis and analysed by gas chromatography as previously described (Brandl et al., 1988). The organic phase was analysed using a Shimadzu GC 17A gas chromatograph (GC) equipped with a DB Waxetr column (30 m x 0.250 mm x 0,25 µm) and flame ionization detector (FID) with hydrogen as the carrier gas (1 mL/min). Initial temperature of 80 ℃ was held for

1 min, before ramping to 200 ℃ at 11 ℃/min and holding for 4 min. The injected volume was 1 µL, manually. The injector temperature was 260 ℃ and the temperature of detector was 280 ℃. Quantitative analysis was performed by internal standardization method using benzoic acid as internal standard. The calibration curve was prepared from P(3HB) (Sigma-Aldrich) and the internal standard. Residual biomass (RB) (g/L) was defined as the difference between DCW (g/L) and P(3HB) (g/L).

2.5 Residual sucrose

Residual sucrose in the growth medium following fermentation was measured in hydrolysed sample by the dinitrosalicylic acid method (DNS) (Miller, 1959).

2.6 Transmission electron microscopy

Cells incubated in Suc 6.5 medium, 28 ºC and 250 rpm during 24 h were washed with saline solution, fixed and then dehydrated in increasing concentrations of alcohol, 30, 50, 70, 90 and 95%(v/v), and P.A. acetone. Finally, the samples were embedded in Epon AB-DinP30 resin and cured for 5 days at 60℃. The P(3HB) granules accumulated after 24 h of cultivation at 28 ºC and 250 rpm were observed as ultra-thin sections using TEM (Zeiss model EM900).

2.7 P(3HB) recovery

For P(3HB) extraction, chloroform was added in the proportion of 10 mL/g of DCW. Samples were placed in closed tubes and heated to 59 ℃ with magnetic shaking for 1h. Cellular debris was removed by filtration and the supernatant was transferred to covered Petri dishes for slow chloroform evaporation inside a gas exhaust chamber. The P(3HB) extraction was carried out using DCW obtained from the cultives in 250 mL medium volume for 24 h, using the following combinations of temperatures and agitations: 28 ℃ and 250 rpm, 28 ℃ and 150 rpm, 30 ℃ and 250 rpm, and 32 ℃ and 250 rpm.

2.8 Polymer characterization

2.8.1 Fourier transform infrared spectroscopy (FTIR)

The identity of the P(3HB)s was confirmed by Fourier transform infrared spectroscopy (FTIR). The precipitated dry polymers produced from *Pseudomonas* strain CMM43 and the P(3HB) control (Sigma-Aldrich) were used to prepare KBr tablets. A Fourier transform infrared spectrometer Shimadzu model IR Prestige 21 was used to obtain the FTIR spectra. The total of 64 images were acquired over a wavelength range of 4500 to 500 cm^{-1} with a resolution of 4 cm^{-1}.

2.8.2 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used to estimate the molecular mass (Viscotek Model VE 2001, equipped with refractive index detector and PS/DVB columns). The samples and the P(3HB) control (Sigma-Aldrich) were solubilized in 2 mL chloroform and filtered (0.45 mm PVDF filter) before injection. The movel fhase used was THF at a flow rate of 1 mL/min. The molecular mass was calculated from a calibration curve based on standard polystyrene.

2.8.3 Differential scanning calorimetry (DSC)

The thermal properties of the P(3HB) samples and the control were investigated using a differential scanning calorimeter (DSC) (Shimadzu, model TA 60WS) in a nitrogen atmosphere at a rate of 50 mL/min. The samples and the P(3HB) control (Sigma-Aldrich) were subjected to the following test conditions: first heating cycle from 30 to 200 °C at a rate of 10 °C/min, isotherm 200 °C for 5 min; cooling cycle from 200 to 30 °C at a rate of 10 °C/min, isotherm of 30 °C for 5 min; second heating cycle from 30 to 200 °C at a rate of 10 °C/min. To assess the effect of thermal history in obtaining the samples, we observed the melting temperature (Tm) during the first heating cycle. However, in order to eliminate the thermal history of the material, the Tm was measured during the second heating cycle. The crystallinity degree (χ_c) was determined from the melting enthalpy of the sample (ΔHm) and the melting enthalpy of pure crystalline P(3HB) (ΔHm = 146 J/g) (Gogolewski et al., 1993; Jianchun et al., 2003).

2.9 Statistical analysis

The experimental data were generated from triplicate and the average and standard deviation calculated by Excel.

3 Results and Discussion

3.1 Cell growth phase conditions and P(3HB) accumulation

Pseudomonas sp. CMM43 presented high DCW and high P(3HB) levels while they were in the cell growth phase. Figure 1 shows DCW values at 0 and 24 h and P(3HB) accumulation (%) at 24 h using different combinations of temperature, pH, and carbohydrate. The DCW results at 24 h are comparable or exceed values normally reported when specific media for P(3HB) production phase are used in a longer time of cultivation. The results for P(3HB) accumulation (%) showed that in 24 h of growth phase an expressive amount of polymer was accumulated. Belal (2013), cultivating *Pseudomonas stutzeri* E114 obtained maxima DCW values and P(3HB) content of 3.01 g/L and 53.2%, respectively when used sucrose as carbon source and at 24 h in the production phase. Ashby, Solaiman and Foglia (2002) achieved 3.4 g/L of DCW with 29% of polymer accumulated after 48 h, cultivating *Pseudomonas oleovorans* NRRL B- 778 in production phase with media containing glucose.

Pseudomonas sp. CMM43 has demonstrated a narrow range of optimal temperature, with the temperature presenting higher influence on the DCW than variations on carbohydrate source and pH. From these data the best carbohydrate, pH and temperature combination was selected, which was Suc 6.5 at 28 ℃, resulting around 7 g/L of DCW and P(3HB) content was 35.5%.

The results obtained from aeration and agitation on cell growth and accumulation of P(3HB) (%) are expressed in Figure 2. The variation of useful volume allowed us to evaluate the aeration influence, since low useful volume in the same total volume bottle results in more high aeration.

The increase in DCW from 0 to 24 h was more influenced by the increasing on agitation than increasing on aeration. The combination of high agitation speed with low aeration (50 mL) was responsible for the DCW greatest increase. It can be said that there was an interaction between aeration and agitation at 24 h of cultivation. When a growing condition with higher aeration (20 mL) was offered, the

highest value for DCW was found using a lower agitation of 150 rpm; and when the volume was increased (50 mL), resulting in decreased aeration, the value of DCW was higher using the high agitation (200 rpm). The bacterium seems to present a level of tolerance to oxygen for cell production, since the maximum values for DCW were achieved in conditions of higher aeration and lower agitation and vice versa.
The lowest aeration (50 mL) favored polymer accumulation in relation to the cultivation at low aeration (20 mL) at both agitations used, whereas the higher agitation favored more the P(3HB) accumulation. The percentage of polymer accumulated in low aeration condition was similar for both tested agitations. The achievement of most high P(3HB) accumulation using low aeration are in agreement with Jung et al. (2005).

Figure 3 summarizes DCW and P(3HB) accumulated results in 24 h by change on temperature, agitation and aeration and increasing on production volume by use 100 and 250 mL useful volume against 500 mL of total flask volume.

The P(3HB) accumulation in the cell at 24 h reached 70%, and this polymer amount is typically achieved after longer fermentation time, from 48 to 96 h (Bourque et al., 1995, Reddy et al., 2008, Jiang et al, 2008) and under special culture conditions, such a nutrient shortage and excess carbon source (Sangkharak and Prasertsan, 2008; Wen et al., 2010; Liu et a.l, 2011). P(3HB) accumulation in a short time is a very important result, because P(3HB) accumulation is usually a slow process (Jiang et al., 2008).

Larger amounts of DCW were achieved using 28 ℃ and 250 rpm, in both used aerations (useful volumes 100 and 250/500 mL). The aeration, however, resulted different polymer accumulation, whose values were 30% and 70%, using high aeration, and low aeration, respectively, confirming the results of Fig. 2. Evaluating agitation speed of 150 rpm, with high aeration (100 mL) and with low aeration (250 mL), the highest value for DCW and the maximum P(3HB) accumulation were achieved, respectively.

Zafar et al. (2012) commented that the effects of oxygen limitation should be considered for P(3HB) accumulation optimization. Piccoli (1995) reported elevated biomass production and 3.5% PHA accumulation in the growth phase cultivating *A. eutrophus* under fed-batch culture. Tay et al. (2010) reported that PHA biosynthesis by *Bacillus* sp. MC1 was growth-associated when it was grown in NB medium for 20

h, and up to 19% of PHA in DCW was verified.

Almeida et al. (2010) obtained higher amounts of DCW using high agitation (500 rpm) in bioreactor, regardless of the substrate used, but the percentage of accumulated P(3HB) was high using glycerol and low agitation (250 rpm) and using glucose and high agitation. Tripathi et al. (2012), cultivating *Pseudomonas aeruginosa* obtained the maximum DCW yield and P(3HB) accumulation using an agitation speed of 175 rpm, whereas higher agitation speed (200 rpm) caused cell lyses and low yield.

Evaluating different temperatures on the same aeration, better results for DCW was achieved at 28 °C, for both aerations. Reddy et al. (2008), using temperature of 28 °C, had lower DCW at 48 h, that was 4.91 g/L from municipal sewage sludge bacteria, that including the genus *Pseudomonas*. This result contrasting with most studies conducted with *Pseudomonas* to obtain PHAs, which use temperature more elevated, as 30 °C (Ni et al., 2010; Wang, Li and Chen, 2009; Das, Chowdhury and Anwar, 2005; Diniz et al., 2004). Fernandéz et al. (2005), however, cultivating *Pseudomonas aeruginosa* at 30 °C in a mineral medium with agro-industrial oily wastes and glucose as carbon source, had lower DCW results than to ours, 3.2 g/L, at 24 h.

The results obtained in this work encouraging, but can be improved. Yamane et al. (1996) produced an inoculum with 13.7 g/L at 16.5 h using a bioreactor and an amount of P(3HB)/DCW of 50% in just 18 h of cultivation by *Alcaligenes latus* was obtained. Our data at 24 h and 28 °C, even had lower DCW than found by Yamane et al. (1996), are highest in relation to P(3HB), being reached 70% at low aeration (250 mL) condition.

3.2. Increasing on incubation time

New experiments with increasing on incubation time of *Pseudomonas* sp. CMM43, yet in orbital shaker, with DCW, P(3HB) accumulation and sucrose consumption determination were conducted (Fig. 4). As expected, the sucrose consumption increased in the course of cultivation time and was more elevate at high agitation (250 rpm) (Fig. 4B), which favored aerobic respiration. At 72 h practically all sucrose was consumed. The maximum amounts of DCW and P(3HB), 10.2 g/L and 6.6 g/L, respectively, were early achieved using high agitation speed.

During the incubation time, decreases in P(3HB) accumulation (g/L and %) and DCW were observed. Probably, polymer accumulated was consumed for cellular metabolism maintenance, hypothesis supported by the significant reduction in the sucrose levels. With low agitation (150 rpm) (Fig. 4A), even P(3HB) accumulation had increased along the time and reached the highest value, almost 70% at 72 h, the P(3HB) yield (g/L) was lower than at high agitation.

In the transmission electron micrographs (Fig. 5) cells with massive amounts P(3HB) inclusions and in division process also could be seen at 24 h, suggesting that the P(3HB) accumulation is growth associated. Both, size and overall inclusions appearance are very similar to P(3HB) granules found in many bacteria (Ramachander et al., 2002; Tian et al., 2005). The granules also present electron lucent appearance, as showed in the Lageveen et al. (1988) and de Loo and Sudesh (2007) works. Micrographs confirm others results showed in this work and becomes evident that the polymer biosynthesis by *Pseudomonas* sp. CMM43 was growth associated cause it is possible to seen the cells filled with P(3HB) and under division.

3.3 Extracted biopolymer characterization

All samples formed thin polymeric films after slow solvent evaporation (Fig. 6). Only the film obtained from cell growth at 28 ºC and 25 rpm (Fig. 6A) was slick and more intact at the edges; the others films were brittle, with powder appearance, very similar to P(3HB) used as control (Sigma Aldrich) in this work. The whitish coloration also conferring with the color presented by polymer control.

