Tesis Doctoral

Development of biorefineries using renewable raw materials for the production of biodegradable polymers

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TITULO: *DEVELOPMENT OF BIOREFINERIES USING RENEWABLE RAW MATERIALS FOR THE PRODUCTION OF BIOFUELS AND BIODEGRADABLE POLYMERS.*

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TÍTULO DE LA TESIS: Development of biorefineries using renewable raw materials for the production of biodegradable polymers

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INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

During her PhD thesis, the student has developed important skills in the field of biotechnology. As a result of her hard work, she has found interesting and innovative results that have been presented in different oral presentations to national and international conferences, i.e.:

IL García, *El concepto de biorefinería: integración de residuos y cambio climático,* I Congreso científico de Investigadores en Formación, ISBN: 9788499270319, pp: 322-323 (2 páginas), Universidad de Córdoba, Spain, 15-16 October 2009

I.L. Garcia; S. Pinzi; J.J. Ruiz; C. Quintana; I. Lopez; F.J. Lopez; M.P. Dorado; A. Koutinas, *R. Eutropha – PHAs Production from waste streams*, MAS XXI, VIª Conferencia Científica Internacional de Medio Ambiente Siglo XXI, ISSN/ISBN: 978-959-250-456-1, Universidad Central "Marta Abreu" de las Villas; Santa Clara, Cuba, 3-6 November 2009

I.L. García, A. Koutinas, S. Papanikolou, M.P. Dorado, Valorisation of by-products from biodiesel production processes for the generation of microbial bioplastics, COST Action CM0903, Utilisation of biomass for fuel and chemicals (UBIOCHEM), Universidad de Córdoba (Spain), 13-15 May 2010

I.L. García, M.P. Dorado Pérez, J.A. López, M.A. Villar, S. Yanniotis, A. Koutinas, Design and techno-economic evaluation fo microbial bioplymer production from food industry wastes and agricultural crops, ICEF11, International Congress on Engineering and Food, Food process engineering in a changing World, ISBN/ISBN: 978-960-89789-6-6 / 978-960-89789-3-5, pp:399-400, Atenas (Greece), 22-26 May 2011

I.L. García, M.P. Dorado Pérez, J.A. López, M.A. Villar, S. Yanniotis, A. Koutinas, Valorisation of confectionary industry wastes for the microbial production of polyhydroxyalkanoates (FEW677), ICEF11, International Congress on Engineering and Food, Food process engineering in a changing World, ISBN: 978-960-89789-6-6 / 978-960-89789-3-5, vol. II, pp: 745-746, Atenas (Greece), 22-26 May 2011

I.L. García, J.A. López, S. Pinzi, S. Papanikolaou, M.P. Dorado, A. Koutinas, Valorisation of byproduct streams from biodiesel production plants for microbial synthesis of polyhydroxybutyrate. RRB, Renewable resources and biorefineries, RRB7,

Seventh International Conference on Renewable Resources and Biorefineries, pp: 42, Bruges (Belgium), 8-10 June 2011

J.A. López, I.L. García, M. Komaitis, S. Papanikolaou, M.A. Villar, A. Koutinas, Bacterial production of polyhydroxybutyrate from Jerusalem artichoketubers through the implementation of a sustainable bioprocess, RRB7, Seventh International Conference on Renewable Resources and Biorefineries, pp: 115 (1 page), Bruges (Belgium), 8-10 June 2011

She has also been involved in different publications derived from her PhD thesis, i.e.:

Apostolis A. Koutinas, Isabel L. Garcia, Nikolaos Kopsahelis, Seraphim Papanikolaou, Colin Webb, Marcelo A. Villar, Jimmy A. López. Production of fermentation feedstock from Jerusalem artichoke tubers and its potential for polyhydroxybutyrate synthesis. Waste and Biomass Valorization, 2012, DOI: 10.1007/s12649-012-9154-2

Some other works from this study have been sent to different international journals to be considered for publication. She has also made a big effort to provide a valuable work and has spent 21 months of her research at the Agricultural University of Athens (Greece), thus giving an added value to the final work.

We believe this thesis meets the standards to get the authorization for the viva. For all these reasons, we allow its presentation for the PhD degree evaluation.

Córdoba and Athens, 26__ of ___July ___ 2012

Co-supervisors signature

Signature:_Mª del Pilar Dorado Pérez_ Signature: _Apostolis Koutinas_

Abstract

The current world demand of 12 millions tons oil per day, for the production of fuels and chemicals, together with the estimated increase of 16 tonnes per day for 2030 and the geographically restricted availability of conventional oil, represent a new human era where cheap secure oil is no more a reality.

Nowadays, petroleum is the main raw material for the production of plastics. The annual capacity of approximately 150 million tons of petroleum-derived plastics produced worldwide is currently disposed mainly in landfills or it is incinerated. In addition, approximately 135 thousand tons of plastics are disposed to the sea on an annual basis. Therefore, petroleum-derived plastic production will be eventually hindered by the depletion of petroleum and the inevitable environmental pollution that is caused by their disposal.

The production of biodegradable plastics is the only solution to the replacement of petroleum-derived plastics. This work focuses on the utilization of wastes from the agro- and food-industries, as well as non-food crops, for the production of polyhydroxyalkanoates (PHAs), a family of biodegradable plastics that are accumulated intracellulary by many bacteria. PHAs have a high potencial for substitution of petroleum derived plastics, such as polyestirene and polypropylene, but a large research on its production is required to optimize fermentation processes and cost-competitiveness and to implement this units in existing industries (such as first generation biodiesel production plants) developing the concept of biorefinery.

In the present work production of PHAs from three different renewable sources was studied in a laboratory scale. A techno economic evaluation of the industrial production of PHAs, from different waste streams and industrial crops, was also perform.

In the first study, polyhydroxyalkanoate (PHB) was produce using Jerusalem Artichoke (JA), a tubercule rich in sugars, that can be cultivated

in marginated lands. Shake flask fermentations, with *Cupriavidus necator* DSM 4058, using JA hydrolysate as fermentation medium showed that PHB production is feasible. However, enrichment of JA hydrolysates with nutrient-rich supplements (e.g. oilseed meal hydrolysates) should be carried out in bioreactor applications.

The second study explored the potential production of PHAs, including P(3HB-co-3HV), with *C. necator* DSM 545 using rapeseed meal hydrolysates and crude glycerol (both residues from the biodiesel industry) as nutrient supplements and carbon sources, respectively. PHA property analysis verified the potential to produce complex PHAs from rapeseed hydrolysates.

Expired flour-based food for infants returned from the market and waste streams generated from the production line of flour-based food for infants (FBFI) were converted into a nutrient-rich fermentation feedstock in the third study of this work. The macromolecules in FBFI (i.e. starch, protein) were hydrolyzed using crude enzymes (i.e. amylase, protease) produced via solid state fermentation of *Aspergillus awamori*. FBFI hydrolysates were tested as fermentation media in shake flask and bioreactor fermentations for the production of PHB. The feasibility of bioplastic production from flour-based waste streams has been demonstrated.

Finally, design and techno-economic evaluation has been employed so as to compare two process flowsheets based on different initial raw materials, microorganisms and downstream separation strategies. All required information used in this study was taken from literature-cited publications. The processing schemes that were evaluated utilize food processing wastes and by-products (i.e. whey) and wheat as raw materials.

Resumen

La actual demanda mundial de petróleo de 12 millones de toneladas de crudo diarias, para la producción de combustibles y productos químicos, junto al incremento estimado para el año 2030 de 16 toneladas al día, y la restringida disponibilidad geográfica de esta materia prima fósil, representa una nueva era para la humanidad donde el acceso fácil al petróleo barato ha dejado de ser una realidad.

Hoy en día el petróleo es la principal material prima para la producción de plásticos. Anualmente, 150 millones de toneladas de plásticos derivados del petróleo (no biodegradables) están siendo depositados, principalmente en vertederos, o incinerados. Además, unas 135 mil toneladas de plásticos son arrojadas anualmente al mar. La producción de plásticos derivados del petróleo se verá dañada, no sólo por el agotamiento de esta material prima, sino además, por las consecuencias medioambientales que su eliminación genera.

La producción de plásticos biodegradables es la única solución viable para el reemplazo de los petro-plásticos. El presente trabajo está centrado en la utilización de residuos de las industrias agraria y agroalimentaria, así como de cultivos no alimentarios, para la producción de polihidroxialcanoatos (PHA), una familia de plásticos biodegradables que son acumulados intracelularmente por numerosas bacterias. Los PHA tienen un alto potencial para el reemplazo de los plásticos derivados del petróleo, como son el poliestireno y polipropileno, pero es necesario profundizar en las investigaciones para optimizar los procesos fermentativos necesarios para su producción, así como su competitividad económica. Se hace necesaria además la integración de unidades de producción de estos biopolímeros en industrias actualmente existentes (como las plantas de producción de biodiésel de primera generación) desarrollando así el concepto de biorrefinería.

En el presente trabajo, la producción de PHA, a partir de tres diferentes materias primas de origen renovable, fue investigada a escala de laboratorio. Además se llevó a cabo la evaluación tecno-económica de la

producción industrial de PHA a partir de residuos de la industria agroalimentaria y de cultivos agrícolas.

En el primer caso objeto de estudio, se investigó la producción de polihidroxibutirato (PHB) empleando pataca (también denominada como topinambur o alcachofa de jerusalem), un tubérculo rico en azúcares que puede ser cultivado en tierras marginales. Se realizaron fermentaciones en matraces Erlenmeyer, empleando la bacteria *Cupriavidus necator* DSM 4058 en un medio de cultivo originado a partir de hidrolizado de pataca, demostrando que la producción de PHB es posible. Sin embargo, para fermentaciones llevadas a cabo en bioreactor se hizo necesario suplementar el medio de cultivo con nutrientes adicionales (e.g. hidrolizado de torta proteica de semillas oleaginosas).

En el segundo caso de estudio se estudió el potencial para la producción de polihidroxialkanoatos, incluyendo el copolímero P(3HB-co-HV), con la bacteria *C. necator* DSM 545 y empleando hidrolizado de torta de colza y glicerina (ambos residuos de la producción industrial de biodiésel) como fuente de nutrientes y carbono respectivamente. El análisis de las propiedades del PHA producido verifica el potencial para producir copolímeros complejos a partir de hidrolizado de torta de colza.

Productos expirados y desechados por el mercado de alimentos para bebés a base de harinas, así como corrientes de desecho de la misma industria, fueron transformados en un medio de cultivo rico en nutrientes en el tercer caso de estudio de este trabajo. Las macromoléculas contenidas en estas materias primas (e.g. almidón, proteina) fueron hidrolizadas a través de enzimas (e.g. amilasa, proteasa) producidas en un proceso de fermentación en estado sólido con el hongo *Aspergillus awamori.* Estos hidrolizados fueron probados como medio de cultivo para fermentaciones en matraces Erlenmeyer y en bioreactor, para la producción de PHB. Con este estudio se ha demostrado el potencial para la producción de bioplásticos a partir de desechos harinosos de la industria agroalimentaria.

Finalmente, se llevó a cabo el diseño y la evaluación tecnoeconómica de dos procesos industriales, basados en diferentes materias primas, microorganismos y técnicas de separación, con el fin de compararlos económicamente. Toda la información empleada en los procesos de simulación fue tomada de bibliografía citada en revistas de

reconocido prestigio. Los esquemas de proceso evaluados emplean residuos y subproductos de la industria agroalimentaria (e.g. suero de leche) y trigo, como materias primas.

Chapter 1

Introduction

1.1 Introduction

The United Nations Development Programme (UNDP), in its third annual report published in 1992, conceptualize sustainable development as "a process in which economic, fiscal, trade, energy, agricultural and industrial policies are all designed to bring about development that is economically, socially and ecologically sustainable. That is, current consumption can not be financed by incurring economic debts that others must repay in the future. Investment must be made in the health and education of today's population so as not to create a social debt for future generations, and natural resources must be used in ways that do not create ecological debts by overexploiting the carrying and productive capacity of the earth" [1].

This concept reflects four dimensions in human development:

- ✓ Economic and social dimension: education, health and employment.
- ✓ Political dimension: political freedom as an option for the individuals and the societies.
- ✓ Environmental dimension: physical environment and

natural resources.

- ✓ Temporal dimension: consecuences of present actions in future posibilities of development.

There is a consensus about the temporary or intergenerational dimension of human sustainable development: it does not mean that a world with the same resources must be inherited by future generations, but there must be an adecuated compensation –of knowledge and infrastructure, on the one hand, and of natural resources, on the other hand- for the use of non-renewable natural resources, such as fossil fuels, and for the degradation of the environment by present generations [2].

In this scenario and regarding to the production and consumption of assets and energy, there are also important differences and relationships between the different regions of the planet [1]. Developed countries produce and consume more assets than necesary in order to sustain high quality live levels and economic growth. For this purpose, this countries use mainly non renewable cheap resources like crude oil. Most of the residues of the world end in developing countries with the environmental problems derived from this toxics depositions. More over, in many cases, the only way of survivance for the population of this countries involve a serious damage and degradation of the natural environment they live in.

Nevertheless, the current world demand of 12 millions tons oil per day, for the production of fuels and chemicals, together with the estimated increase of 16 tonnes per day for 2030 [3] and the geographically restricted availability of conventional oil, represent a new human era where cheap secure oil is no more a reality.

1.2 Social and economic considerations

In this chapter, externalities cost and its environmental and social consecuences, regarding the production of energy and goods, is to be discussed.

1.2.1 Climate change, environmental pollution and effects on human health

The World Health Organization (WHO) estimates that every year 800,000 people die prematurely from illnesses by outdoor air pollution worldwide [4]. Among the most urgent global problems it is to emphasize those asociated with anthropogenic climate change derived from a productive, energetic and transport system highly based on fossil carbon sources. The WHO states that 23% of prematurely deaths worldwide are caused by environmental factors and, in Europe, 20% of total sickness incidences are due to these expositions. Children below 5 years old (10% of human population) are more sensitive to this sickness, accounting with more than 40% of this global statistics of pollution-derived sickness. 65% of infant illness is a consecuence of contamination and degradation of the environment. In Spain, it is estimated that, every year, 16,000 people die

because of the transport and industry contamination.

The United Nations Conference on Sustainable Development, Rio+20, celebrated in June 2012 in Brasil, adopted a series of measures that have the potential to contribute to a more equitable, cleaner, greener, and more prosperous world, and recognizes the important linkages between health and development [5]. Dr Margaret Chan, Director-General of the World Health Organization, asserts that the link between health and sustainable development is critical, thus "healthy people are better able to learn, be productive and contribute to their communities. At the same time, a healthy environment is a prerequisite for good health". Health-related development issues include:

- ✓ Access to better energy services;
- ✓ Greater focus on urban planning measures including more sustainable, energy efficient housing and transport;
- ✓ Better sanitation in cities and villages;
- ✓ Sustainable food systems that combat hunger and contribute to better health and nutrition;
- ✓ More sustainable water usage, meeting basic needs for safe drinking-water, and stewardship of water supplies to grow food;
- ✓ Assurance that all jobs and workplaces meet minimum safety and health standards to reduce cancer, chronic lung diseases, injuries and early deaths.

The United Nations Environment Programme (UNEP) estimates that, in the last 25 years, world economy has quadrupled, wich means a significant benefit for hundreds of millions people [6]. Nevertheless, in the same period of time, 60% of main assets and services from the world ecosystems, from wich the human being depends strongly for its sustenance, have been degradated o used in a non-sustainable way. At the same time, and for the first time in human history, more than a half of the planet population lives in urban zones. Nowadays, cities hoard 75% of energetic

consumption and are responsible for 75% of the carbon emmisions.

Over the period 1997 to 2008, 13 to 62% of Europe urban population was exposed to air pollution concentrations exceeding the EU air quality limits or targets. This consideration regards to concentrations of particulate matter (PM_{10}, less than 10 micrometres diameter), ozone (O_3) or nitrogen dioxide (NO_2). As emissions can range from year to year, together with pollution build-up and dispersion/deposition conditions (cotrolled mainly by weather processes), this means that the number of people affected also varies [7].

According to the European Energy Agency, transport related emissions were responsible for aprox. 24% of all EU greenhouse gas (GHG) emissions in 2009 [8]. In Spain, 80% of the energy consumption in the transport sector belongs to road movility [9]. The emissions resulting from this consumption can be sorted in two big groups: greenhouse gasses, that contribute to global warming and climate change (mainly CO_2), and local scale contaminants, that affect life quality and health of urban population (NO_x, solid particules, volatile hydrocarbons, CO and SO_2 among others).

The 12th of June, 2012, and after a week-long meeting of international experts, the International Agency for Research on Cancer (IARC), which is part of the World Health Organization, classified diesel engine exhaust as carcinogenic to humans (Group 1), based on sufficient evidence that exposure is associated with an increased risk for lung cancer [10]. In the published document, the IARC defines the evaluation groups as follows:

- ✓ Group 1: The agent is carcinogenic to humans; this category is used when there is sufficient evidence of carcinogenicity in humans.
- ✓ Group 2: includes agents for which, the degree of evidence of carcinogenicity in humans is almost sufficient (Group 2A) as well as the other extreme, there are no human data but there is evidence of carcinogenicity in experimental animals (Group 2B).
- ✓ Group 3: the agent is not classifiable as to its

carcinogenicity to humans.

- ✓ Group 4: the agent is probably not carcinogenic to humans.

In view of the above data, it is obvious that there is a special and urgent need for changing our transport and production system towards a more sustainable and health friendly one. Biorefinery systems for the production of biofuels and goods, might be one of the possible solutions to our global health problems.

1.2.2 Social and economic benefits of renewable industrial production systems: biofuels production

For the global biofuels industry, key drivers should be the desire to develop alternative sources of energy (in response to the araising crude oil prices and environmental problems), generate increased revenue for farmers through the production of value-added biofuel products, mitigate climate change and to stimulate agricultural production and rural development.

The production and use of biofuels (biodiesel and bioethanol) displaces the crude oil needed to manufacture petrofuel and distillates and, at the same time, reduces the world dependence on foreign oil. The second is of vital importance especially for major importers, such as the U.S and EU, and rapidly growing emerging markets such as China, India and Brazil. Only in 2010, 1.2 billion barrels of crude oil (valued $135.4 billion at 2011 prices) were the equivalent for the production of 110.8 billion liters of ethanol and biodiesel. The displacement of crude oil with biofuels is projected to increase to nearly 2.3 billion barrels by 2020 valued at $253.6 billion [11].

The increased consumption of EU-produced ethanol creates jobs and investment in Europe but it also reduces Europe oil bill. The EU spent 315 billion euro alone in 2011 to import foreign oil, an expense that is almost the size of the entire debt of Greece. Without biofuels, the EU oil account deficit would have been 6.3% higher. Decreasing this oil bill is good for the EU economy, keeping more money inside the EU and reducing the EU overall trade deficit of 115 billion euro [12].

The report "Contribution of Biofuels to the Global Economy" [11], published by the Global Renewable Fuels Alliance, reveals that biofuel industry contributed $277.3 billion (€213.0 billion[1]) -including all the production derived costs: seeds, farming, fertilizers, phytosanitaries, transport, processing, manufacture, etc.- to the global economy in 2010 and supported 1.4 million jobs in all sectors. This amounts to 0.4% of the globe Gross Domestic Product (GDP). Table 1 summarizes the implications of biofuels for economic output and employment in 2010, besides the prediction for 2020.

Table 1. Global economic impact of biofuels (Source: [11])

Gross Output	**2010 (Mil $)**	**2020 (Mil $)**	**% Change**
Ethanol	301,480	525,088	74.2
Biodiesel	72,952	154,663	112,0
Total	374,432	679,751	81.5
Employment	**2010 (Jobs)**	**2020 (Jobs)**	**% Change**
Ethanol	1,088,229	1,594,315	46.5
Biodiesel	291,129	673,380	131.3
Total	1,379,358	2,267,695	64.4

The report highlights that the biofuels sector sustained 1,379,358 jobs worldwide, and estimates more than 2 millions jobs in 2020. Brasil, accounting 470,000 workers in this sector, leads the list followed by USA (420,000) and EU (222,000). Raw material-producing countries, such as Malaysia (main productor of palm oil) remain with only 14,000 jobs.

"This report shows that biofuels industry is contributing substantially to the global public good. As global biofuels production increases, the economic benefits of biofuels are being further maximized", said Mr. Rob Vierhout, Secretary-General of ePURE (European Renewable

[1] European Central Bank Exchange rate of 1 Euro = 1,3132 USD

Ethanol Association) [13]. Particulary, ePURE remarks that ethanol production sustained nearly 70,000 jobs in Europe in 2010, and this could rise to about 190,000 jobs in 2020. Currently in 2012, with more ethanol production plants having been constructed in Europe during the past 2 years, the sector supports 100,000 direct and indirect jobs, mainly in the agricultural sector. However, in Europe, biodiesel industry is the most powerfull one, accounting with 151,840 empoyers and with an estimation of 283,000 in 2020. Thus, Europe is the world leader in biodiesel production followed by Argentina, in the second position, with only 26,057 jobs.

Individual country results and estimations for ethanol and biodiesel (including employment rates) are detailed in Table 2 and Table 3, respectively.