Infrared spectroscopy is widely used for characterization of bacterial polyesters (Lundgren et al., 1965; Pal and Paul., 2002; Rodrigues,Parra and Lugão, 2005; Lugg et al., 2008.). The IR spectra show characteristic bands of P(3HB) for all samples analyzed (Fig. 7) and no other stranger band to P(3HB) was found. The band found at 1454-1459 cm^{-1} corresponding to the asymmetric deformation of CH bond in CH_2 groups, while the band found at 1378 cm^{-1} is equivalent to this deformation of the CH3 groups, also described by Oliveira et al. (2007). The bands around 3000 cm^{-1} also refer to symmetric and asymmetric axial deformation of CH bond (Khardenavis et al., 2007). The presence of strong absorption bands at 1724-1727 cm^{-1} and 1281 cm^{-1} are assigned to the axial deformation of the carbonyl (C = O) and C-O stretching of ester group, respectively (Xu et al., 2002; Berekaa and

Thawadi, 2012). The series of bands located between 1228-1057 cm^{-1} also corresponds to the C-O stretching of the ester group (Khardenavis et al., 2007; Lugg et al., 2008). The band around 3440 cm^{-1} corresponds to the OH group (Oliveira et al., 2007; Arun et al., 2009). All characteristic peaks were confirmed by comparison with the P(3HB) control.

The molecular weight is the main primary property that provides interesting and useful to the physical and mechanical properties to polymers. Physical properties of P(3HB) as crystallization and high tensile strength depend on the molecular weight, that is influenced by the species of microorganism used, growth conditions and samples purity (Punrattanasin, 2001). The extracted P(3HB)s were analyzed by Gel Permeation Chromatography (GPC) to estimate the molecular ratio of the biopolymers weight (Table 1).

Wild bacteria normally produce P(3HB) with a molecular weight (Mw) range 1.10^4-3.10^6 and polydispersity (Mw/Mn) around 2.0 (Khanna and Srivastava, 2005). The Mw values of samples are in the minor zone reported in the literature and may be related to culture conditions and substrate used (Chen and Page, 1994). Extraction methods can also cause serious damage to the granules, leading to loss of polymer molecular weight (Dawes and Senior, 1973; Holmes, 1988). The Mw/Mn values are higher than those usually reported (Galego et al.,2000; Oliveira et al., 2007; Tanadchangsaeng and Yu, 2012), but are in agreement with the value found by Fiorese et al. (2009). Both the molecular weight and the polydispersity are important parameters to evaluating P(3HB) application.

The industrial application of high molecular weight P(3HB) is wide, but some specific applications, as for preparation of certain graft and block copolymers, require low molecular weight P(3HB) (Yalpani et al., 1991; Yu and Marchessault, 2000). Applications which the polymer degradation inside the body is important, as suture materials, bone graft and controlled release drug delivery systems require low molecular weight polymer for its manufacture (Ramkumar and Bhattacharya, 1998). To obtain a material with low molecular mass, copolymers are used, such as P(3HB)-PHV (Holland et al., 1987). The *Pseudomonas* sp CMM43 produced naturally a P(3HB) with low molecular weigth.

The thermal analysis results are showed in Table 2. The melting temperature of different P(3HB) samples are lower than those reported in the concernment

literature, such as 177.3 °C described by Hong et al. (2008) and 177.2 °C according to Chaijamrus and Udpuay (2008), but similar to P(3HB) control (Sigma Aldrich). At the same agitation, the polymers obtained using more high incubation temperature had highest Tm at second cycle of analysis (Tm 2). The low values may be related to the extraction technique used or with external contaminants such as residual biomass that can affect P(3HB) thermal properties (Kim et al., 2006). Meantime Tm values found are in agreement with those obtained by the same strain *Pseudomonas* sp. CMM43 Tm (1) 164 °C and Tm (2) 155 °C, using another medium but the same carbon source (Crochemore et al., 2012), and with those extracted with chloroform from *Bacillus cereus* by Valappil et al. (2007), that had Tm 160.83 °C.

The X_c verified to produced P(3HB) (30 - 45%) were lower than usually reported. X_c values around 40 % are more closer to the P(3HB)/PHV blends (Buzarovska et al., 2009). Value of 60% was found by El-Hadi et al. (2002) using a Brazilian commercial P(3HB) produced by *Alcaligens euthrophus* and sugar cane broth, a very rich on sucrose substrate, and by Dobroth et al. (2011) using mixed microbial consortia. P(3HB) have been reported to be excessively stiff and brittle, as a result of their relatively high crystallinity, resulting in very poor mechanical properties (Savenkova et al., 2000). The results achieved to X_c in this study are desirable because they make the polymers more flexible and processable at lower temperatures, protecting the polymer from thermal degradation (Köse et al., 2003).

4 Conclusion

The *Pseudomonas* strain CMM43 is able to utilize sucrose for growth and P(3HB) production in short period of time, indicating that the polymer production is growing-associated and it can be performed in a single step, thus reducing the process costs. The maximum levels were achieved in lowest aeration condition and in a temperature not usual for *Pseudomonas*. Although FTIR analysis revealed characteristics P(3HB) peaks for all samples, the values for molecular weight, melting temperature and crystallinity were lower than those normally reported to P(3HB), but which are desirable for biomedical and pharmaceutical applications.

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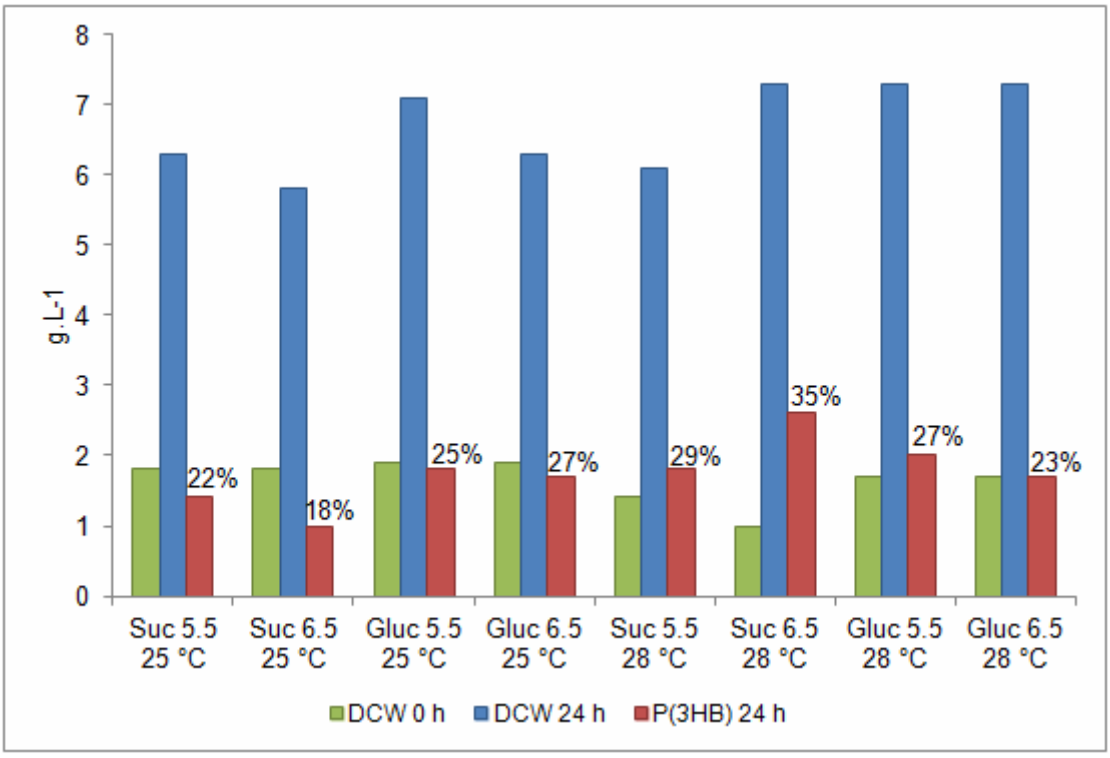

Figure 1. DCW variation (g/L) and P(3HB) accumulation in the growth phase using different combinations of carbohydrate source, (Suc) Sucrose or (Gluc) glucose, pH 5.5 or 6.5 and temperature of 25 or 28 ℃. Volume of 50 mL in 250 mL Erlenmeyer flasks and agitation speed of 150 rpm. Analysis were done in triplicate with a standard deviation less than 2%.

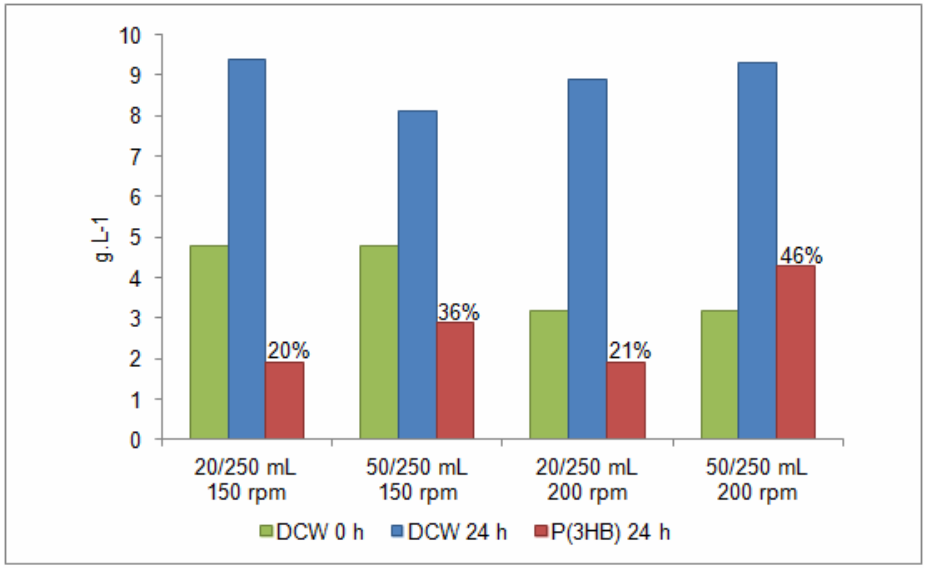

Figure 2. Indirect aeration (by volume modification) evaluation and agitation speed on the increase in DCW and P(3HB) accumulation in the growth phase using Suc 6.5 medium and 28 ℃. Analysis were done in triplicate with a standard deviation less than 3%.

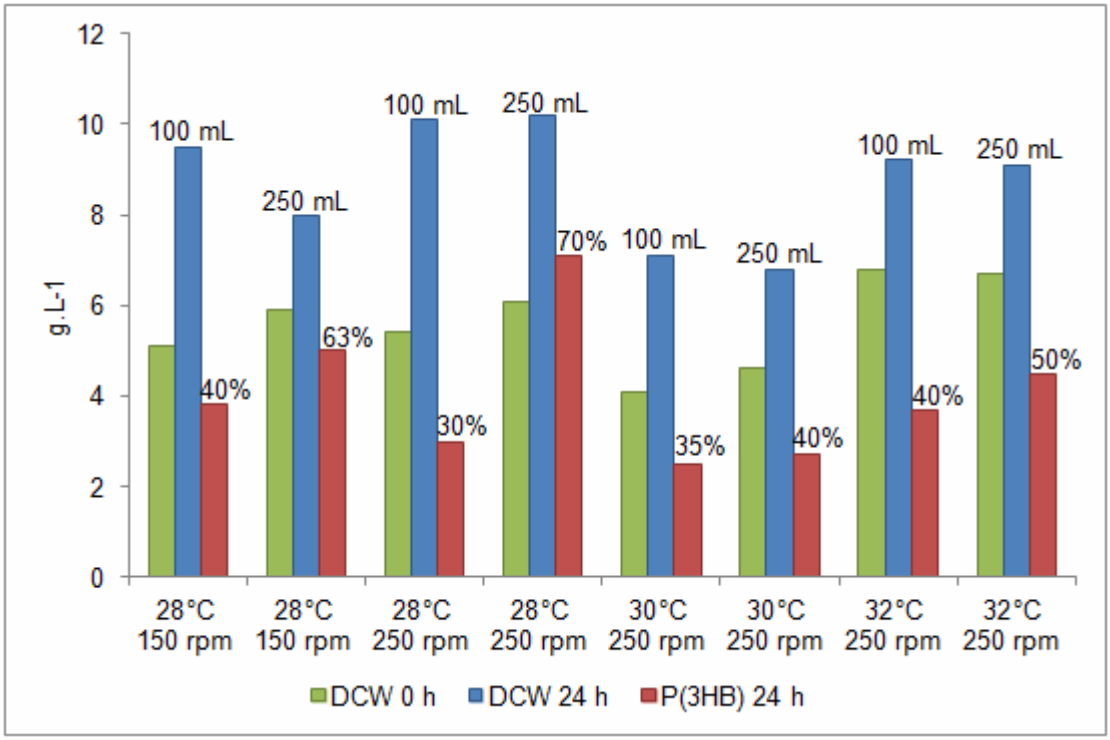

Figure 3. DCW amounts and biopolymer accumulation in the growth phase, changing temperature, agitation and aeration. Analysis were done in triplicate with a standard deviation less than 2%.