Table 2. Economic impact of ethanol production by country, 2010 and 2020 (Source: [11])

Region	2010 Ethanol output (Mil liters)	2020 Ethanol output (Mil liters)	2010 Gross output (mil $)	2020 Gross output (Mil $)	2010 Employment (Jobs)	2010 Employment (Jobs)
US	50,333	63,961	129,191	170,983	400,644	434,997
Brazil	26,200	50,393	111,353	208,121	444,378	676,213
EU27	4,455	16,316	17,376	63,180	69,343	205,280
China	2,048	7,930	5,191	25,437	20,714	82,648
India	1,892	2,204	8,041	9,102	32,088	36,358
Canada	1,363	2,359	3,454	8,199	13,784	26,639
Thailand	672	2,111	1,704	6,771	6,798	22,001
South Africa	384	421	1,373	1,350	5,481	5,394
Columbia	310	587	1,316	2,424	5,252	7,877
Japan	307	946	777	3,034	3,102	9,859
Argentina	303	470	1,019	1,634	4,065	5,308
Australia	299	492	1,956	1,710	7,807	5,556
Indonesia	210	248	894	1,024	3,568	4,091
Viet Nam	150	423	637	1,747	2,542	5,676
Philippines	118	603	503	2,490	2,006	8,091
Perú	71	217	302	896	1,205	2,912
Malaysia	66	74	279	306	1,112	1,221
Turkey	64	88	163	282	651	917
Mexico	64	90	163	289	650	938
Tanzania	29	55	123	176	490	572
Mozambique	25	59	106	189	424	614
Other	3,880	4,916	15,559	15,743	62,092	51,152
TOTAL	**93,242**	**154,962**	**301,480**	**525,088**	**1,088,229**	**1,594,315**

Table 3. Economic impact of biodiesel production by country, 2010 and 2020 (Source: [11])

Region	2010 Biodiesel output (Mil liters)	2020 Biodiesel output (Mil liters)	2010 Gross output (Mil $)	2020 Gross output (Mil $)	2010 Employment (Jobs)	2020 Employment (Jobs)
EU27	9,184	17,610	38,049	64,977	151,840	282,901
Argentina	1,576	3,231	6,530	11,922	26,057	51,908
Brazil	1,550	3,139	6,423	11,583	25,633	50,430
US	1,192	4,002	4,940	14,767	19,713	64,294
Malaysia	765	1,331	3,170	4,910	12,650	21,378
Australia	627	719	2,597	2,653	10,366	11,551
Thailand	584	1,697	2,420	6,262	9,658	27,263
Indonesia	369	811	1,530	2,992	6,105	13,028
Columbia	302	768	1,253	2,833	4,999	12,333
Canada	236	594	979	2,191	3,907	9,541
China	227	500	940	1,845	3,753	8,032
India	179	3,293	743	12,149	2,967	52,896
Peru	174	130	720	480	2,873	2,089
Philippines	158	271	653	999	2,606	4,349
Turkey	62	52	257	192	1,025	836
South Africa	57	100	235	369	939	1,605
Mozambique	51	80	212	297	848	1,291
Tanzania	50	61	205	224	820	977
Viet Nam	8	100	21	269	128	1,606
Other	257	3,428	1,063	12,649	291,129	673,380
Total	**17,608**	**41,917**	**72,952**	**154,663**	**291,129**	**673,380**

Moreover, not only operatives but also highly skilled jobs have been created in the fields of scientific research, sustainability certification, technology development, engineering and consultancy, as well as throughout the whole value chain of renewable biofuel production.

Taking into account the economic potential of next-generation renewable ethanol, up to one million jobs could be created in Europe by 2020 in all sectors, mainly in rural areas [14]. It is estimated that 230,000 employments would be generated in new EU27 member states, wich means that biofuels production is also a powerfull tool for the recovery and

development of the already-abandoned rural areas in Europe, with the social, economic and environmental benefits that a distributed population model (contrary to the present human concentration in huge cities) is associated with.

Nevertheless, not all the locations are of the same interest in order to establish such an industry. An interesting method to select the proper location of an ethanol-based biorefinery was report by Luk *et al.* [15]. The Preference Ranking Organization Method for Enrichment and Evaluations (PROMETHEE) method was applied to the selected data, ranking the alternative sites. Twelve criteria were taken in account grouped in four categories that could be modified for future location analysis of biofuel production systems:

1. Regional resources: feedstock availability; byproduct demand; water resources;

2. Economic condition: construction costs; labor costs; miscellaneous costs; local competition;

3. Goverment support: legislated demand; production incentives; carbon credit;

4. Social indicators: education level and unemployment level.

An EU research project on the impact of renewable energy policy on economic growth and employment in the European Union also revealed that the liquid biofuels industry (together with wind and solar technologies) is the most promising renewable technology that will contribute to additional employment in Europe [16]. The creation of jobs and new investment of capital, which has been stimulated by an ever-growing European biofuels industry, is expected to lead to an increase in the EU GDP of at least 0.17% (about €25 billion) by 2020 [17].

1.3 Synthetic polymers and the necessity of biodegradable polymer

Plastics have been produced by the chemical industry since the early 1930s and they have become something common in our daily life. Since the discovery of petroleum and gas-derived polymers, and due to their relatively low cost, ease of manufacture, strength, lightness, durability, resistance to degradation, versatility and impermeability to water, synthetic plastics are used in a wide range of products: fibers, textiles, bottles (for water, carbonated drinks, detergents, etc.), jars, film, microwavable packagging, supermarket bags, food packaging, plumbing pipes and guttering, shower curtains, window frames, flooring, car fenders, electronic equipment cases, etc. In 1950, the total global capacity of commodity plastic production was 1,5 million tonnes. This production capacity increased with an annual growth rate of 9%, reaching 245 million tons in 2008 [18].

Scientific research carried out in the last years on the field of polymeric materials has developed polymers with excellent physical properties and durability. However, most of the commodities made of synthetic polymers are only for a single-use application and after its usage

they are discharged in landfills (40% of the global production), incinerated (150 million tons a year) or disposed to the sea and accumulated in oceanic regions (135,000 tons every year) [19]. Only in the US, 19.2 million tons of plastics were produced in 1993, according to the Environmental Protection Agency [20], from wich only 0.7 million tons were recycled.

Because of their non biodegradable nature, they accumulate in the ecosystem resulting in an enormous waste management problem. Contrary to biodegradable polymers, petroleum-derived plastics can remain in the surface of the planet for hundreds of years, without important changes in their structure, thus they are not affected by microbial degradation, maybe because of their huge molecular size [21].

There are three main processes to treat this kind of wastes, but all of them present significant problems for the environment and human health, or a practical difficulty to be implemented [22]:

- ✓ Bio/photo degradation;
- ✓ Incineration. During the combustion of acrylonitrile-based plastics, hydrogen cyanide, a very toxic compound, can be formed. The toxicity is caused by the cyanide ion, which halts cellular respiration by inhibiting an enzyme in mitochondria called cytochrome c oxidase;
- ✓ Recycling is a good option but it results in a very tedious and time-consuming process, thus there is a wide variety of discarded plastics that must be sorted. Moreover, the presence of additives (pigments, coatings, fillers, etc.) limits the use of the recycled material.

Because synthetic oil-based polymers have become an important commodity to enhance the comfort and quality of life, it is almost impossible to reduce their consumption, but their production will be eventually hindered by the depletion of petroleum and the inevitable environmental pollution caused by their disposal. An interesting solution to this problem is their replacement with alternative materials that have polymer-like properties and can degrade after being discarded. In such scenario, biodegradable plastics offer the best solution to the environmental

hazard posed by conventional plastics.

1.3.1 Goverment policies for non-biodegradable polymers

Goverments worldwide have already established policies against pretroleum-based packaging. In Europe and the US some eco-taxes have been proposed for plastic bags, carbon-based packaging, etc. where compostable packaging materials are exempted (EN 13432 [23]). Another example are French policies for agriculture promotion that require disposable retail bags to be biodegradable by 2010 [18]. In Table 4 some global policies and measures for the control of non-biodegradable packaging materials and for the promotion of bio-based ones are shown.

Table 4. Global policies and measures for non-biodegradable materiasl (Source: [18, 24])

Country	National policies and measures
Germany	German packaging directive (2005). Compostable packaging is exempted from the requirements in P.6 of the Directive
France	Law for the promotion of French agriculture (2006), requirement for biodegradability of disposable retail carry bags by 2010
Italy	Markets in Florence charging €0.10-0.20 per plastic bag (2009)
Ireland, Scotland, Denmark & Sweden	Levies and taxes on non-degradable plastic bags already imposed
UK	Ecotax per plastic bag in Durham (2003)
US	San Francisco Board of Supervisors: law of non-biodegradable plastic bags in large supermarkets and pharmacies (March 2007)
Canada	Toronto City Council: charge of minimum $0.05 for each plastic retail shopping bag (2008)
Japan	Law on promoting green purchasing and law on recycling (2001)
India	Plastic if officially banned in Ladakh
Australia	South Australia prohibited non-biodegradable plastic bags in May, 2009.
Bangladesh	Use of plastic bags in Dhaka is banned from January 2002.
Spain	Residues law that considers a gradually disappearance of plastic bags (2011)

1.3.2 Biopolymers classification

The structure of biodegradable materials can be reduced or hydrolyzed to simpler substances by the action of microorganisms such as fungi, yeasts and others. The biodegradability of polymers depend mainly on the chemical structure of monomers, molecular mass and cristalinity [25].

Due to the relevance that bio-materials are gaining in the last years, it is difficult to find a unique definition for biopolymers. The most relevant classifications are reported in the following pharagraphs.

In Europe, the criteria for biodegradability are set out within the standard EN13432: 2000[2] [23]. In the US the Institute for Standards Research defines compostability with similar criteria under the ASTM D6400-99 Specification for Compostable Plastics [26]. Both are equivalent to the international standard ISO17088 from the International Organization for Standardization [27].

Attending to their biodegradable characteristics, biopolymers can be classified into three groups [18]:

1. Biopolymers that are not bio-based plastics but have biodegradable or compostable properties, including synthetic biodegradable aliphatic–aromatic copolyesters: polybutylene terephthalate adipate (PBTA), polybutylene terephthalate succinate (PBTS) and polybutylene terephthalate glutarate (PBTG).

2. Bio-based polymers that are biodegradable or compostable: the polyester polylactide (PLA) and its compounds and blends, starch-based materials, cellulose-based materials, and PHAs.

[2] Identic to the UNE-EN 13432:2001

3. Bio-based resources that are manufactured into non-biodegradable polymers: corn sugar bio-based 1,3 propanediol (PDO) and bioethanol-based linear low-density polyethylene (LLDPE).

Attending to their components, biopolymers can be classified into four groups [18]:

1. Biopolymers produced directly by living organisms such as cotton, silk, wool, other natural fibers, cellulose, starch, lignin, oil proteins, natural rubber and PHAs.

2. Biopolymers produced by polymerization of monomers that either exist in nature or are derived from materials that exist in nature: PLA, polytrimethylene glycol, soy-based polyols, and their derivatives.

3. Combinations of monomers produced from renewable resources with petrochemical-derived monomers: isosorbate-containing polycarbonates and soy-based urethanes.

4. Polymers produced from blends of renewable resources and petroleum-based materials: starch-polyvinyl alcohol.

Khanna and Srivastava reported a very similar classification for biodegradable plastics into three categories, attending to their biodegradability [22]:

1. Chemically synthesized polymers, susceptible to enzymatic or microbial attack. They are not commercially viable because they do not match all the properties of petroleum derived plastics;

2. Starch-based biodegradable plastics, wich are actually a blend of starch and plastic. Soil micro-organisms degrade the starch but, because of the non-biodegradable fraction contained in this polymers they remain in the environment for a long time. Moreover, the production of starch for

commodity purposes compite with the food market.