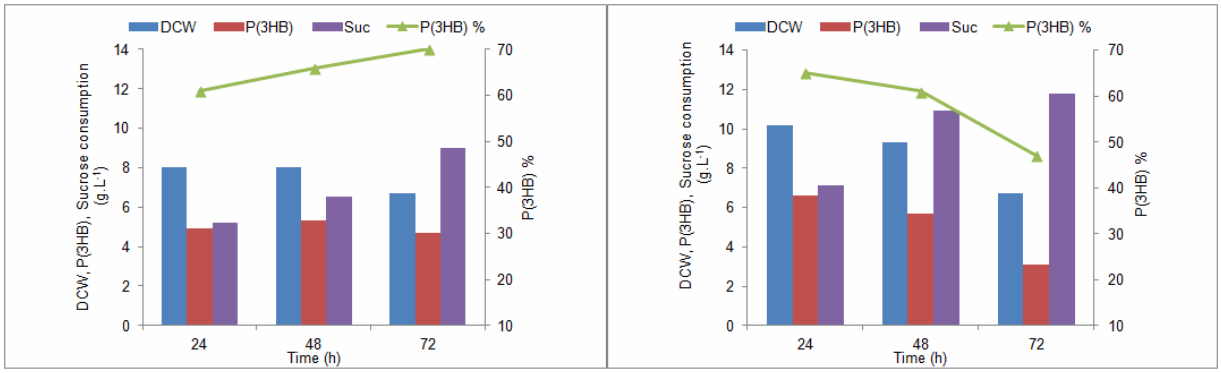

Figure 4. DCW and P(3HB) yield (g/L), P(3HB) accumulation (%) and sucrose consumption, using Suc 6.5 medium and 28 ℃ and two agitation speed: (a)150 rpm and (b) 250 rpm. Analysis in triplicate with a standard deviation less than 2% were done.

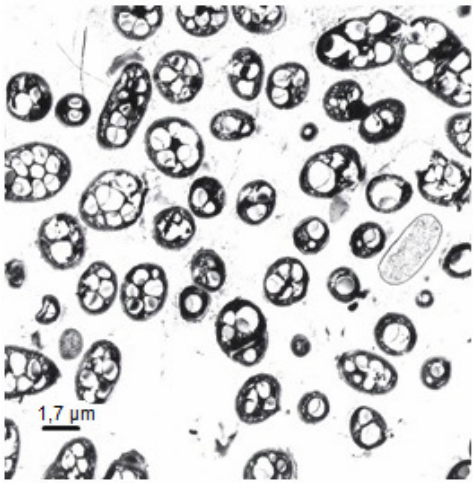

Figure 5. Transmission electron micrograph of the P(3HB) accumulation by *Pseudomonas* sp. CMM43 grown in Suc 6.5 medium, 28 ℃ and 250 rpm, during 24 h.

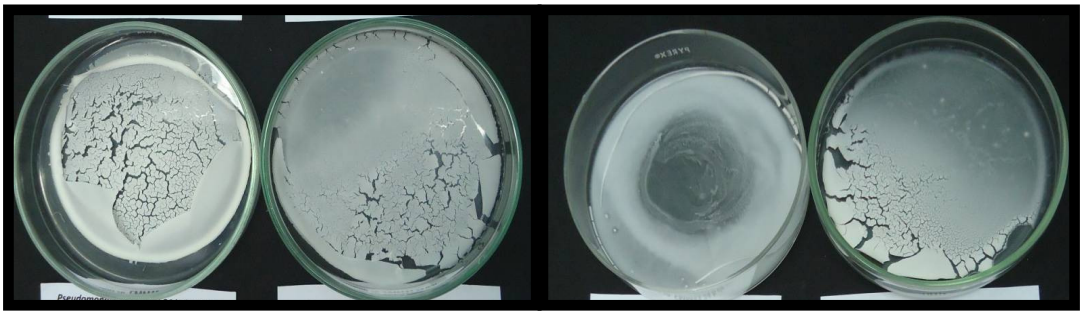

Figure 6. P(3HB) films obtained by chloroform slow evaporation from dry cell weight in growth phase cultivated in Suc 6.5 medium during 24 h at (A) 28 ℃ and 250 rpm, (B) 28 ℃ and 150 rpm, (C) and 30 ℃ and 250 rpm (D) 32 ℃ and 250 rpm.

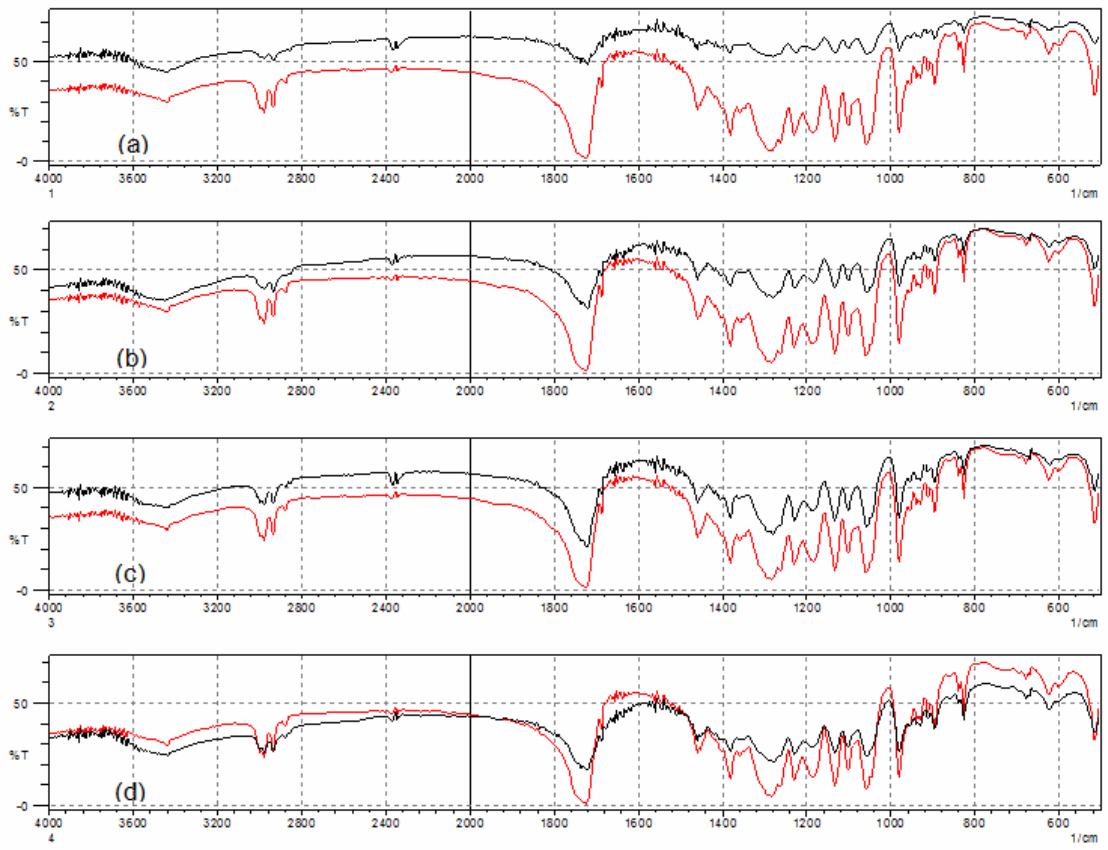

Figure 7. ▬ Infrared spectra of P(3HB) produced in different conditions of temperature and agitation combinations: (a) 28 ℃ and 250 rpm (b) 28 ℃ and 150 rpm (c) 30 ℃ and 250 rpm (d) 32 ℃ and 250 rpm. ▬ P(3HB) control (Sigma Aldrich).

Table 1. P(3HB) molecular weight obtained in different temperature and agitation combinations determined by GPC.

Sample	Mw (Da)	Mn (Da)	Mw/Mn (polydispersity)
A	$4.6x10^4$	$1.3x10^4$	3.6
B	$4.7x10^4$	$1.3x10^4$	3.6
C	$4.7x10^4$	$1.3x10^4$	3.5
D	$3.8x10^4$	$1.2x10^4$	3.0
P(3HB) control	$2.5x10^5$	$1.1x10^5$	2.2

(A) 28℃ and 250 rpm, (B) 28℃ and 150 rpm, (C) 30℃ and 250 rpm and (D) 32℃ and 250 rpm. Condition Sac 6.5. Mw: weight average molecular weight, Mn: number average molecular weight, Mw/Mn: polidispersity

Table 2. Thermal properties of P(3HB) obtained from cultives temperature and agitation speed modifying.

P(3HB) samples	Tm 1 (°C)	Tm 2 (°C)	X_c %
A	156.5	143.8	38
B	158.1	140.6	30
C	158.0	148.3	45
D	156.4	150.6	42
Control P(3HB)	167.2	146.9	40

(A) 28 °C and 250 rpm, (B) 28 °C and 150 rpm, (C) 30 °C and 250 rpm and (D) 32 °C and 250 rpm.

4.3 Manuscrito 2 – High accumulation of Poly (3-hidroxybutyrate) with low thermal properties and molecular weight by *Pseudomonas* sp. CMM43

Manuscrito será submetido à revista *PLOS ONE*

High accumulation of Poly (3-hidroxybutyrate) with low thermal properties and molecular weight by *Pseudomonas* sp. CMM43

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Abstract

Polyhydroxybutyrate P(3HB) is a polyester with similar properties to petrochemical derived plastics and a wide range of microorganisms can produce it. However, there are few reports of *Pseudomonas* producing poly(3-hydroxybutyrate) [P(3HB)]. *Pseudomonas* spp. strain CMM43 was cultivated with sucrose as the carbon source under non-limiting conditions, different inoculum concentrations and media were evalueted. Under optimal conditions, the media with 1% carbohydrate concentration and using a 10% inoculum, P(3HB) content reached levels of 83% dry cell weight. This high level, confirmed by transmission electron microscopy, surpassed the desired minimum level for industrial production. Further characterization by FTIR, GPC, DSC and TGA showed that, under all the testing conditions, the accumulated P(3HB) had an unusually low molecular weight and a melting temperature lower than its degradation temperature. These properties meet the requirements for several medical and pharmaceutical applications.

Keywords: P(3HB), *Pseudomonas*, shake flask production, mineral and complex media, molecular weight analysis, thermal analysis

1. Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that accumulate in response to stress and act as an energy reserve (Anderson and Dawes, 1990). PHAs have properties that make them competitive with petrochemical plastics and with the additional benefits of total biodegradability, biocompatibility and production from renewable sources (Braunegg et al., 1998; Khardenavis et al., 2006). Poly(3-hydroxybutyrate) P(3HB) was the first PHA discovered, and is the most extensively studied and characterized PHA (Lee and Chang, 1995; Lemoigne, 1926). P(3HB) has properties similar to those of polypropylene, but with a low thermal stability. However, the high production cost of these biopolymers limits their applications. The high cost of the process is due to the substrates used in the fermentation and recovery stages of polymer extraction. Current research has focused on strategies to make the production of P(3HB) economically viable. The utilization of low-cost carbohydrates including: olive oil mill wastewater, cane molasses, glycerol, Jatropha biodiesel byproduct has been explored (Arun et al., 2009; Ntaikou et al., 2009; Shrivastav et al, 2010; Tripathi et al., 2012). New extraction methods that reduce or

replace the use of the toxic solvents (Fiorese et al., 2009; Mantelatto et al., 2005) which pollute the environment and the search for new wild-type PHA-producing strains, as *Bacillus*, *Burkolderia*, *Pseudomonas* spp. have been reported (Brämer et al., 2001; Ayub et al., 2009; Rodríguez-Contreras et al., 2013). *Pseudomonas* spp. that accumulate medium chain length PHAs have been evaluated (Fernández et al., 2005). The accumulation of P(3HB) in *Pseudomonas* spp. was initially reported in the nineties (Daniel et al., 1992), however, there are no additional published reports on the subject for more than a decade (Ayub et al., 2006; Crochemore et al., 2012; Jiang et al., 2008; Li et al, 2013; Lopez et al, 2009). Most *Pseudomonas* spp. are capable of biosynthesizing medium chain length (MCL) PHAs (Haba et al., 2007). Of note, they seldom produce blends of P(3HB) (short chain) and MCL PHAs (Hang et al., 2002), and they rarely accumulate P(3HB) (Jiang et al., 2008; Mohan et al., 2010) and more rarely they accumulate P(3HB) when sucrose is used as the carbon source, using preferentially glucose and fatty acids (Andersen et al., 2000; Li et al, 2013). P(3HB) has a melting temperature (Tm) of approximately 170 °C, a χc crystallization percentage of 70% and a molecular weight in the range from 10 to 3000 kDa (Holmes, 1985; Noda et al., 1999; Sudesh et al., 2000). These thermal properties and high molecular weight are not ideal for applications in the biomedical area, e.g. graft and block copolymers (Lauzier et al., 1994; Zhang et al., 1997). These applications require a P(3HB) with differentiated thermal properties and with a low molecular weight. In the present work, the objective was to investigate P(3HB) production by the *Pseudomonas* strain CMM43 and to evaluate the influence of the production medium and inoculum concentration on polymer accumulation and its characteristics. In the present work, the objective was to evaluate the influence of the production medium and inoculum concentration on polymer accumulation by the *Pseudomonas* strain CMM43 and its characteristics.

2. Materials and methods

2.1. Microorganism and P(3HB) production

The pesticide degrading *Pseudomonas* strain CMM43 was from the Embrapa Temperate Agriculture Multifunctional Microorganism Culture Collection. The fermentation process was carried out in two phases, inoculum phase followed by

P(3HB) production. In all experiments, the classical YM medium (Jeanes, 1974) was modified to include sucrose (yeast extract 3.0 g/L; malt extract 3.0 g/L; peptone 5.0 g/L and sucrose 10.0 g/L) and was used in the inoculum phase. A volume of 100 mL of this medium, named YM (1), was inoculated in a 500 mL Erlenmeyer flask, and incubated at 28 °C with shaking at 250 rpm for 24 h, to a density of approximately 3 x10^8 CFU/mL.

Two different media and three inoculum concentrations were evaluated in this study. In the first experiment a defined medium named mineral salt medium (MSM) (Tripathi et al, 2012) was used. MSM medium comprising: sucrose 40 g/L; urea 0.8 g/L, KH_2PO_4 2.0 g/L; Na_2HPO_4 0.6 g/L; $MgSO_4.7H_2O$ 1.0 g/L; yeast extract 0.1 g/L and trace element solution (10 mL/L) comprising: $ZnSO_4.7H_2O$ 3.0 mg/L; $CaCl_2$ 20.0 mg/L; $FeSO_4.7H_2O$ 3.0 mg/L, $(NH4)_6Mo_7O_{24}.4H_2O$ 3.0 mg/L and H_3BO_3 6.0 mg/L. Inoculum concentrations of 10 and 20% (v/v) were evaluated. The second experiment was carried out using three different medium for P(3HB) production: YM (1); YM (2), prepared from YM (1) plus sucrose (20 g/L); and YM (3), prepared from YM(1) supplemented with sucrose (20 g/L) plus $MgSO_4$ (1 g/L). An inoculum concentration of 10% (v/v) in relation to the production media were evaluated. In the third experiment YM (1) medium and inoculum concentrations of 20 and 50% (v/v) were evalueted. For preparation of the production media MSM, YM (2) an YM (3), the carbon sources were sterilized separately at 121 °C for 15 min and then aseptically added into the flasks containing the others components. In all experiments, total volumes of 250 mL, composed by different proportions between inoculum and production medium, were dispensed into 500 mL Erlenmeyer flasks, and incubated at 28 °C with shaking at 150 rpm. Incubation time to first and second experiments was 72 h, while the incubation time for the third experiment was 48 h.

2.2. Residual sucrose, dry cell weight (DCW) and P(3HB) determination

Residual sucrose in the growth medium following fermentation was measured in hydrolysed sample by the dinitrosalicylic acid method (DNS) (Miller, 1959). Dry cell weight (DCW) was determined by gravimetry and the amounts of P(3HB) in 20 mg DCW were determined by acid methanolysis and analysed by gas chromatography as previously described (Brandl et al., 1988). The organic phase was analysed using a Varian GC 430 gas chromatograph (GC) equipped with a CP7485/CP-FFAPCB 9

column (25 m x 0.32 mm x 0.45 µm) and flame ionization detector (FID) with helium as the carrier gas (1 mL/min). Initial temperature of 60 °C was held for 1 min, before ramping to 200 °C at 11 °C/min and holding for 4 min. The injector temperature was 260 °C and the temperature of detector was 280 °C. The injected volume was 1 µL, manually. Quantitative analysis was performed by internal standardization method using benzoic acid as internal standard. The calibration curve was prepared from P(3HB) (Sigma-Aldrich) and the internal standard. Residual biomass (RB) (g/L) was defined as the difference between DCW (g/L) and PHA (g/L).

2.3. Transmission Electron Microscopy (TEM)

The cells were washed with saline solution, fixed and dehydrated in increasing concentrations of alcohol, 30, 50, 70, 90 and 95% (v/v) and P.A. acetone. The samples were embedded in Epon AB-DinP30 resin and cured for 5 days at 60 °C. The P(3HB) granules accumulated after 24 h, 48 h and 72 h of cultivation were observed as ultra-thin sections using TEM (Zeiss EM900).

2.4. Polymer extraction and obtainment of films

For P(3HB) extraction, chloroform was added in the proportion of 10 mL/g of DCW. Samples were placed in closed tubes and heated to 59 °C with magnetic shaking for 1h. Cellular debris was removed by filtration and the supernatant was transferred to covered Petri dishes for slow chloroform evaporation inside a gas exhaust chamber.

2.5. Analytical procedures for polymer identification and characterization

The identity of the P(3HB)s was confirmed by Fourier transform infrared spectroscopy (FTIR). The precipitated dry polymers produced from *Pseudomonas* strain CMM43 and the P(3HB) control (Sigma-Aldrich) were used to prepare KBr tablets. A Fourier transform infrared spectrometer Shimadzu model IR Prestige 21 was used to obtain the FTIR spectra. A total of 64 images were acquired over a wavelength range of 4500 to 500 cm^{-1} with a resolution of 4 cm^{-1}. The thermal properties of the P(3HB) samples and the control were investigated using a differential scanning calorimeter (DSC) (Shimadzu, model TA 60WS) in a nitrogen atmosphere at a rate of 50 mL/min. The samples were subjected to the following test

conditions: first heating cycle from 30 to 200 °C at a rate of 10°C/min, isotherm 200 °C for 5 min; cooling cycle from 200 to 30 °C at a rate of 10 °C/min, isotherm of 30 °C for 5 min; second heating cycle from 30 to 200 °C at a rate of 10 °C/min. To assess the effect of thermal history in obtaining the samples, we observed the melting temperature (Tm) during the first heating cycle. However, in order to eliminate the thermal history of the material, the Tm was measured during the second heating cycle. The crystallinity degree (χc) was determined from the melting enthalpy of the sample (ΔHm) and the melting enthalpy of pure crystalline P(3HB) (ΔHm = 146 J/g) (Gogolewski et al., 1993; Jianchun et al., 2003).

To determine thermal stability the samples were heated from 30 to 500 °C at a heating rate of 10 °C/min under a nitrogen atmosphere with a flow of 50 mL/min. Gel permeation chromatography (GPC) was used to estimate the molecular weight (Viscotek Model VE 2001, equipped with refractive index detector and PS/DVB columns). The samples were solubilizing in 2 mL chloroform and filtered (0.45 mm PVDF filter) before injection. The solvent used was THF at a flow rate of 1 ml/min. The molecular weight was calculated from a calibration curve based on standard polystyrene.

2.6 Statistical analysis

The experimental data were generated from triplicate and the average and standard deviation calculated by Excel.

3. Results and discussion

3.1. P(3HB) production in shaking flasks

The results from the first experiment are presented in Fig. 1, and include: P(3HB) content, DCW, residual biomass (RB) and sucrose consumption (SUC) during growth in MSM supplemented with sucrose (40 g/L) and comparing two inoculum concentrations, 10 and 20%. Using inoculum concentrations of 10% and 20% (Fig. 1a and 1b) the biopolymer content (g/L) was optimal at 48 h. Using 20% inoculum a significantly higher content (relative to the DCW) was achieved and the direct and relative P(3HB) yield was improved. The DCW during the first 72 h remained practically constant when inoculum concentration of 20% (v/v) was used.

Meantime this DCW increased by more than 1 g/L of P(3HB) in all growing times analysed, resulting in a maximum P(3HB) content of 80% of the DCW. This coincided with a decreased RB, probably because the carbon flux switched towards polymer synthesis rather than protein formation or cell multiplication (Koller et al., 2007). Using a 10% inoculum, the DCW increased across the cultivation time. At 72 h incubation, the P(3HB) content decreasing irrespective of the inoculum was verified . These results suggest that the bacterium was degrading and consuming the accumulated P(3HB). This, together with DCW increase that occur when 10% inoculum was used, resulted highest increase in RB (Fig. 1A). Sucrose was consumed during the incubation time, with 6.5 g/L remaining after 72 h in when a 20% inoculum was used, approximately half in relation to the 10% inoculum. However, even with twice of amount of sucrose available as a carbon source, P(3HB) underwent degradation. These observations were expected, because double the amounts of cells were used to inoculate the production medium (Fig. 1B). The maximum yield coefficient of P(3HB) on the sucrose ($Y_{P/S}$) and higher productivity were observed after 24 h for both inoculum concentrations, 0.133 and 0.095 g/L/h, respectively for the 20% inoculum, 0,089 and 0,051 g/L/h, respectively for the 10% inoculum. This is in agreement with the observations of Patwardhan and Srivastava (2008) found similar results to ($Y_{P/S}$) and productivity comparable to that obtained in 20% of inoculum, which were 0.19 and 0.09 g/L/h cultivating *Wautersia eutropha* for 60 h. However, the productivity achieved with the 10% inoculum was lower than the 0.09 g/L/h reported by Nath et al. (2008) and by Fernández et al (2005) who obtained 0.08 g/L/h using *P. aeruginosa* strain 42A2. The values of ($Y_{P/S}$) and productivity were similar to those found during the growth of *R. eutropha* ATCC 1769, 0.57 and 0.062 g/L/h, respectively (El-Sayed et al., 2009). Comparing the inoculum concentrations the best results were achieved using the 20% inoculum. In both cases the sucrose was converted to P(3HB) but the concentration used in the media was not totally metabolized for P(3HB) synthesis, after 72 h sucrose was still present in the medium, showing a decrease in the amount of P(3HB).

The profiles of the DCW and P(3HB) concentration, as well as the consumption of sucrose and the RB of bacterial growth in variations of the classical YM medium using inoculum 10% are presented in Fig. 2. The maximum P(3HB) content was obtained in YM (1) after 48 h incubation, equivalent to 83% of the DCW

and a yield of 3.2 g/L (Fig. 2A). It was possible to observe the accumulation of P(3HB) granules inside the cell after 48 h of cultivation in YM (1) (Fig. 3B). TEM at 24 h showed the presence of large P(3HB) granules within the cytoplasm and in cells undergoing division (Fig. 3A). The P(3HB) granules were similar to those observed in *W. eutropha* H16 (Tian et al., 2005) and in *Bacillus megaterium* (Gouda et al., 2001). At 72 h of cultivation time in the medium YM (1) and when the sucrose had been completely consumed, a decrease in P(3HB) content (50% of the DCW) was observed (Fig. 2A), sugesting that biopolymer was being used as carbon source by the cell, as confirmed by TEM (Fig. 3C).