3. Polyhydroxyalkanoates (PHAs) is the only family of biopolymers that is totally biodegradable. They are polyesters of various hydroxyalkanoates (HAs), which are synthesised intracellulary by numerous microorganisms.

1.4 Polyhydroxyalkanoates (PHAs)

Several biological polymers could fulfill the requirement of biodegradability that our societies require. This work focuses on polyhydroxyalkanoates (PHAs) because of their challenging and price-competitive with petroleum-based plastics.

Polyhydroxyalkanoates can be degradated to carbon dioxide and water, by many microorganisms present in the environment (see Figure 1) and thus, their carbon cycle is completely closed [28]. Another advantage of PHA production is based on the fact that renewable resources (e.g.carbohydrates fatty acids or even amino acids) can be used as main sources of carbon and energy. These raw materials are synthesized via carbon dioxide and water transformation through sun light fixation. For this reason, PHAs are considered as renewable materials [29].

PHAs can be biodegradated both intracellulary and extracellulary. Biodegradation experiments in *Wautersia eutropha*, carried out by Sudesh *et al.* have shown that this process can take 10 times more time than the biosynthesis of the polymer [30]. Nevertheless, the most relevant process is extracellular biodegradation, carried out by many microorganisms such as bacteria and fungi, that secrete PHA-hydrolyzing enzymes known as PHA-hydrolases and PHA-depolymerases [28, 31].

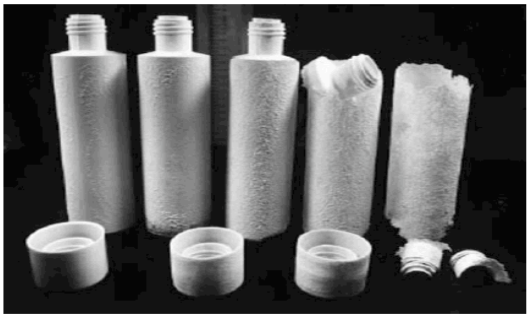


Figure 1. Biopol^{TM} PHAs bottles biodegradation by incubation in mud for 0, 2, 4, 6 and 8 weeks (Source: [30])

PHAs are natural polyesters that many microorganisms synthesize and accumulate as carbon and energy storage materials or as a sink for redundant reducing power, under conditions of limiting nutrients (N, P, S, O or Mg) and in the presence of excess carbon source [32]. Once the supply of the limiting nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy source. To date, more than 100 microoganism are known to accumulate this microbial polyesters up to 90% of their dry weight [33]. The polymers are accumulated in the bacterial cells as defined granules [34]. This granules can be easily observed through electron microscopy (Figure 2), where PHB granules in *Wautersia eutropha* cells were photographed by Tian et al [35].

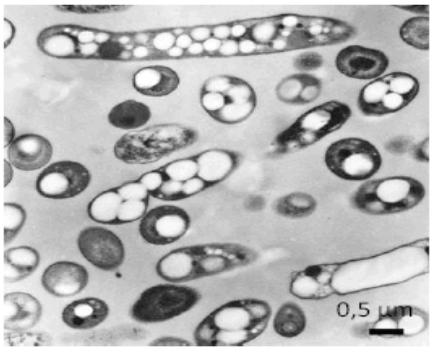
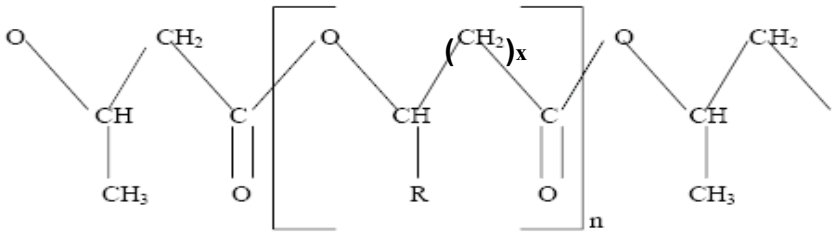


Figure 2. PHB granules in *Wautersia eutropha* bacterial cells (Source: [35])

PHAs can be classified into three groups depending on the number of carbon atoms in the monomer units: shortchain-length (SCL) PHAs, which consist of 3–5 carbon atoms, mediumchain-lenght (MCL) PHAs, which consist of 6–14 carbon atoms and longchain-lenght (LCL) with more than 14 carbon atoms (i.e. poly(3-hydroxy-7cis-tretradecanoate)). The molecular weights of these biopolymers range from 2×10^5 to 3×10^6, and the number of monomers (n) varies between 1,000 and 30,000 depending on the specific PHA, the microorganism that accumulates PHAs and the growth conditions [36].

Even though polyhydroxybutyrate (PHB) is the most famous member of the PHAs family, to date more than 100 different monomeric units have been identified as constituents of this family of polyesters. This variety of copolymers PHAs results in enormous versatility regarding physical properties and potential applications. The chemical structure of polyhydroxyalkanoates and some of the identified monomers are presented in Figure 3.



x = 1	R = hydrogen	Poly (3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
x = 2	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
x = 3	R = hydrogen	Poly(5-hydroxyvalerate)
	R = methyl	Poly(5-hydroxyhexanoate)
x = 4	R = Hexyl	Ply(6-Hydroxydodecanoate)

Figure 3. General chemical structure of Polyhydroxyalkanoates and some identified copolymers; n = 100 to n = 35,000 (Source: [22, 37])

The presence of several monomers in a PHA copolymer results in different physical properties that imply a multitude of applications. Thermophysical properties of selected PHAs are presented in Table 5, together with a comparison with the properties of petroleum-derived polypropylene.

Table 5. Physical properties comparison: PHAs *vs* polypropylene (Source: [36-38])

Physical properties	P(3HB)	P(3HB/3HV) 90:10	P(3HB/3HV) 80:20	P(4HB)	P(3HB/4HB) 90:10	P(3HB/4HB) 10:90	PP
Melting point (°C)	179	150	135	53	159	50	170
Tensile strength (Mpa)	40	25	20	104	24	65	34.5
Young's modulus (Gpa)	3.5	1.2	0.8	149	-	1100	1.7
Elongation to break (%)	3.0	20	100	1000	242	1080	400

P(3HB), poly(3-hydroxybutyrate; P(3HB/3HV), poly(3-hydroxybutyrate-co-3-hydroxyvalerate); P(4HB), poly(4-hydroxybutyrate); P(3HB/4HB), poly(3hydroxybutyrate-co-4-hydroxybutyrate); PP, polypropylene.

1.4.1 History of the industrial producción of PHAs

Eventhough many bacteria have been screened to produce PHAs, not all of them are suitable for biotechnological production of these polyesters on a large scale. There are several factors that contribute to the selection of specific bacteria for an industrial proces, i.e. stability and safety of the organism, growth and accumulation rates, achievable cell densities and PHA contents, extractability of the polymer, molecular weights of accumulated PHA, range of utilizable carbon sources, costs of the carbon source and other nutrients, and occurrence of by-products [36].

The biodegradable polymer industry lacks the advantagies of economies of scale that is easily achieved in the case of petroleum-derived plastics. For instance, polyethylene production has a capacity of 300,000 tons per year, while biopolymers reach a capacity of 1,000 to 20,000 tons per year [18].

In 1959, W. R. Grace and Company (W.R. Grace & Co., New York, USA) [39] was the first to produce PHB for commercial applications but

due to low production efficiency and a lack of suitable purification methods the company shut down.

Later on, in 1976, Imperial Chemical Industries (ICI Ltd., Bellingham, UK) produced PHB and P3HB/3HV, a copolymer that was comercialized under the tradename Biopol™ [40]. However, these biopolymers were relatively expensive compared to petrochemical plastics, and because the expected rise in oil prices did not occurred at that time, the production of these biopolymers was not economically sound until the 90's.

In 1990, the German company Wella released a new shampoo packaged in bottles made of Biopol™. In the production plant of ZENECA BioProducts at Billingham (UK) PHB and P3HB/3HV were produced at scales up to 200,000 L [38]. A two 48h-step fed-batch fermentation process, with a mutant of *Hydrogenomonas* H16 (*Ralstonia eutropha*), based on the technology developed by Schlegel *et al*. [41], was carried out. During the first step, biomass was grown in a mineral salt medium with glucose and a specific amount of phosphate. In the second step, under phosphate limiting conditions, the fermentation was continued until a total dry weight of 100 g/L was achieved. To produce the copolymer P3HB/3HV, a mixed feed of glucose and propionic acid (ratio depending on the desired content of 3-hydroxyvalerate in the copolymer) was added in the polymer accumulation phase [42].

Meanwhile, the company Biotechnologische Forschungsgesellschaft in Linz (Austria) developed a one-step industrial process with the strain *Alcaligenes latus* DSM1124 [43, 44], which can accumulate PHB during balanced cell growth up to 80% of the cellular dry matter. Using a mineral salts medium with sucrose as sole carbon source, a biomass of 60 g/L was achieved. The company stopped the production in 1993 [36].

In Brazil [45], in 1992 a joint venture started between a sugar producer (Irmaoes Biagi) and an alcohol producer (the Balbo Group) to create PHB Industrial S.A. that produce 50 tons of PHB per year under the trade name Biocycle™.

In 1993, the German company Biomer Inc. (Kraaling, Germany) acquired, from the Austrian company Petrochemia Danubia, the technology and microbial strains for commercial production of PHB-based products.

Later on, in 1995, the company registered the trade name Biomer™ [46]

In 1996, the Biopol™ technology from ZENECA was sold to Monsanto (USA) that started a process of generating transgenic plants (i.e., soybean and rapeseed) for agricultural production of PHB and PHB/ HV, expecting a lower price of the final product [36]. Finaly it was sold to the biotech-company Metabolix, Inc., in Cambridge, Massachusetts (USA), founded in 1992.

In 2004, Metabolix, Inc. and Archer Daniels Midland Company (ADM), one of the world largest processors of soybeans (soy meal and oil), corn (sweeteners and flour), wheat, cocoa, ethanol, and world leader in agricultural processing and fermentation technology, announced a strategic alliance to commercialize the Metabolix proprietary PHA technology. Clinton, Iowa (USA) was the selected location for the first commercial plant for PHA from corn, with an initial annual capacity of 50,000 tons per year [47]. Finally in March 2010, Telles (the joint venture company formed by ADM and Metabolix, Inc.) opened the first commercial-scale plant to produce a corn syrup-based PHA resin under the comercial name of Mirel™. Even though it was expected to begin shipping PHA resin in April of 2010 and because of technical and commercial problems, by mid January 2011 Mirel™ was not commercialised yet [48]. Up to date, Mirel™ is being commercialized in the form of plastic bags and film by companies like UCA or Lakeside.

In 2008, Metabolix, Inc. announced the construction of a second technology platform, with a combined production of PHA directly in non-food crop plants such as switchgrass with the associated biomass being used for energy production, after the plastic is extracted [49]. The main target was to obtain PHA from switchgrass at a level of 20% of dry cell mass, 75% of which could be recovered. Thus, if switchgrass yields are 10 to 15 tons per acre, then each acre will yield 1.5 to 2.25 tons of PHA bio-based polymers or derived chemicals, and one million acres will yield 3.3 to 5 billion pounds of PHAs [50].