The DCW, P(3HB) and RB in YM (2) increased with time and the biopolymer concentration was higher at 72 h (2.82 g/L) reaching a polymer content of 68% of DCW (Fig. 2B). Although sucrose consumption increased during the experiment, the sucrose concentration was 11.8 g/L after 72 h. In the experiment using the YM (3) medium (Fig. 2C) the highest P(3HB) concentration, (2.56 g/L), and P(3HB) accumulation, 75% of DCW, was obtained at 72 h. The RB and DCW did not change significantly, which means that under these conditions P(3HB) accumulation was associated with growth. An increase in sucrose consumption was observed, however, the sucrose concentration was 12.24 g/L after 72 h. This suggests that is not necessary to use 20 g/L of sucrose in the culture medium. In addition, when less than 50% of available carbohydrate was used, P(3HB) accumulation was reduced. Furthermore, the addition of $MgSO_4$ to the medium not improved DCW or P(3HB) accumulation. The ($Y_{P/S}$) maximum was achieved after 48 h incubation in YM 1 (0.3) and YM 2 (0.93), and after 24 h in YM 3 (0.46). Productivity was highest after 24 h incubation in all the three media evaluated in this study, 0.08 for YM (1); 0.07 for YM (2) and 0.08 for YM (3). The ($Y_{P/S}$) results reported in this work were higher than those observed by Aremu et al. (2011), ($Y_{P/S}$) of 0.0257, cultivation *P. putida* KT2440 after 72 h. In addition, the productivity rates were lower than those reported by Kanjanachumpol et al. (2013), (1.73 g/L/h), however, the P(3HB) content, 45.84%, was higher. Du et al (2001) reported 1.23 g/L/h to P(3HB) productivity. In the present work, using sucrose and non-limiting conditions, the amounts of accumulated P(3HB)/%DCW surpassed the desired minimum level (60%) recommended for industrial production (Ramsay et al., 1990). However, it will be necessary to further optimize the culture conditions in order to increase the final DCW in order to further

improve the P(3HB) yield.

The third experiment, using YM (1), compared 20 and 50% inoculum concentrations. Increasing the inoculum size did not result in improved P(3HB) accumulation (Fig.4). Using a 20% inoculum, the DCW, P(3HB) and RB results increased from 24 to 48 h (Fig. 4A). The optimal P(3HB) yield was 2.18 g/L at 48 h, corresponding to 53% DCW content, confirmed by an increase in sucrose consumption. The use of a 50% inoculum resulted in high DCW values at 24 h, followed by a decrease at 48 h; the same was observed for the RB and P(3HB) levels (Fig. 4B). Under these conditions, the highest P(3HB) yield was observed (2.95 g/L), however it did not justify the use of such a large inoculum (50 %). The gain in yield was not proportional when compared to that observed with the 20% inoculum. The amount of sucrose consumed from 24 to 48 h was only 0.7 g/L, this result explained the decrease in DCW, P(3HB) and RB. The maximum Y(p/s) using a 20 or a 50% inoculum at 24 h, were 0.32 and 0.36, respectively. Similar values were reported by Du et al. (2001) using glucose as the carbohydrate source. The P(3HB) yield was 0.07 g/L/h with the 20% inoculum compared to 0.012 g/L/h with the 50% inoculum. Nath et al. (2008) reported a P(3HB) yield of 0.09 g/L/h in fed batch fermentation. Compared all results obtained, for P(3HB) production in all experiments, the YM (1) medium, that contained only 10g/L carbohydrate and did not have sucrose or magnesium salt added, presented the best results for P(3HB) accumulation with a 10% inoculum.

3.2. Fourier transform infrared (FTIR) spectroscopy

The main absorption bands of the FTIR spectra from all of the polymers recovered from the cultures corresponded to those characteristic of the P(3HB) homopolymer (Fig. 5). The band at 1454 to 1459 cm^{-1} corresponded to C-H asymmetrical stretching for the CH_2 groups, while the band at 1378 cm^{-1} matched the CH_3 group deformation. These bands were comparable to those observed by Oliveira et al. (2007). The bands near 3000 cm^{-1} signifed the asymmetrical stretching of the C-H bond in the aliphatic CH_3 and CH_2 groups (Khardenavis et al., 2006). The most prominent marker band for the identification of P(3HB) is the ester carbonyl band (C=O) at 1724 to 1727 cm^{-1}. The bands at 1281 cm^{-1} represented the carboxylic (C-O) bonds. The series of intense bands at 1228-1057also corresponded to the C-O of

the ester group (Arun et al., 2009; Oliveira et al., 2007). The wide band around 3440 cm^{-1} corresponded to hydroxyl end groups (Arun et al., 2009; Oliveira et al., 2007). The FTIR spectra for P(3HB) extracted from *Pseudomonas* strain CMM43 were compared with the spectrum obtained from the P(3HB) control sample, and this confirmed the identity of the extracted polymer as P(3HB).

3.3. Molecular weight by GPC

The molecular weight (Mw) is an important index, determining the physicochemical properties of P(3HB). It depends on physiological, biochemical, and genetic features of the producing strain. Wild-type bacteria produce P(3HB) with a Mw ranging between 10 and 3000x10^3 Da (Volova, 2004) and the polymers can be classified into three groups: low, high and ultra-high Mw (Bastioli, 2008). Ultra-high-Mw P(3HB) have an Mw higher than 3x10^6 Da (Kusaka et al., 1999). The results of GPC analysis to determine the molecular weight distribution of the produced polymers under the different conditions of the present study and the control P(3HB) are presented in Table 4. The Mw values, including the control value ranged from 2.8x10^4 to 2.5x10^5 Da. The produced polymer with the highest Mw (2.3x10^5Da) was produced in the organic medium YM with a 20% inoculum. The polymer with the lowest Mw (2.8x10^4 Da) was produced in the YM (3), a organic medium with magnesium salt added, with 10% inoculum. The values found to Mw for the P(3HB)s produced by the *Pseudomonas* strain CMM43 put them in the low-Mw group when compared to other P(3HB)s (Chen and Page, 1994; Myshkina et al., 2008; Oliveira et al, 2007). The Mw of the P(3HB) produced in this work are unusual and are more similar to poly(3-hydroxyvalerate) (PHV), which has a Mw of 6x10^4 Da or less (Lee, 1996). This may suggest that the P(3HB) produced in the present study could have copolymer applicability. However, the low molecular weigth of P(3HB) may be related to the microorganisms, substrates used and culture conditions (Chen and Page, 1994; Van der Walle et al., 2001). Alternatively, some extraction methods cause severe damage to the granules, leading to a low polymer molecular weigth (Dawes and Senior, 1973; Holmes, 1988). P(3HB) with lower molecular weight (14000 Da) was also produced by *B. subtilis*, *Azotobacter vinelandii*, and *Streptomyces lividans* (Reusch and Sadoff, 1988; Reusch, 1999) in association with polyphosphate and calcium ions. Penloglou et al. (2012) explained that P(3HB) accumulation in the

Alcaligenes latus (now *Azohydromonas lata*) cells and the build-up of the polymer chain length are two competitive mechanisms, when the P(3HB) concentration increased the Mw of P(3HB) decreased.

The polydispersity (Mw/Mn) of P(3HB) is in the range of 2.2 - 3 (Brandl et al., 1988) and demonstrated that the production conditions have an influence on polydispersity. The polymers with highest polydispersity had lowest molar mass.

High Mw P(3HB)s have limited industrial application, however, some specific applications in the medical and pharmaceutical field e.g. preparation of suture materials, bone graft and controlled drug release systems and block copolymers, require a low molecular weight P(3HB) (Yu and Marchessault, 2000). Kawaguchi and Doi (1992) produced spheres consisting of low Mw P(3HB) ($6{,}5x10^3$ Da) and another from high molar mass P(3HB). Both spheres were impregnated with a drug but the drug was released faster from the spheres of lower molecular weight, showing the applicability of this differentiated polymer.

3.4. Thermal properties (DSC and TGA)

The P(3HB) produced by *Pseudomonas* strain CMM43 displayed thermal properties different to the majority of P(3HB)s. The P(3HB) demonstrated the necessary characteristics for use in biomedical applications or packaging materials. The thermal properties results are presented in Table 2. The melting temperature (Tm) of all of the polymers obtained in the present study were below 160 ºC after the second heating cycle. The P(3HB) produced using YM (3), the classic organic medium YM enriched with sucrose and Mg salt, had thermal properties very similar to the P(3HB) control sample. Overall, the Tm values were lower than majority those previously reported, e.g. 177.3 °C (Hong et al., 2008) and 177 °C (Yezza et al., 2007). Low Tm values have been related to external contaminants, such as RB and organic components from the fermentation process, which can affect the thermal properties of P(3HB) (Kim et al., 2006). There is also reports that a low Tm can be related to the extraction technique. For example, Valappil et al. (2007) used three different techniques to extract the P(3HB) accumulated by *B. cereus* and the lowest Tm was 160.83 °C. The soxhlet and the chloroform–hypochlorite dispersion methods had similar Tm values (169.71 and 171.71 °C, respectively) but the chloroform extraction technique resulted in the extraction of the P(3HB) with the lowest Tm.

However, the low Tm was found to be a property of the P(3HB) produced by *B. megaterium* uyuni S29 rather than the extraction method used (Rodriguéz-Contreras et al., 2013). The Tm values presented were in agreement with those reported previously (Tm (1) 164° C and Tm (2) 155°C), for a P(3HB) from the same bacterium and carbon source but using a different growth medium (Crochemore et al., 2012).

The polymers crystallinity (χc) can affect the mechanical, physical, thermodynamic and optical properties among others. From the pharmaceutical point of view, polymer crystallinity affects the degradation properties. Normally, polymers with a low crystallinity exhibit increased biodegradability and improved drug release than P(3HB)s with a high crystallinity. This is because bacterial enzymes degrade more easily polymer on amorphous phase (Lee, 1996; Pouton and Akhtar,1996). The χc of the P(3HB)s varied from 29 to 58% and depended on the production medium used. These values were higher than the P(3HB) control sample and yet they were lower than the usual range of crystallinity previously reported. Khanna and Srivastava (2005) found a χc of between 60 and 80%. The crystallinity of the samples were closer to those of P(3HB) produced by *B. megaterium* strain OU303A which ranged between 42 and 50% (Reddy et al., 2009). These results are desirable because high levels of crystallinity are associated with a rigid and brittle P(3HB), resulting in very poor mechanical properties (Savenkova et al., 2001). Low crystallinity represents more flexibility and processability at low temperatures, protecting the polymer from thermal degradation (Köse et al., 2003). The degradation temperatures (Tonset and Tmax) of the P(3HB)s obtained in this work were well above melting temperatures.

A low crystallinity and Tm are generally only achieved by altering the monomer composition of the P(3HB). This is achieved through the use of special promoters in the culture media or special fermentation conditions which promote the incorporation of hydroxyvalerate into the P(3HB) (Pouton and Akhtar,1996; Sudesh et al., 2000). Therefore, the present work showed a synthesized P(3HB) by *Pseudomonas* strain CMM43 with a reduced Tm and a lower crystallinity without needing to use special culture conditions. According to Rodriguéz-Contreras et al. (2013), low Tm and low χc values can result from a blend of different P(3HB) molecular weight fractions, with were detected by GPC analysis. In the present study, there was no correlation between Tm and χc, and different P(3HB) fractions were detected in GPC analysis (data not shown).

Thermal stability is defined as the ability of the substance to maintain their properties as close as possible to their initial characteristics, considering the environment imposed on the material and the functions it must perform. The thermal degradation behaviour of the polymer is presented in Table 3.

The polymers obtained had initial degradation temperatures between 192-207 ℃ and final degradation temperatures between 261-269 ℃. These values were low compared to those reported previously (Barud et al., 2010; Bordes et al., 2009;Wang et al., 2009). The one major drawback of P(3HB) is its low fusion stability due to early degradation near its Tm. All of the extracted P(3HB)s presented a Tm lower than the degradation temperature, a characteristic that allows it to be easily transformed into films and coatings. Conversely, the P(3HB) obtained had good thermal stability on processing because they displayed a relatively low Tm and high Tmax. The P(3HB)s from the MSM with a 10% inoculum and the YM with a 20% inoculum showed a loss of weight lower than the others samples. All the P(3HB) films exhibited thermal degradation in a single stage weight loss, that seemed to follow the same mechanism (data not shown). It was observed that the P(3HB) sample from YM(3) had the highest difference between the Tm and the degradation temperatures (Tonset and Tmax), suggesting that these P(3HB)s samples were more thermally stable. The biopolymers produced by *Pseudomonas* strain CMM43 exhibited thermal resistance without being brittle (Noda, Satkowski and Satkowski, 2002), demonstrating that they are candidates for medical applications that require rate-controlled degradation.