The Nodax™ technology, with a wide range of applications for PHB and PHBH (Poly-hydroxybutyrate-co-hydroxyhexanoate), was released by the multinational corporation Procter and Gamble, in partnership with Kaneka Corporation, Tsinga University in China and the

Riken Institute in Japan but it was sold in 1993 [18]. In 2010, Kaneka Corporation announced its plan to launch the production of a plant-derived soft polymer called Kaneka PHBH, with a production capacity of 1,000 tons per year at Takasago City, Hyogo, Japan [51]. Also in Japan, the company Mitsubishi Gas Chemical has made progress on the production of PHB from methanol fermentation under the commercial name BioGreen™ [52].

A very interesting option for cheap biopolymer production was proposed in Canada, where the company Biomatera Inc. specializes in the manufacture of PHA by fermentation of agricultural residues. These biopolymers found applications in the manufacture of creams and gels used as slow-release agents in drug manufacturing and as cosmetic agents, and tissue matrix regeneration [18].

PHAs find a large number of commercial, industrial and medical applications. At the beggining of its introduction to the market, bottles, cosmetic cases, pencils, diapers and even golf t-shits were produced [39]. But the greatest applications for these biopolymers are those where large amounts of residues are generated, such as plastic bags, bottles, food-films, hygiene products, etc [37, 53].

They find a wide spectrum on medical applications due to their biocompatibility, thus PHAs can be digested by human body without any risk for the helth [53] because of the presence of 3-hydroxybutyric acid in our blood. In this sense, PHB has been largely used for drugs delivery, knitting, base for medicins and many biomedical products such as syringes, thread, etc [54].

Another important application for PHAs is the production of different molecules through hydrolysis. Chemical and enzymatic hydrolysis of PHAs releases monomers that can be transformed in high interest commercial molecules such as 3-hydroxyacids, 2-alkanes, 3-hydroxyalkanols, 3-acillactones, β-amynoacids and 3-hydroxyacids-esters [55].

Table 6 shows the most important, current and potencial, PHAs production plants worldwide.

Table 6. Current and potential big scale production plants of polyhydroxyalkanoates

Polymer	Microorganism	Carbon source	Trade name	Manufacturer	Capacity (tons)	Price (€/kg) in 2010	References
PHB & P(3HB/3HV)	*Ralstonia eutropha* mutant (H16)	Glucose	- Biopol™	ICI / ZENECA BioProducts (UK)	20,000	-	[40-42]
PHB	*Alcaligenes latus* (DSM1124)	Sucrose	-	Biotechnologische Forschungsgesellschaft (Austria)	-	-[b]	[36, 43, 44]
PHB P(3HB/3HV)	Alcaligenes sp.	Sugar cane	Biocycle(R) -	PHB Industrial Company (Brazil)	50	-	[45]
PHB P(3HB/3HV)	-	-	Biomer(R)	Biomer Inc. (Germany)	50	3.00-5.00	[18, 46]
PHB	-	-	Mirel™	Telles (US)	50,000	1,50	[18, 48, 56]
PHB	-	Methanol fermentation	BioGreen(R)	Mishubishi Gas Chemical Company Inc. (Japan)	10,000	2.5 - 3.0	[18, 52]
PHBH	-	-	Nodax™	P&G (US)	20,000-50,000	2.50	[18]
PHBH	-	-	Nodax™	Lianyi Biotech (China)	2000	3.70	[18]
PHBH	-	-	Kaneka PHBH	Kaneka Corporation (Japan)	1000	-	[18, 51]
P(3HB/4HB)	-	-	Green Bio	Tianjin Gree Bio-Science Co /DSM	10,000	-	[18]
P(3HB/3HV) + Ecoflex blend	-	-	Enmat(R)	Tianan Biologic, Ningbo (China)	10,000	3.26	[18]
PHA from P&G	-	-	Meredian	Meredian (US)	272,000 (2013)	-	[18]

1.4.2 Polyhydroxybutyrate (PHB)

The PHB homopolymer is the most well-known member of the family of PHAs. Low molecular weight oligomers of PHB (120-200 monomers) appear not only in microorganisms but also in plants (plant tissues) and animals (beef heart, chicken liver, porcine kidney), including humans (human blood plasma) [36]. Nevertheless, only the high molecular weight PHB has a potential use as industrial biodegradable plastic.

Polyhydroxybutyrate is a stereoregular thermoplastic, with all asymmetric carbon atoms in the (R)-configuration, which results in high cristalinity (55-80%) that makes it relatively stiff and brittle. Regarding thermal properties, glass transition temperature (T_g) lies between 5 and 9 ºC, melting temperature (T_m) ia within the range of 173-180 ºC and decomposition temperature is around 200 ºC. Its solid-crystalline state, in the form of a right-handed helix, allows PHB extraction from biomass in chloroform solutions [36]. Chemical structure of PHB is shown in Figure 4.

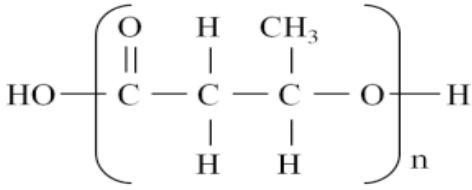


Figure 4. Chemical structure of PHB; n = 100 to n = 30,000

Because of its thermophysical properties and mechanical properties, (Young's modulus 3.5 Gpa; tensile strength 40 Mpa) PHB can be a suitable substitute for polypropylene [40]. However, the elongation to break for PHB is about 3%, which is significantly lower than that of polypropylene (400%) [36].

This biopolymer can be produced from many renewable sources

such as sugars (e.g. glucose, sucrose, fructose), agricultural products (e.g. wheat, corn, oilseeds), by-products from various industries (e.g. glycerol from biodiesel production, sweet-cane molasses), agricultural residues (e.g. straw) and industrial waste streams (e.g. whey). It is considered a promising biopolymer for commercialisation because of its main qualities: biodegradable, non toxic, biocompatible, with a high degree of polymerization, insoluble in water, highly crystaline (if extracted from its natural environment), optically active, isotactic and piezoelectric [57].

The cost of producing PHB depends highly on the substrates price (among 28 - 50% of the total production cost [58]) followed by the productivity of the fermentation stage and the extraction and purification of the biopolymer [59]. The search for economic renewable carbon sources for fermentation processes, as well as new microbial strains, and extraction/purification systems, are crucial in order to boost industrial production of PHB and improve its properties.

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

The flexibility of the PHB homopolymer is greatly improved when 3-hydroxyvalerate units are incorporated, thus Young's modulus and tensile strength decrease below 0.7 and 30 Mpa, respectively, with increasing percentage of comonomer in the polymer. Depending on the comonomer fraction (3-hydroxyvalerate content), the elongation to break also increases (getting a bit closer to polypropylene) and melting temperature is reduced significantly up to approximately 130 ºC. Eventhough the degradation temperature is not affected significantly, the reduction of melting temperature allows thermal processing without degradation of the material and increases processing possibilities [36]. Mechanical properties of P(3HB-*co*-3HV), with different 3-hydroxyvalerate content, and its comparison with petrochemical plastics, are shown in Table 7.

Table 7. Mechanical properties of P(3HB-*co*-3HV) with different comonomer content compared with petroleum-based plastic bags (Source: [18])

PHAs composition	Thoughness (Mpa)	Young's modulus (Mpa)	Tensile strength (Mpa)	Elongation (%)
P(3HB)	-	3500	40	0.4
P(3HB-*co*-3HV)				
3% 3HV	-	2900	38	-
9% 3HV	-	1620	190	37
14% 3HV	-	1500	150	35
20% 3HV	-	1450	120	32
25% 3HV	-	1370	70	30
Petroleum based plastic				
HDPE	62	640	19	576
PP	64	590	27	435
LDPE	15	156	13	126
UV degradable bag	60	674	24	384

As mentioned before, thermal properties of P(3HB-*co*-3HV) copolymers are also improved in terms of industrial production and range of manufactured products. Thermal properties of P(3HB/3HV) with different 3HV content are presented in Table 8.

Table 8. Thermal properties of P(3HB/3HV) with different comonomer content (Source: [18, 36])

PHAs composition (mol%)	T_g (ºC)	T_m (ºC)	M_N	PDI
PHB	10	177	$7.82x10^5$	1.8
P(3HB-*co*-3HV)				
10% 3HV	-	150	-	-
24% 3HV	-6	138	$1.38x10^5$	1.9
45% 3HV	-10	75	$4.00x10^5$	2.4
71% 3HV	-13	87	$2.54x10^5$	2.0

P(3HB/3HV) copolymers with different 3-hydroxyvalerate content, ranging from 0 to 30%, are commercially available as Biopol™ resins [36], Biocycle100 and Biocycle24005 [18].

1.4.4 Microbial pathways for PHAs production

PHAs producing bacteria can be divided into two groups, depending

on their cultivation conditions. The first group contains bacteria that require nutrient limitation (such as N, P, Mg or S) and an excess of carbon source for accumulation of PHAs. *Cupriavidus necator* (previously designated as *Ralstonia eutropha* and *Alcaligenes eutrophus*), *Bacillus* sp., *Protomonas extorquens* and *Protomonas oleovorans* among others, belong to the first group of bacteria. Fed-batch fermentations, where cultivation takes place in two stages, is the most suitable operation mode for the production of PHAs in the first group. In the first stage, no nutrient limiting conditions are applied and high microbial cell concentrations, without polymer production, are achieved. In the second stage, through the limitation of any nutrient, cell growth is inhibited and PHA biosynthesis is activated [60]. The microorganisms (e.g. *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii* and recombinant strains of *E. coli* among others) that belong in the second group do not need any nutrient limitation for PHAs synthesis, fermentations take place in batch or continuous mode [32].

The proper selection of PHA producing microorganism depends on several factors such as its ability to consume cheap carbon sources, its growth rate, biopolymer synthesis rate and maximal polymer accumulation capacity. This work will focus on the production of PHAs by the bacteria *C. necator*. The metabolic pathway leading to PHB production in *C. necator*, using glucose or similar sugars, takes place through three enzymatic reactions (Figure 5). In this metabolic scheme, β-ketothiolase (acetyl-CoA acetyltransferase) catalyzes the reversible condensation reaction of two molecules of acetylcoenzymeA (acetyl-CoA) to produce the complex acetoacetyl-CoA. The β-ketothiolase is negatively regulated by the product coenzymeA (HSCoA), which is also a product when acetyl-CoA enters the tricarboxylic acid cycle (TCA) under non-limited conditions that promote microbial growth. The product is subsequently stereoselectively reduced to (R)-3-hydroxybutyryl-CoA (an enzymatic complex related to the monomer of PHB) in a reaction catalyzed by NADPH-dependent acetoacetyl-CoA reductase. The third and final step is the polymerisation reaction catalyzed by PHB synthase [22, 32, 36, 57]. The genes phbA, phbB, and phbC of *C. necator* are located in one operon and code for the β-ketothiolase, acetoacetyl-CoA-reductase and PHB synthase, respectively.