4. Conclusions

In summary, all of the media and inoculum concentrations used in the present study resulted in P(3HB) accumulation by *Pseudomonas* strain CMM43 from sucrose under non-limiting conditions. The strain accumulated P(3HB) up to 83% of its DCW and using a 10% inoculum. Well above the recommended minimum for industrial production of 60%. The general characteristics of the P(3HB)s included a low molecular weight, low Tm and crystallinity, and a Tm lower than the degradation temperature. This suggested that these characteristics were strain dependent, however, the production conditions influenced the P(3HB)s. These P(3HB)s represent promising candidates for use in the production of films, coatings and in

specific pharmaceutical and biomedical applications. Further work will include scale up to growth in a bioreactor, making it possible to directly evaluate these P(3HB)s in medical applications.

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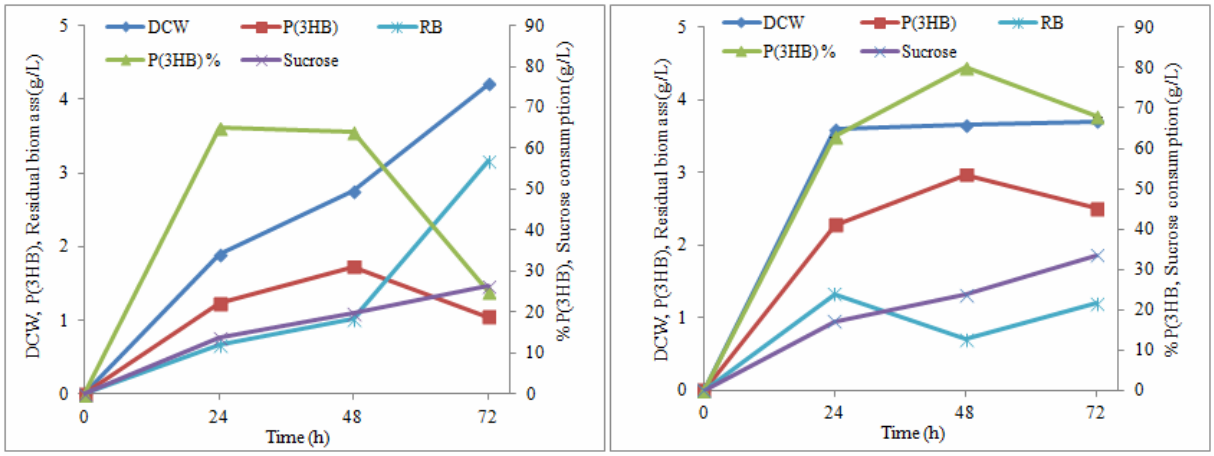

Fig. 1. P(3HB) content, dry cell weight (DCW), residual biomass (RB) and sucrose consumption by *Pseudomonas* strain CMM43 grown in the MSM medium at 28 ℃, 150 rpm; (a) 10% inoculum (b) 20% inoculum. The values shown are the mean of three experiments and the standard deviation less than 1%.

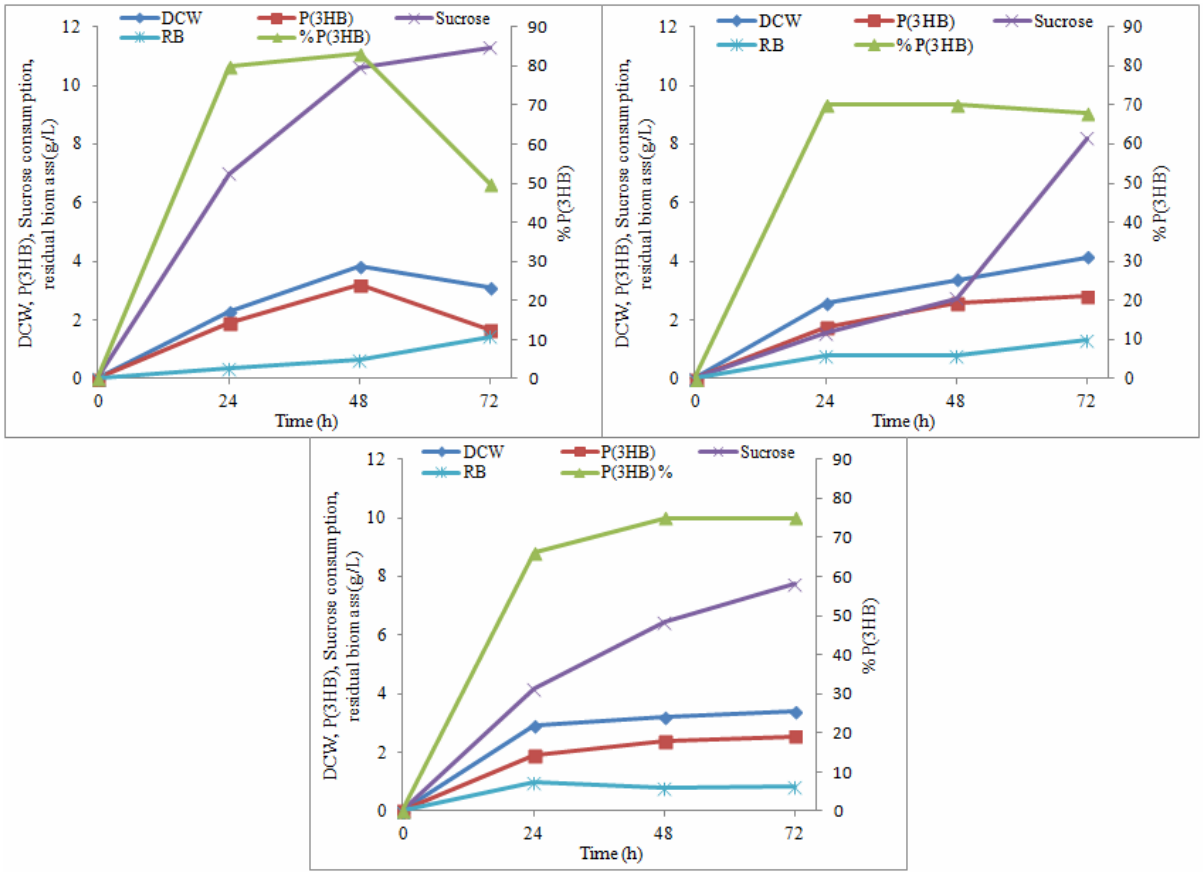

Fig. 2. P(3HB, dry cell weight (DCW), residual biomass (RB) production and sucrose consumption by *Pseudomonas* strain CMM43 using a 10% inoculum, 28 ℃ and 150 rpm using different variations of YM: (A) YM (1), (B) YM (2) and (C) YM (3). The values are the mean of three experiments with a standard deviation less than 1%.

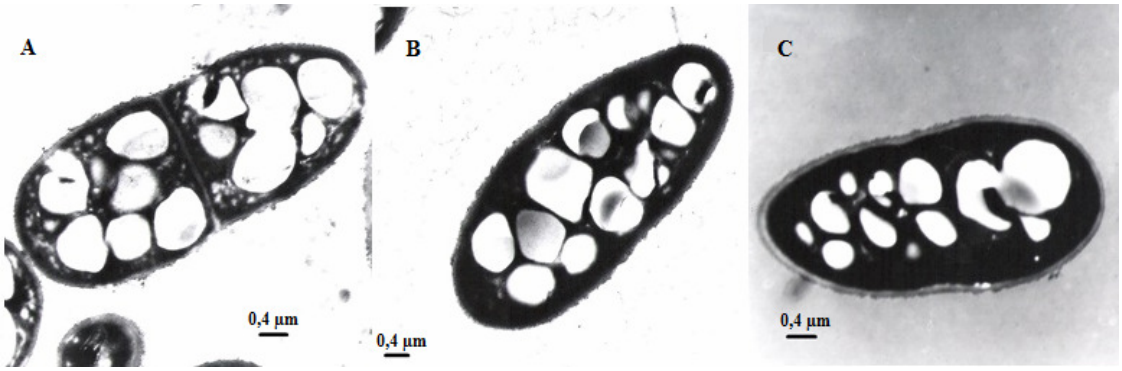

Fig. 3. Transmission electron micrograph of *Pseudomonas* strain CMM43 cells accumulating P(3HB) granules: (A) 24 h, (B) 48h and (C) 72 h cultivation time in YM (1) (10% inoculum).

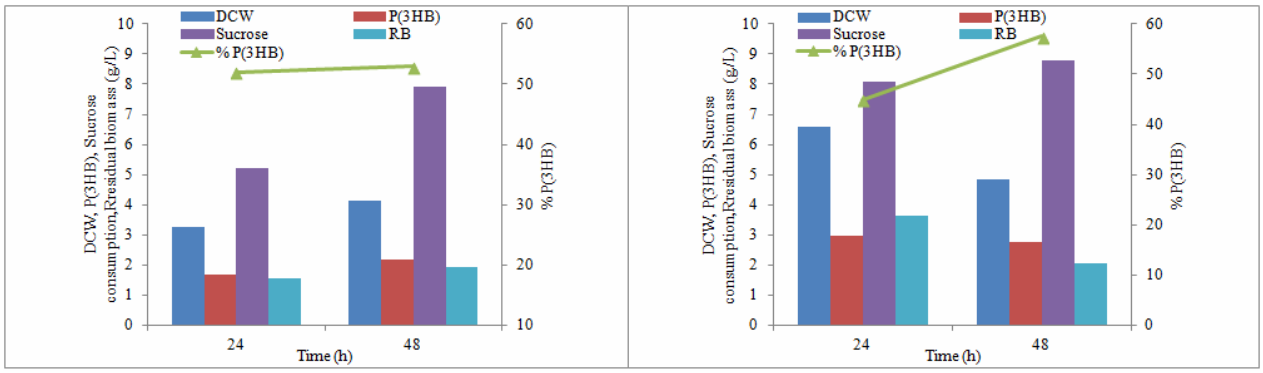

Fig. 4. The DCW, P(3HB), and RB production and sucrose consumption under the conditions of 150 rpm and 28 ℃ using different inoculum concentrations in YM (1): (A) 20% inoculum and (B) 50% inoculum. Analyses were done in triplicate with a standard deviation less than 2%.

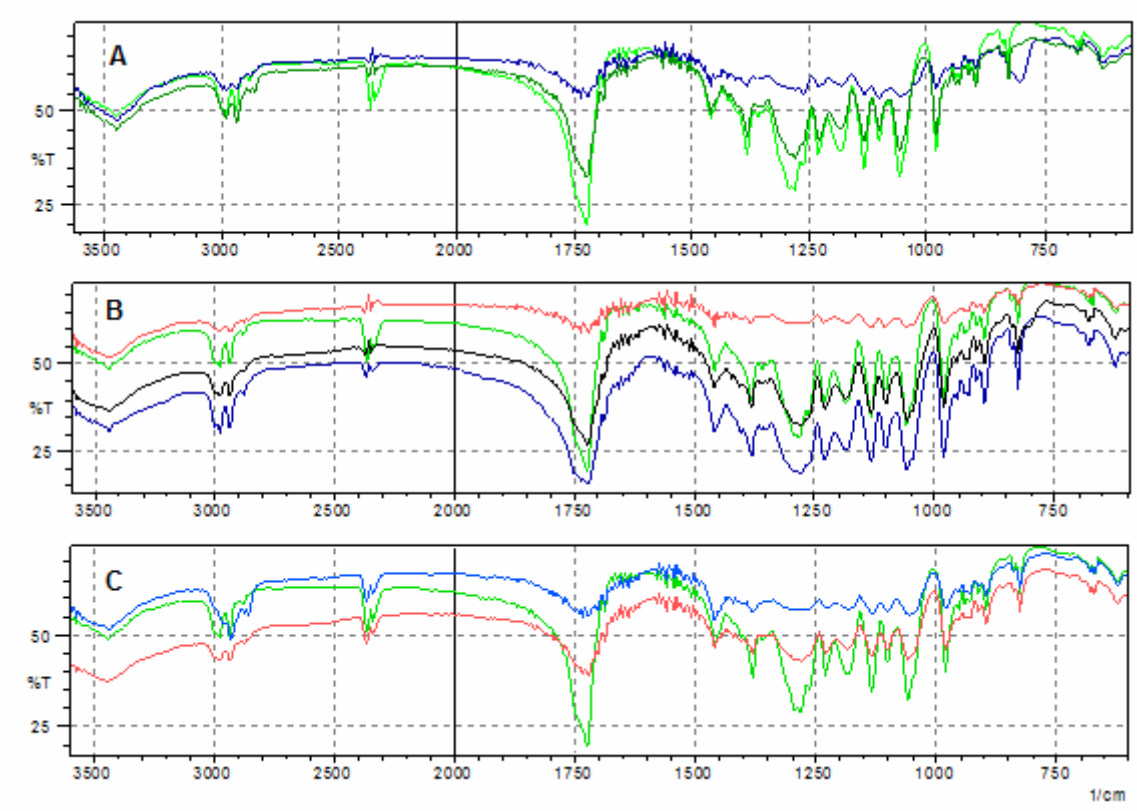

Fig. 5. FTIR spectra of the P(3HB)s produced in from different media and different inoculum conditions. (A) P(3HB) samples extracted from MSM (10% inoculum) (blue) and MSM (20% inoculum) (red), (B) P(3HB) samples extracted from YM (1) (black), YM (2) (blue) and YM (3) (red), and (C) YM (20% inoculum) (blue) and YM (50% inoculum) (red). The control P(3HB) sample (Sigma-Aldrich) is shown in green.