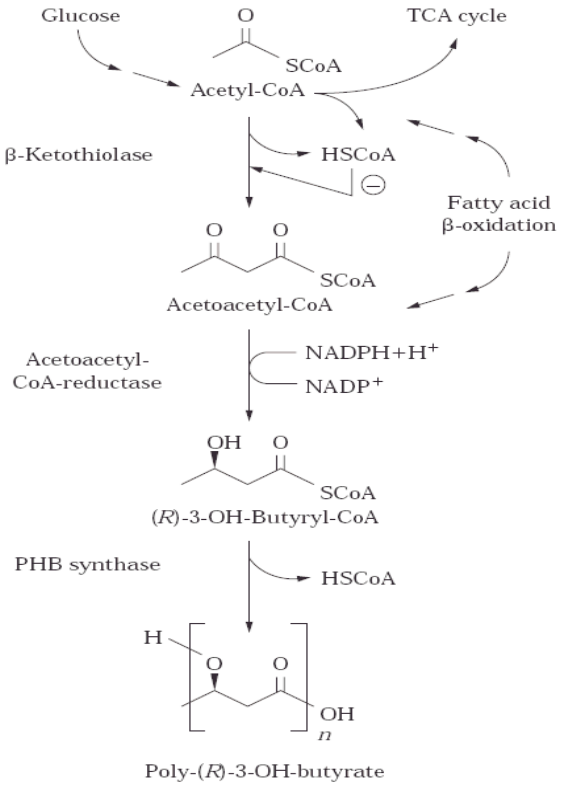


Figure 5. Methabolic path way for the synthesis and breakdown of PHB *in R. eutropha (Source:* [36])

Synthesis of P(3HB-co-3HV) occurs in many P(3HB)-accumulating bacteria only from substrates that can be converted into propionyl-CoA, 3-ketovaleryl-CoA or R(-)-3-hydroxyvaleryl-CoA (e.g. propionic or valeric acids). Nonspecific β-ketothiolases and acetoacetyl-CoA reductases allow the synthesis of R(-)-3-hydroxyvaleryl-CoA, which is a substrate for many PHA synthases. In Figure 9, the biosynthetic pathway of PHB and

P(3HB/3HV) is shown. Hydroxyalkanoic acids, with a hydroxyl group at the 4', 5' or 6' carbon atom, are only incorporated into PHA if the corresponding alkanoic acids, diols or lactones are used as substrates [57].

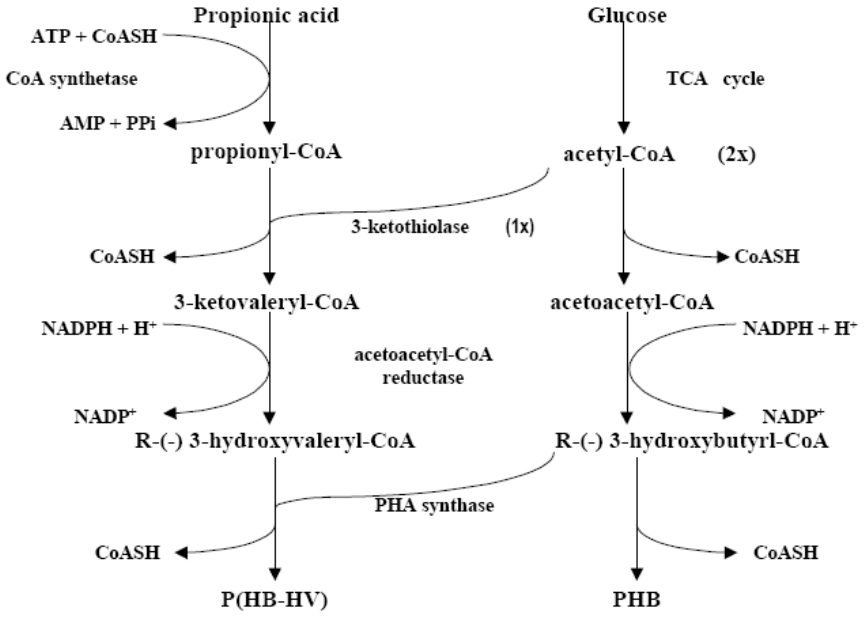


Figure 6. Biosynthetic pathway of PHB and P(3HB/3HV); (Source: [61])

As mentioned before, for the production of PHA-copolyesters, precursors have to be fed to the medium during the PHA production phase. More than 150 precursors are known, leading to a wide range of copolyesters differing in their thermophysical and mechanical properties including liquid, crystalline or amorphous forms and a variety of piezoelectric, thermoplastic and elastomeric properties among others [58]. Some of these precursors that have been used in the case of PHA production by *C. necator* are shown in Table 9. It is of vital importance to control the chemical nature and concentrations of these precursors in order to avoid inhibition of bacterial growth and PHA accumulation, and reach a desirable percentage of comonomer in the copolyesters.

Table 9. Some examples for precursors for PHAs production in *C. necator* (Source: [58])

Precursor	Copolyester
Propionate	Poly-3-hydroxybutyrate-co-3-hydroxyvalerate
γ-Butyrolactone	Poly-3-hydroxybutyrate-co-4-hydroxybutyrate
1,4-Butandiol	Poly-3-hydroxybutyrate-co-4-hydroxybutyrate
4-Hydroxybutyrate	Poly-3-hydroxybutyrate-co-4-hydroxybutyrate

One of the problems in the production of PHAs copolymers, is the high price of these substrates. This factor prevents their use in industrial proccesses. Moreover, the comonomer yields from the precursors will be rather low if specific conditions are not applied during PHA accumulation.

Only a limited number of strains is known to store copolyesters without addition of special precursors [58]. Some examples are shown in Table 10. Nevertheless, to date, production of PHAs copolymers by *C. necator* has only been reported with addition of precursors such as sodium propionate [62, 63] and aminoacids. In this report (see Chapter 4) it is shown how the accumulation of a copolymer conformed by monomers of P3HB and P3HV can be stored under certain cultivation conditions by *C. necator* DSM545, in the presence of rapeseed cake hydrolysates (that contains aminoacids) and biodiesel-derived glycerol as sole carbon source, without any commercial precursors addition.

Table 10. Examples for copolyester production without precursor addition (Source: [58])

Strain	Copolyester	Carbon sources
Alcaligenes sp. SH-69	Poly-3-hydroxybutyrate-co-3-hydroxyvalerate	Glucose, sucrose, sorbitol, mannitol and glutamate
Pseudomonas cepacia	Poly-3-hydroxybutyrate-co-3-hydroxy-4-pentenoate	Gluconate and sucrose
Pseudomonas sp. (NCIMB 40135)	Copolyester from 3-hydroxydecanoate (mainly) and 3-hydroxyoctanoate	Acetate, glycerol, lactate, succinate, glucose, gluconate and n-octanoate

1.5 The biorefinery concept

Around 95% of worldwide chemical production is based on non-renewable crude oil and natural gas. The primary building blocks produced from crude oil are hydrocarbons, containing no oxygen or nitrogen in their molecular formula, such as ethylene, propylene, butadiene, butenes, benzene, toluene, xylenes or methane [64]. This substances are called platform , basic or bulk chemicals, thus most of the chemical products we use nowadays are derived from these primary building blocks (e.g. monomers for polymer synthesis such as ethylene or propylene, precursors, etc.) and some of them can also be used as endproducts (e.g. benzene as solvent). Figure 7 presents the most important production routes of various chemicals derived from crude oil.

The depletion of cheap and easily-explored petroleum, coupled with a gradually increasing governmental and social awareness about the effect of greenhouse gas emissions to global climate, is developing a consensus about substitution of petrochemical processes by sustainable chemical processes utilising renewable resources as feedstocks.

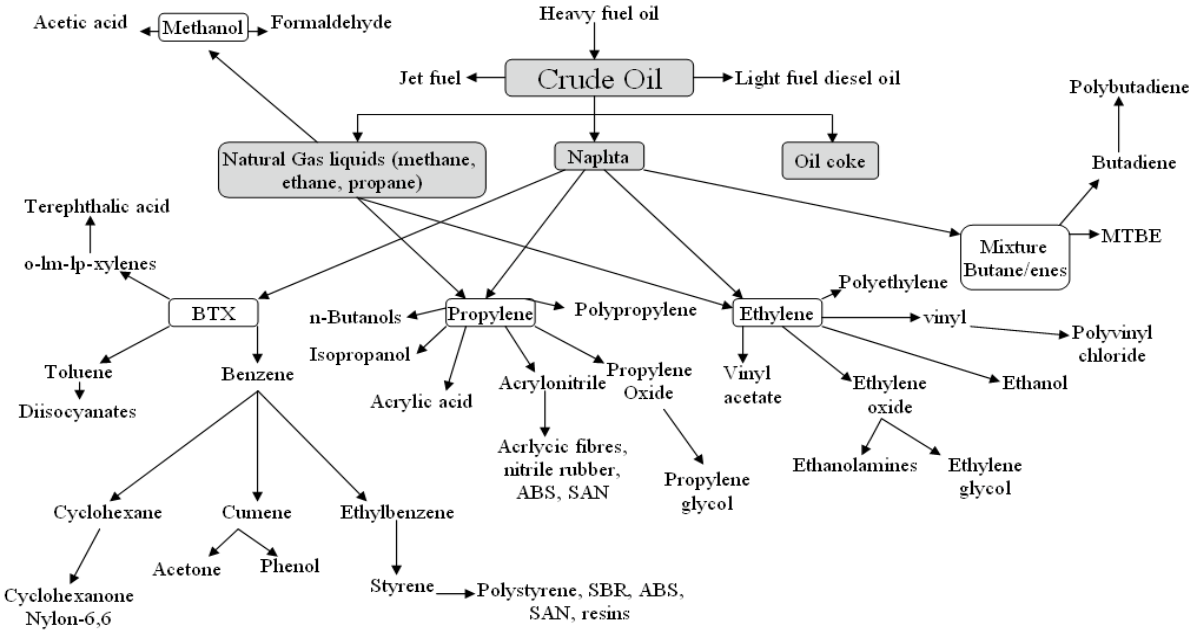


Figure 7: Predominant production routes, capacities and unit cost of major organic chemicals and plastics (Source: [64])

In 2007, Koutinas *et al.* [65] introduced the term *biochemurgy* to define a new concept that "exploits fundamental principles from biochemical engineering, biochemistry and chemistry to develop clean and sustainable technologies that combine physical, chemical and biological processing to convert agricultural or organic raw materials into chemical products". It is obvious that the implementation of such technologies is not going to be neither easy nor cheap. Nevertheless, chemical products must be produced in the future from renewable resources, such as agricultural, forest and marine residues and industrial organic waste streams. It should be stressed that there is an important difference between energy and chemical production. There are several alternative sources for energy production such as wind, sun, water or biomass, while the production of materials and materials should rely on biomass [66].

1.5.1 Definition and classification of biorefineries

The biorefinery system is based on the treatment of all biomass components as the primary building blocks for the manufacture of multiple bio-based products and intermediates. The product palette of a biorefinery includes not only those produced in a traditional petroleum refinery, but also particular products that cannot be produced by (petro)chemical processes. This production systems can create a completely new chemical industry and, at the same time, will reduce environmental concerns, create new job opportunities and minimize waste production [64].