Table 1. Molecular weight of P(3HB) under different growth conditions.

P(3HB) samples	Inoculum (%)	Mw (Da)	Mn (Da)	Mw/Mn
MSM	10	9.1×10^4	5.4×10^4	1.68
	20	1.3×10^5	9.2×10^4	1.44
YM (1)	10	9.3×10^4	1.3×10^4	1.89
YM (2)*	10	9.0×10^4	3.7×10^4	2.41
YM (3)**	10	2.8×10^4	4.0×10^3	7.10
YM	20	2.3×10^5	1.2×10^5	1.95
	50	4.5×10^4	5.8×10^3	7.70
Control P(3HB)	N/A	2.5×10^5	1.1×10^5	2.20

*YM (2) = YM (1) plus sucrose; ** YM (2) = YM (1) plus $MgSO_4$; Mw: weight average molecular weight; Mn: number average molecular weight; Mw/Mn: polydispersity; N/A: not applicable

Table 2. P(3HB) melting temperature (Tm), melting enthalpy (ΔHm) and degree of crystallinity (χc %) based on DSC curves (1º and 2º heating cycle).

P(3HB) samples	Inoculum (%)	1 ° heating cycle			2º heating cycle		
		Tm (ºC)	ΔHm (J/g)	χc (%)	Tm (ºC)	ΔHm (J/g)	χc (%)
MSM	10	164.0	40.0	27.4	156.5	69.0	47.3
	20	165.8	47.5	32.5	158.6	42.9	29.4
YM (1)	10	166.2	57.4	39.5	158.6	53.3	36.5
YM (2)*	10	160.2	56.4	38.6	149.1	63.0	43.1
YM (3)**	10	157.0	114.3	78.3	153.8	137.4	54.1
YM	20	161.3	55.8	38.3	157.5	54.4	37.3
	50	154.9	77.31	53.0	146.8	84.4	57.8
Control P(3HB)	N/A	165.8	32.1	22.0	152.6	30.0	20.5

*YM (2) = YM (1) plus sucrose; ** YM (3) = YM (2) plus $MgSO_4$; N/A: not applicable

Table 3. Degradation onset temperature (Tonset), maximum degradation temperature (Tmax) and percentage of weight loss determined from TGA curves of the recovered P(3HB) films from different media and inoculum concentration.

P(3HB) samples	Inoculum (%)	Tonset (°C)	Tmax (°C)	Loss weigth (%)
MSM	10	192	265	78
	20	207	263	87
YM (1)	10	199	265	88
YM (2)*	10	200	265	92
YM (3)**	10	198	264	90
YM (1)	20	206	268	61
	50	194	269	97
Control P(3HB)	N/A	197	261	98

*YM (2) = YM (1) plus sucrose; ** YM (3) = YM (2) plus $MgSO_4$; N/A: not applicable

4.4 Produção de P(3HB) por *Pseudomonas* sp. CMM43 em biorreator

1. Introdução

Polihidroxialcanoatos (PHAs) representam um grupo interessante de bioplásticos ecológicos adequados a diversas aplicações técnicas. A sua aplicação é favorável porque são biodegradáveis e podem ser produzidos a partir de recursos renováveis. A produção de bioplásticos em larga escala ainda é limitada devido ao custo do substrato e da obtenção de culturas de elevada densidade celular, principalmente (ANDERSON & DAWES, 1990; REDDY et al., 2003).

PHAs podem ser produzidos em grandes quantidades a partir de fontes renováveis por meio de processos conhecidos de fermentação sob imposição de condições particulares de cultivo, em regime de batelada, semibatelada ou contínuo (FULLER & LENZ, 1990 *apud* BRAUNEGG et al., 1998).

Estratégias de cultivo são empregadas para obter alta densidade celular para produção de polihidroxialcanoatos, principalmente por tratar-se de polímeros intracelulares. Alta densidade celular é exigência para alcançar uma elevada produtividade (RIESENBERG & GUTHKE, 1999). Além da obtenção de cultivos com alta densidade celular é de suma importância que a bactéria acumule níveis desejáveis de P(3HB) na célula pois, segundo Ramsay et al. (1990), para um microrganismo ser rentável para aplicação industrial é necessário que consiga acumular no mínimo 60 % de sua massa celular seca em polímero. Em resumo a produção industrial de PHAs só se torna economicamente viável quando altas densidades celulares e produtividades elevadas de polímero são alcançadas com custos baixos (IENCZAK, SCHMIDEL & DE ARAGÃO, 2013) .

O objetivo do estudo foi avaliar a produção P(3HB) em biorreator, utilizando as mesmas condições testadas em agitador orbital, como meio com sacarose, velocidade de agitação de 150 rpm e temperatura de 28℃, que resultaram em altos níveis de P(3HB). Este trabalho também avaliou propriedades como temperatura de fusão, massa molecular e temperatura de degradação dos polímeros recuperados, identificados por FTIR, para verificar se essas propriedades diferenciadas reportadas nos demais trabalhos como temperatura de fusão, grau de critalinidade e massa molecular menores que as usuais encontradas para P(3HB), e temperatura de fusão menor que a de degradação, que parecem ser características da bactéria,

permanecem as mesmas.

2 Materiais e Métodos

2.1 Microrganismo, meio de cultivo e condições de cultivo

Pseudomonas sp. CMM43 degradadora de agrotóxico, isolada de solo de arroz irrigado e preservada na Coleção de Culturas de Microrganismos Multifuncionais da Embrapa Clima Temperado, foi utilizada neste estudo.

As fermentações foram realizadas em duas fases, uma de crescimento celular (inóculo) e outra de produção de P(3HB). O meio utilizado foi YM (Jeanes, 1974) modificado pela mudança de glicose por sacarose, contendo (g/L): 3,0 de extrato de levedura; extrato de malte 3,0; peptona 5,0 e 10,0 sacarose foi utilizado nas duas fases. O inóculo foi preparado em frasco Erlenmeyer de 500 mL contendo 100 mL de meio em triplicata, e incubado a 28 °C sob agitação de 250 rpm por 24 h, atingindo cerca de 3 $x10^8$ UFC/mL.

O cultivo para a produção de P(3HB) foi conduzido a 28 °C, 150 rpm e 1vvm constante em biorreator de 5 L (Discovery 100, Inceltech ®) com 3 L de volume útil, inoculados com 10 % (v/v) de inóculo. Foram utilizados 5 mL de óleo de silicone como antiespumante. O pH foi ajustado inicialmente em 6,5 e não mais controlado. Três cultivos foram realizados.

2.2 Sacarose residual, massa celular seca e determinação de P(3HB)

A sacarose residual presente no caldo fermentado foi medida em amostra hidrolisada por meio do método do ácido dinitrossalicílico (DNS) (Miller, 1959). A massa celular seca (MCS) foi determinada por gravimetria. Os valores de P(3HB) foram calculados em %, em relação à MCS, sendo determinados em 20 mg de MCS, que foram sujeitos à metanólise ácida e analisados por cromatografia gasosa de acordo com Braunegg et al. (1978), método modificado por Brandl et al. (1988). A fase orgânica foi analisada por cromatógrafo gasoso Varian GC 430 equipado com coluna CP7485/CP-FFAPCB 9 (25mx0,32mmx0,45mm) e detector de ionização de chama (FID), com hélio como gás de arraste (1 mL/min). P(3HB) adquirido da Sigma Aldrich® foi utilizado para preparar a curva de calibração e o ácido benzoico foi utilizado como padrão interno. A biomassa residual (BR) (g/L) foi definida como a diferença entre MCS (g/L) e PHA (g/L).

2.3 Microscopia Eletrônica de Transmissão (MET)

As células foram lavadas com solução salina, banhadas em dois fixadores e, em seguida, desidratadas em concentrações crescentes de álcool, 30, 50, 70, 90 e 95% (v/v) e acetona PA. Finalmente, as amostras foram embebidas em resina Epon® AB-DinP30 e secas durante 5 dias a 60 °C. Os grânulos de P(3HB) acumulados após 24 h e 72 h de cultivo foram observados em secções ultrafinas usando microscópio eletrônico de transmissão Zeiss® EM900.

2.4 Extração do polímero e obtenção dos filmes

Para a extração do polímero foi adicionado clorofórmio na MCS, na proporção de 10 mL/g de MCS. As amostras foram colocadas em tubos fechados e aquecidos a 59 °C, com agitação magnética durante 1 h. Em seguida, as amostras foram filtradas para remover os detritos celulares e transferidas para placas de Petri. As placas foram tampadas e deixadas em capela de exaustão para lenta evaporação do clorofórmio.

2.5 Procedimentos analíticos para a identificação e caracterização dos polímeros

Os P(3HB)s produzidos foram identificados pela técnica de espectroscopia de infravermelho (FTIR). Os polímeros precipitados e secos produzidos pela *Pseudomonas* sp. CMM43, os P(3HB) controles, um adquirido da Sigma Aldrich® e outro cedido pela P(3HB) Industrial S.A. (P(3HB)ISA) foram usados para preparar pastilhas de KBr. Um espectrômetro de infravermelhos com transformada de Fourier Shimadzu ® modelo Prestige 21 foi usado para obter os espectros de FTIR. Adquiriram-se 64 imagens durante um intervalo de comprimento de onda de 4500-500 cm^{-1}, com uma resolução de 4 cm^{-1}.

As propriedades térmicas das amostras produzidas e dos P(3HB) controles foram investigados em calorímetro diferencial de varredura (DSC) Shimadzu®, modelo TA 60WS, utilizando atmosfera de nitrogênio a uma taxa de 50 ml/min para análise. As amostras foram submetidas às seguintes condições de teste: primeiro ciclo de aquecimento entre 30 e 200 °C, a uma taxa de 10 °C/min, isotérmica de 200 °C durante 5 min; ciclo de arrefecimento de 200 a 30 °C a uma taxa de 10 °C/min, isoterma de 30 °C durante 5 min; segundo ciclo de aquecimento entre 30 e 200 °C, a

uma taxa de 10 ℃/min. Para avaliar o efeito da história térmica na obtenção das amostras, observou-se os valores de temperatura de fusão (Tm) no primeiro ciclo de aquecimento. No entanto, a fim de eliminar a história térmica do material, a TF foi verificada no segundo ciclo de aquecimento. O grau de cristalinidade (X_c) foi determinado a partir da razão entre a entalpia de fusão da amostra (ΔHF) e a entalpia de fusão do P(3HB) cristalino puro (ΔHF = 146 J/g) (Gogolewski et al , 1993; Jianchun et al, 2003). Para determinação da estabilidade térmica, as amostras e os controles foram aquecidos de 30 a 500 ℃ a uma taxa de aquecimento de 10 ℃/min, sob uma atmosfera de nitrogênio com um fluxo de 50 mL/min.

A análise de cromatografia de permeação em gel (GPC), para estimar a massa molecular, foi realizada utilizando um equipamento Viscotek modelo VE 2001, equipado com um detector de índice de refração e colunas de PS/DVB. O cromatograma foi obtido por solubilização da amostra em 2 mL de clorofórmio seguida de filtragem através de um filtro de PVDF 0,45 mm antes da injeção. O solvente utilizado foi o THF, a uma taxa de fluxo de 1 ml/ min. A massa molecular foi calculada a partir de uma curva de calibração com poliestireno padrão.