Several authors have provided overviews of the biorefinery concept [66-68], but this is a young working field and therefore is still an open field of knowledge, which is reflected in the search for an appropriate description and classification.

Among the several definitions of biorefinery, the US National Renewable Energy Laboratory (NREL) defines a biorefinery as "a facility that integrates biomass conversion processes and equipments to produce fuels, chemical, value-added products and power" [69]. Also, the International Energy Agency (IEA Bioenergy Task 42) gives a the following definition for biorefineries: "Biorefining is the sustainable

processing of biomass into a spectrum of marketable products and energy" [70]. Ragauskas *et al.* [71], emphasize that "the power of the biorefinery is supported by economies of scale and efficient use of bioresources. The imbalance between commodity chemical needs and transportation fuels is key to the biorefinery concept".

To date a standard classification for biorefinery systems is still under discussion. In 2009, Cherubini *et al.* [72] reported a wide approach for biorefinery system classification, based on the needs of IEA Bioenergy Task 42 for a common way to describe energy-driven biorefineries. **Error! Reference source not found.** shows the classification proposed by Cherubini *et al.*, in which each biorefinery can be classified according to four main aspects: platforms, products, feedstock and processes.

1.5.2 Classification of biorefineries by employed feedstocks

Many authors associate the idea of biorefineries with the use of different feedstocks, such as crops and crop residues, forest residues, green grasses, lignocellulosic biomass and industrial waste [66, 67, 73-75]. Renewable carbon-based raw materials for biorefineries are available from four different sectors:

- ✓ Agriculture (dedicated crops and residues);
- ✓ Forestry;
- ✓ Industry (process residues and leftovers) and households (municipal solid waste and wastewaters);
- ✓ Aquaculture (algae and seaweeds).

A further distinction can be made between feedstocks derived from dedicated crops as well as residues from agricultural, forestry and industrial activities, which can be available without upstream concerns.

The main biomass feedstocks can be grouped in three categories: carbohydrates and lignin, triglycerides and mixed organic residues [68]. Figure 8 represents a general scheme for precursor-containing biomass.

Table 11. Features and relative subgroups used in biorefinery classification (Source: [72])

Platforms	Products	Feedstocks	Processes
C5 sugars	**Energy products**	**Dedicated crops**	**Thermochemical**
	Biodiesel	Oil crops	Combustion
C6 sugars	Bioethanol	Sugar crops	Gasification
	Biomethane	Starch crops	Hydrothermal upgrading
Oils	Synthetic biofuels	Lignocellulosic crops	Pyrolysis
	Electricity and heat	Grasses	Supercritical
Biogas		Marine biomass	**Biochemical**
	Material products		Fermentation
Syngas	Food	**Residues**	Anaerobic digestion
	Animal feed	Lignocellulosic residues	Aerobic conversion
Hydrogen	Fertilizer	Oil-based residues	Enzymatic processes
	Glycerin	Organic residues & others	**Chemical**
Organic juice	Biomaterials		Catalytic
	Chemical and building blocks		Pulping
Pyrolytic liquid	Polymers and resins		Esterification
	Biohydrogen		Hydrogenation
Lignin			Hydrolysis
			Methanization
Electricity and heat			Steam reforming
			Water electrolysis
			Was gas shift
			Mechanical/Physical
			Extraction
			Fiber separation
			Mechanical fractionation
			Pressing/disruption
			Pretreatment
			Separation

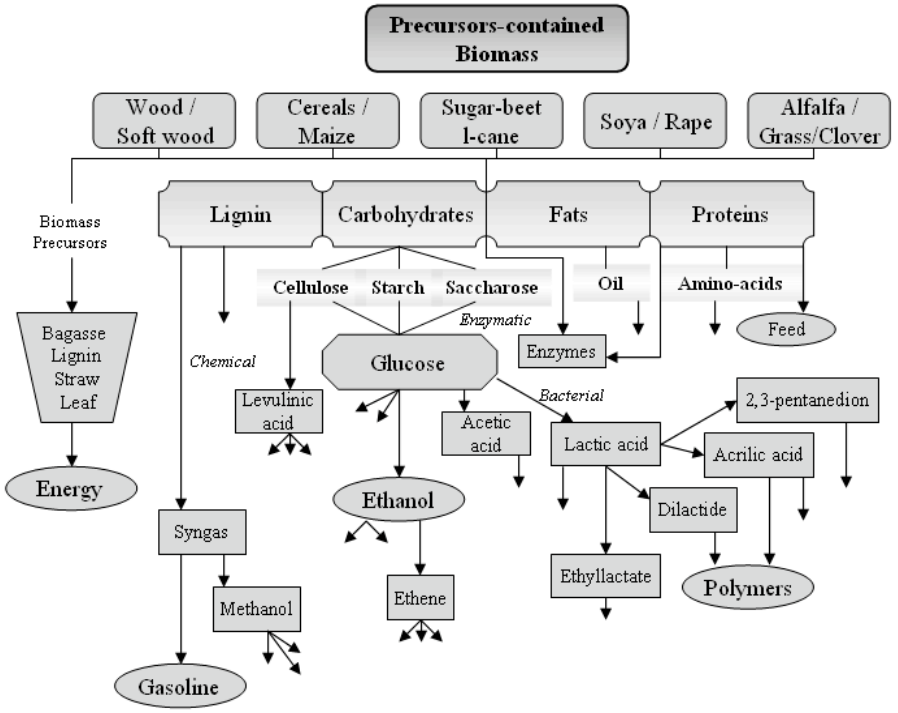


Figure 8. General scheme of a biorefinery: precursors and products (Source: [66]

Carbohydrates based biorefinery

Carbohydrates are the main raw materials for the biorefinery production units of the future [64]. Polysaccharides (such as cellulose, hemicellulose and starch) must be broken down into their constitutive monomers (via enzymatic hydrolysis, thermochemical degradation or a combination of these two) before their utilization as feedstocks for biofuel and chemical production.

Monossacharides such as glucose, xylose and arabinose, derived mainly from cellulose, starch and hemicellulose, could be converted to various bulk chemicals through different processes.

There are 12 main platform molecules considered as the most promising ones: 1,4-diacids (succinic, fumaric and malic), 2,5-furandicarboxylic acid, 3-hydroxy-propionic acid, aspartic acid, glucaric acid, glutamic acid, itaconic acid, levulinic acid, 3-hydroxybutyrolactone, glycerol, sorbitol and xylitol/arabinitol [76]. Glucose-based fermentation media could produce half of these molecules: succinic acid, fumaric acid, malic acid, glutamic acid, itaconic acid and aspartic acid [64].

One of the main derivatives from various saccharides (mainly glucose) via fermentation is lactic acid, that has both a carboxylic group and a hydroxyl group, which allows it to take part in many chemical reactions such as polymerization, esterification, dehydrataion, etc. Polymers, solvents and fine chemicals (i.e. 1,2-propanediol, propionic acid) can be produced from lactic acid [77]. Succinic acid has also a wide range of possibilities in the chemical industry but its production is not yet as efficient and economic competitive as lactic acid [78].

Lignocellulosic biomass conversion to chemicals

Cellulose, hemicellulose and lignin are the main constituents of lignocellulosic biomass and can be found in nature in many forms: agricultural and forestry residues, wood, energy crops and industrial waste streams. Integrated physical, chemical and biochemical processes may be used for the conversion of these materials, to fractionate them into their components and to transform monosaccharides into desirable products such as ethanol (mainly from glucose and xylose) or chemicals [64].

The xylose contained in hemicellulose could be converted into xylitol, for the production of a wide range of chemicals such as xylaric acid, propylene glycol, ethylene glycol, polyesters, etc. [76].

From cellulose, carboxylic acids or levulinic acid (for gasoline or diesel oxygenates, polymers, pesticides and solvents production) could be produced [67]. Other usefull platform chemicals derived from cellulose are formic acid, furfural and carbonaceous char for syngas production obtained through a novel process called “Biofine” [79].

Lignin could be a suitable feedstock for production of lignin-based adhesives and other products such as fibres for the production of light fibre

composites for vehicles [80]. Through lignocellulosic material pyrolysis and gasification technologies, fuels and chemicals can also be produced, but additional development of these technologies is required in the long term [76].

Vegetable oil-based biorefinery

Vegetable oils are mainly used in two processes as raw materials for the production of chemicals and biofuels: hydrolysis, for the production of fatty acids and glycerol; and transesterification, for the production of fatty-acid alkyl esters (mainly used as biofuel called FAME or biodiesel) and glycerol.

Glycerol is a by-product of the biodiesel production and considering the increase in biodiesel production capacity of the last decade, the price of glycerol could decrease to $0.45-1.12/kg [64], which open the possibility of its use as a major building block for bulk chemical production through green chemical transformation and enzymatic, chemical or microbial conversion. The main products that can be obtained from glycerol are propylene glycol, propanol, branched polyesters, nylons, mono-, di- or tri-glycerate, diglyceraldehyde, glycerol carbonate and other oxidation products [76]. Also ethylene glycol and propylene glycol (from the hydrogenolysis of glycerol), acrylic acid (by combined dehydratation and oxidation processes), acrolein (by dehydratation), chlorinated compounds, hydrogen and alkanes (by aqueous-phase reforming) are considered as feasible products by many authors [64].

Glycerol consumption by microorganisms in fermentation processes can lead to the production of many chemicals that could be used either as end products or as important building blocks. Glycerol fermentation products are reported to be succinic acid [81], polyhydroxyalkanoates [82-85], 3-hydroxypropionaldehyde, citric acid, 3-hydroxypropionic acid, butanol and propionic acid [64].

From vegetable oils and their fatty acids, many surfactants, lubricants, dicarboxylic acids, resins, stabilizers, plasticizers, secondary alcohols and polyols can be produced.

Proteins for chemical production

Plant proteins (such as wheat gluten) and the aminoacids they are consisted of, have many applications for the production of polymers, coatings, composites, inks, cosmetics, encapsulation materials, vaccines or antibodies. They are a high valuable by-product of the first generation biofuels industry (biodiesel and bioethanol) [64, 76].

Green chemical extraction of biomass for chemical production

Green extraction processes can be employed for the extraction of minor constituents like metabolites and oils, mainly through supercritical fluid techniques. Protein-rich rapeseed (a very common residue from biodiesel production in Europe) is rich in phenolic compounds, glucosinolates and phytic acid, that could be used as antioxidants in cosmetic and pharmaceutical formulations. Anticarcinogenic agents, biopesticides and flavours are high valued products from the transformation of glucosinolate [64]. Also arabinoxylans could be estracted from biomass and have numerous applications in bread manufacture, animal feeding and medical applications [86].

1.5.3 Classification of biorefineries by process flexibility

Another proposed classification for biomass-processing production plants is related to the process flexibility of the installation [66]. According to this classification, phase I biorefinery is the one that uses only a certain feedstock, has a fixed processing capability and produces a fixed amount of products and co-products. An example of a phase I biorefinery could be a dry-milling ethanol plant, with almost no flexibility in processing.

As phase II biorefineries could be regarded those that produce several end-products, depending on product demand. This factor opens numerous possibilities for the connection of industrial product lines with existing agricultural production units. An example of phase II biorefinery is a conventional sugar plant transformed for the production of biodegradable plastics (PHAs), sugar and ethanol.