3. Resultados

3.1 Acúmulo, rendimento e produtividade de P(3HB)

Os cultivos em biorreator foram realizados visando elevar o acúmulo e o rendimento de P(3HB), em relação aos valores obtidos em incubador agitador orbital. A Fig.3 resume os resultados obtidos nos três cultivos, para acúmulo de polímero, teores de MCS e BR e consumo de sacarose.

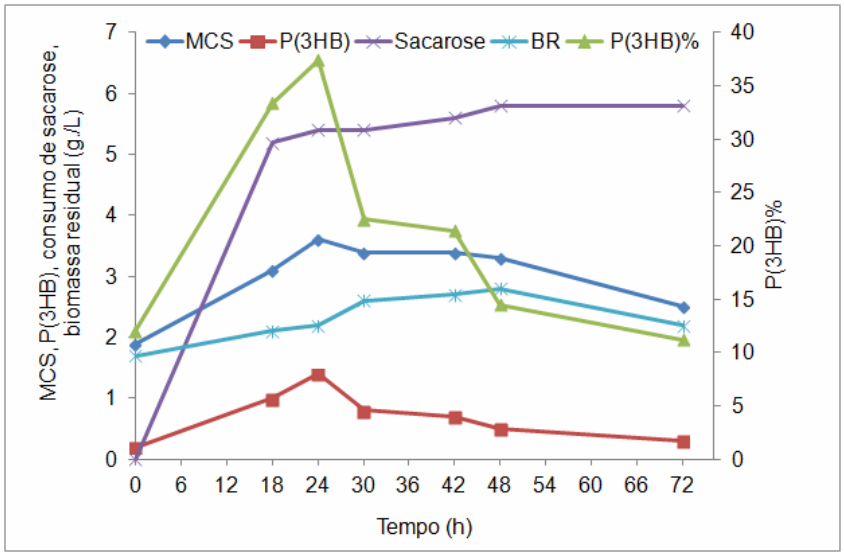

Figura 3. Produção de massa celular seca (MSC) (%), acúmulo (%) e rendimento (g/L) de P(3HB), biomassa residual (BR) (g/L) e consumo de sacarose (g/L) durante cultivo de *Pseudomonas* sp. CMM43 em biorreator a 28 ℃, 150 rpm e 1vvm. Os valores são a média de três experimentos com desvio padrão menor que 2%.

Foi percebido aumento de MCS de 18 para 24 h, seguido de pequena variação nos valores até 48 h e decréscimo em 72 h. A produção máxima de MCS e P(3HB) foi atingida em 24 h de cultivo, 3,6 g/L e 1,4 g/L, respectivamente. A quantidade de P(3HB) acumulado em relação à MCS foi de 37,4%, valor esse muito inferior ao encontrado em experimentos anteriores em incubador agitador orbital, quando os níveis de polímero chegaram a 70-80% em relação à MCS, utilizando o mesmo meio de cultivo. Algumas explicações para a diminuição no acúmulo de P(3HB) pela *Pseudomonas* sp. CMM43 seriam o não controle do pH, baixa agitação (foi utilizada a mesma do incubador agitador orbital) e/ou a falta de um excesso de sacarose no meio de cultivo. Rodriguez-Contreras et al. (2013) mostram em seus resultados uma diminuição no percentual de P(3HB) fazendo transposição de escala de incubador agitador orbital para biorreator, diminuindo de 70% para 30% os níveis de polímero na célula, cultivando *Bacillus megaterium* uyuni S29.

A sacarose foi consumida no decorrer do tempo de cultivo, em 18 h foi percebido um consumo da metade da sacarose disponível no meio (10 g/L), porém a partir de 30 h o consumo foi baixo, com diferença de 0,2 g/L de um horário para outro, tendo restado 40 % do total do açúcar ao final do processo. O baixo consumo de sacarose juntamente com o decréscimo de acúmulo de P(3HB) na célula durante o cultivo sugerem que a bactéria passou a usar preferencialmente o polímero como

fonte de carbono e energia em detrimento ao carboidrato. O aumento da BR comprova que a bactéria canalizou seu metabolismo para manutenção celular e não para acúmulo de polímero.

Baixos valores de produtividade (g P(3HB)/L/h) foram obtidos (18 h - 0,05; 24 h - 0,06; 30 e 42 h - 0,02; 48h - 0,01 e 72 h - 0,004). Nath et al. (2008) e Fernández et al (2005) obtiveram valores de produtividade de 0,09 g/L/h e 0,08 g/L/, respectivamente. Yamane et al. (1996) cultivando *Alcaligenes latus* atingiu alta densidade celular e uma produtividade de 4.0 g P(3HB)/(L/h). Os rendimentos obtidos relativos à conversão de açúcar em polímero Y(p/s) foram maiores aos reportados por Rodriguéz-Contreras et al. (2013) em 18 e 24 h, 0,19 e 0,26, respectivamente. Ienczak, Schmidel & de Aragão (2013) comentam que quando a produtividade e o rendimento de estirpes são muito baixas, o aumento de escala torna-se difícil.

A fig. 4 mostra o acúmulo de grânulos de P(3HB) em 24 h (Fig. 2). Os grânulos de P(3HB) são semelhantes aos observados em *W. eutropha* H16 (Tian et al., 2005) e em *Bacillus megaterium* (Gouda et al., 2001). Os grânulos apresentam-se na forma de corpos eletrônicos densos, como mostrados também no trabalho de Loo & Sudesh (2007).

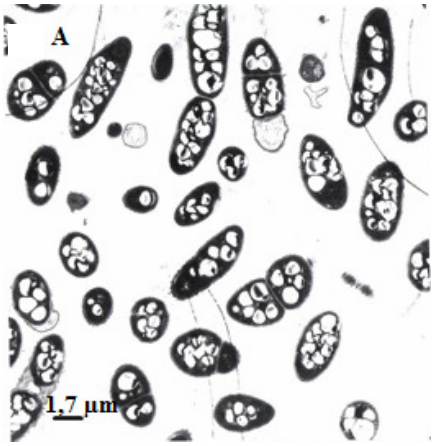

Figura 4. Micrografia eletrônica de células de *Pseudomonas* sp. CMM43 contendo grânulos de P(3HB) acumulados em 24 h de cultivo a 28 ℃, 150 rpm e 1vvm.

3.2 Identificação do polímero por FTIR

As principais bandas de absorção dos espectros de FTIR dos polímeros

extraídos correspondem às características do homopolímero P(3HB) (Fig. 5). A banda em 1454 a 1459 cm^{-1} corresponde ao alongamento assimétrico de CH por grupos CH_2, enquanto que a banda de 1378 cm^{-1} é equivalente à deformação do grupo CH_3. Essas bandas são comparáveis àquelas encontradas por Oliveira et al. (2007). As bandas perto de 3000 cm^{-1} derivam do alongamento assimétrico de CH em grupos alifáticos CH_3 e CH_2 (Khardenavis et al., 2006). A banda de marcação mais proeminente para a identificação de P(3HB) é a relativa à carbonila do éster (C = O), em 1724-1727 cm^{-1}. As bandas a 1281 cm^{-1} representam ligações carboxílicas (C-O). A série de bandas intensas entre 1228-1057cm^{-1} também correspondem ao CO do grupo éster (Arun et al., 2009; Oliveira et al., 2007). A ampla faixa em torno de 3440 cm^{-1} refere-se a grupos terminais hidroxila (Arun et al., 2009; Oliveira et al., 2007). Os espectros FTIR para o P(3HB) extraído produzido por *Pseudomonas* sp. CMM43 comparado com os espectros obtidos a partir dos controles, P(3HB) Sigma Aldrich e PHBISA lote 151, confirmam que o polímero extraído é P(3HB) e que apresenta maior similaridade com P(3HB) Sigma Aldrich.

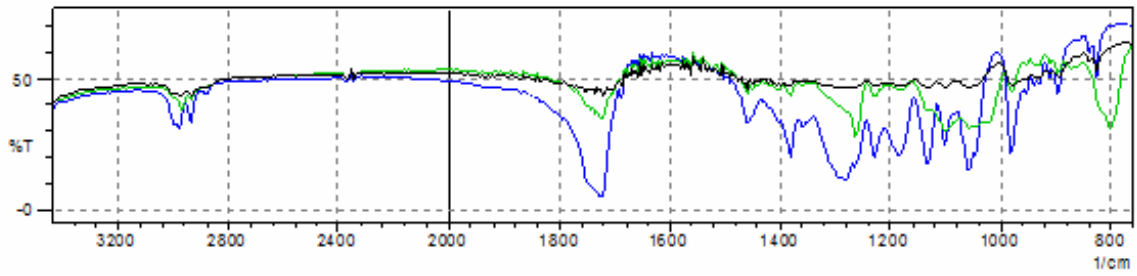

Figura 5. Comparação dos espectros de FTIR: P(3HB) produzido a partir da três fermentações ( ), P(3HB) Sigma Aldrich ( ) e P(3HB) da PHBISA ( ).

3.3 Propriedades térmicas e massa molecular

Os resultados de análise térmica e massa molecular provenientes do P(3HB) produzido no cultivo utilizando YM, 28 ℃, 150 rpm e 1vvm estão expressos na tabela 1. O valor de Tf encontrado foi semelhante ao obtido no trabalho anterior em agitador orbital, utilizando o mesmo meio e as mesmas condições de temperatura e agitação. O grau de cristalinidade foi o mesmo e a massa molecular foi um pouco maior. Porém essas propriedades seguem um padrão, apresentando valores inferiores a maioria dos reportados para P(3HB).

Tabela 1. Temperatura de fusão (Tf), entalpia de fusão (ΔHf) e grau de cristalinidade (χc %) determinados a partir de curvas de DSC (1º e 2º ciclo de aquecimento), e massa molecular determinada por GPC do P(3HB) extraído do cultivo de *Pseudomonas* sp. CMM43 em YM, 28℃, 150 rpm e 1vvm.

Amostras P(3HB)	1° ciclo de aquecimento			2º ciclo de aquecimento			Mw (Da)	Mn (Da)	Mw/Mn
	Tf (ºC)	ΔHf (J/g)	χc (%)	Tf (ºC)	ΔHf (J/g)	χc (%)			
Biorreator*	174,7	53,2	36,4	154,6	53,3	36,5	$1.16x10^5$	$4.56x10^4$	2.5
Sigma Aldrich	165,8	32,1	22,0	152,6	30,0	20,5	$2.68x10^5$	$1.97x10^5$	1,4
P(3HB)ISA	168,26	61,8	42,4	167,0	70,98	48,6	$1.63x10^5$	$8.27x10^4$	1,9

*Mix do P(3HB) extraído das 3 fermentações

A tabela 2 apresenta dados da estabilidade térmica do P(3HB) produzido no cultivo utilizando YM, 28 ℃, 150 rpm e 1vvm. A temperatura de degradação continua menor que a de fusão, confirmando característica já descrita nos trabalhos anteriores.

Tabela 2. Temperatura de degradação inicial (Ti), temperatura máxima de degradação (Tmax) e perda de massa (%) determinadas a partir das curvas de TGA do P(3HB) obtido e controles.

Amostras de P(3HB)	Ti (℃)	Tmax (℃)	Perda de massa (%)
Biorreator*	223	286	83
Sigma Aldrich	205	269	98,7
P(3HB)ISA	221	290	100

*Mix do P(3HB) extraído das 3 fermentações.

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5 CONCLUSÃO GERAL

Das três *Pseudomonas* degradadoras do pesticida carbofurano identificadas como P(3HB) positivas, a *Pseudomonas* sp. CMM43 foi escolhida como a mais promissora para otimização da produção do biopolímero. O estudo realizado na fase de crescimento celular (inóculo), além de registrar altos níveis de polímero acumulado, evidenciou que a produção é associada ao crescimento. Nos cultivos em incubador agitador orbital o polímero produzido atingiu 83% em massa celular seca, bem acima do mínimo recomendado para a produção industrial, que é de 60%. Os cultivos em biorreator registraram quedas no acúmulo de P(3HB), porém as caracteristicas térmicas e a massa molecular mantiveram-se semelhantes.

Pseudomonas sp. CMM43 apresenta a característica de acumular P(3HB) sem a necessidade de limitação nutricional, a partir de sacarose como fonte de carbono e em curto intervalo de tempo, características incomuns para o genêro. E ainda sintetiza P(3HB) com baixa massa molecular e propriedades térmicas diferenciadas permitindo, assim, especícificas aplicações na área médica e farmacêutica.

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