Phase III biorefinery should be the target of every biorefinery

system thus is not only able to produce a variety of chemicals, fuels, intermediates and end-products, but can also use various types of feedstocks and processing methods, with high adaptability toward changes in demand and supply of feedstocks.

Biorefineries will most probably encompass a whole range of different sized installations, thus several bio-industries can combine their material flows in order to reach a complete utilization of all biomass components giving rise to integrated bio-industrial systems [87, 88].

1.5.4 Biorefinery technologies

In order to develop biorefineries comparable to petroleum refineries, it is necessary to produce a broad variety of bio-based products in an efficient construction set system. Each biorefinery fractionates and converts biological raw materials into a multitude of valuable products. The change from petroleum hydrocarbons to bio-based ones will create notable opportunities for the chemical processing industry. For example, the use of carbohydrates as chemical raw materials will eliminate the need for several capital-intensive oxidative processes used in the petroleum-based industry [71].

The biorefinery concept embraces a wide range of technologies able to separate biomass resources, such as wood, grass, corn, etc. into their building blocks (carbohydrates, proteins, triglycerides) which can be converted into value added products, biofuels and chemicals [87].

As humankind progresses from the oil refinery to the biorefinery, challenges associated with the separation processes will change and grow in importance. In the petroleum industry, distillation is the unit operation that dominates the refinery separation scheme. For chemicals derived from biomass, this dominance will be transferred to solvent-based extraction. This is a result of the non-volatile nature of the majority of biomass components [71].

Future biorefinery operations will first extract high value-added chemicals already present in biomass, such as fragrances, flavoring agents, food-related products, and high value nutraceuticals that provide health and

medical benefits. The biorefinery will then focus on processing plant polysaccharides and lignin into feedstocks for bio-derived materials and fuels. This requires the development of innovative separation and depolymerization processes [71].

Although future trends are leading the scientific community to develop and implement sustainable energy systems like the biorefinery concept, the best policy to preserve the environment will remain better energy efficiency and resources conservation through a reduction in energy dependence.

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Chapter 2

Producing Polyhydroxyalkanotares from waste streams with *Cupriavidus necator*

2.1 Introduction

The viability of microbial large-scale production of polyhydroxyalkanoates (PHAs) is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics.

There are three important limitations in the bulk production of bioplastics [1]:

1. the special growth conditions required for the synthesis of these compounds (usually unbalanced nutrient conditions that promote PHA accumulation)

2. the difficulty involved in synthesizing them from inexpensive feedstocks

3. the high cost of their recovery.

There seems to be two challenging targets to achieve industrial production of microbial PHAs, especially copolymers containing the 3HB monomer as major constituent. One target is to gain cost competitiveness of PHA production against petrochemical-based polymers such as polyethylene, polypropylene and polystyrene (the prices of PHAs are about

10 times higher than those of synthetic counterparts). The other, is to verify or support the environmentally friendliness of the PHA production process. These targets could be fulfilled if a carbon source and a bacterial strain are properly selected and a process for efficient PHA production is developed [2].

Over 300 microorganism species have been characterized as PHA producers but only a small number of them are capable of synthesizing short-chain (SC) and medium-chain (MC) length PHAs. The bacteria *Cupriavidus necator* (previously designated as *Ralstonia eutropha* and *Alcaligenes eutrophus*) synthesize predominately SC-PHAs of diverse composition with high yields (up to 80–90%), from a wide variety of substrates such as H_2–CO_2 mixtures, sugars, organic acids, alcohols, and industrial or agricultural wastes [3]. Therefore, these bacteria are among the most promising PHA producers.

Hydroxybutyrate (HB) is the predominant monomer in PHAs produced by *Cupriavidus necator* but some bacterial strains have been reported to produce also hydroxyvalerate (HV) to ratios that reach 80–90 mol %, while hydroxyhexanoate (HH) and hydroxyoctanoate (HO) levels do not exceed 1–2 mol % [3]. This means that it is possible to produce a copolymer with good thermophysical and mechanical properties from *C. necator* and some inexpensive carbon sources.

Several studies on economic evaluation of P(3HB-co-3HV) production indicate that the substrate cost makes an important contribution to the total production cost. As an example, Marangoni *et al.* [4] reported that it can represent more than 38% of the total cost in some cases. Also Eggink *et al.* [5] stated that, for a glucose-based large-scale production of poly-3-hydroxybutyrate (PHB), the raw material cost rises to 30% of the total production costs. Glucose is the most common carbon source for the production of PHB with *C. Necator* and approximately 3.5 kg of glucose or sucrose is necessary to produce 1 kg of PHB. The price per kilo of these sugars is approximately €0.60 to €0.80 [5].

Besides sugars, PHAs can be produce from other carbon sources. The ability of some bacterial strains to utilize a wide spectrum of carbon and nitrogen sources, and the existence of different metabolic pathways, among other factors, promote a large diversity of fermentation processes for

the production of PHAs. Therefore, inexpensive renewable resources such as wastes, residues or by-products from the agricultural sector or food-industry (among other industrial sectors), could be used as fermentation feedstocks [6].

2.2 PHAs production from agroindustrial and municipal wastes

2.2.1 Glycerol from the biodiesel industry

Vegetable oils, due to their high viscosity, among other properties, are not suitable to be used directly as fuel in diesel engines. Therefore, they must be first converted into fatty acid methyl-esters (FAME), also known as biodiesel, in a process that produces as by-product large ammounts of glycerol (approximately 1 kg of glycerol per 10 kg of vegetable oil). The current large-scale production of biodiesel by the oleochemical industry is resulting in an excess of glycerol, thus it is of great importance that new, environmentally friendly and cost competitive industrial applications are developed for glycerol utilisation [5].

One of the most promising options is to investigate the potential of this industrial by-product with respect to microbial PHA production. However, few studies have been published dealing with the production of polyhydroxyalkanoates (PHAs) from this carbon substrate [7-11]. There are some studies dealing with the production of PHB from glycerol [12-14].

Cavalheiro *et al.* [11] focused on the utilization of crude glycerol from a biodiesel plant as carbon source in bacterial fermentations using

Cupriavidus necator DSM 545 for the production of short-chain length PHAs (poly-(3-hydroxybutyrate-*co*-4-hydroxybutyrate) or P(3HB-*co*-4HB) and poly-(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate or P(3HB-*co*-4HB-*co*-3HV)). Incorporation of 4HB monomers was promoted by γ-butyrolactone (GBL). Propionic acid (PA), a stimulator of 4HB accumulation, increased the 4HB molar ratio 2-fold, but also acted as 3HV precursor. Dissolved oxygen (DO) and cultivation time were key parameters for PHAs accumulation and volumetric productivity, yielding P(3HB-*co*-4HB) with 11.4 - 21.5 molar % of 4HB and P(3HB-*co*-4HB-*co*-3HV) with 24.8 - 43.6 molar % of 4HB monomer and 5.6 - 9.8 molar %. of 3HV. The molecular weight of the copolymers varied between 5.5 10^5 and 1.37 10^6 Da[1].

Cavalheiro *et al.* [14] also reported two strategies to enhance PHB production: the increase in PHA volumetric productivity in high density cultures and the use of waste glycerol as primary carbon source. The selected strain was again *Cupriavidus necator* DSM 545, that accumulates PHB from pure and crude glycerol. The cultivation on pure commercial glycerol resulted in productivities of 0.6 – 1.5 g_{PHB} L^{-1} h^{-1}, a maximum cell dry weight of 82.5 g L^{-1} and a PHB content of 62% (w/w). The use of crude glycerol from the biodiesel industry as the sole carbon source for cell growth and accumulation of PHB attained a lower cell dry weight of 68.8 g L^{-1} with a PHB content of 38% (w/w). The final productivity in the case of crude glycerol was 0.84 g_{PHB} L^{-1} h^{-1}. Fermentation performance was improved by decreasing the biomass concentration in the moment that PHB accumulation was triggered and a productivity of 1.1 g_{PHB} L^{-1} h^{-1} (50% PHB, w/w) was attained. The homopolymers produced had molecular weights ranging from 7.9 10^5 to 9.6 10^5 Da.

Posada *et al.* [15] also understood the need for development of biorefineries to convert crude glycerol to value added products. In this publication a techno-economical analysis for PHB production from crude glycerol (purities between 88 and 98 wt%) with *C. necator* JMP 134 (DSM 4058) was studied. The fermentation process was carried out in two stages and three downstream processes, to isolate and purify the PHB, were considered. Economical assessment results showed that the most appropriate technological scheme requires purifying the crude glycerol until

[1] Da: Dalton; the unit of weight is the **dalton**, one-twelfth the weight of an atom of C^{12}.

98 wt%, with a downstream process involving heat pretreatment, enzymatic-alkaline digestion, centrifugation, washing, evaporation, and spray drying.

Sangkharak *et al.* [16] studied the potential of using wastewater and crude glycerol (both from biodiesel refinery) for the production of PHB through fermentation of *Cupriavidus necator* TISTR 1095. No cell growth was obtained from biodiesel-wastewater due to the presence of high Na^+ cation concentration. When crude glycerol was used as carbon source, microbial growth and PHB accumulation was observed. The optimal parameters for PHB accumulation were a glycerol concentration of 60 g L^{-1}, an ammonium sulfate concentration of 1.32 g L^{-1} and a trace element concentration of 2.0 g L^{-1} . Under these optimal conditions, the strain *C. necator* TISTR 1095 produced the highest biomass (46.25 ± 2.10 g L^{-1}) and PHB (24.98 ± 1.87 g L^{-1}) concentrations with a PHB content of 54.01% of the DCM. Aeration rate and agitation speed were also optimized at 2 vvm and 150 rpm, respectively, resulting in a slightly increased bacterial mass (46.96 ± 0.28 g L^{-1}) and PHB production (25.32 ± 0.20 g_{PHB} L^{-1}) with a PHB content of 53.92 % (w/w).

Crude glycerol was also examined, by Mothes and co-workers [12], for its possible use as a cheap feedstock in the biotechnological synthesis of poly(3-hydroxybutyrate). The glycerol samples of various manufacturers differ in their contamination with salts (NaCl or K_2SO_4), methanol or fatty acids (see Table 1). At high cell density fermentation, these pollutants could possibly accumulate to inhibiting concentrations. Mothes *et al.* used the bacterial strain *Cupriavidus necator* JMP 134 (DSM 4058), which synthesize PHB up to 70 % of cell dry mass from pure glycerol as well as from glucose or fructose. When crude glycerol containing 5.5 % NaCl (10 g L^{-1}) was used, PHB content was reduced to 48 %, for a bacterial dry mass of 50 g L^{-1}. Nevertheless, the effect of glycerol contaminated with K_2SO_4 was less pronounced and PHB content was reduced only to 58–60 % of cell dry mass. Moreover the negative effect of NaCl containing substrates could be alleviated by mixing with K_2SO_4 contaminated glycerol. The molecular weight of PHB produced with *C. necator* from crude glycerol varies between 620,000 and 750,000 g mol^{-1} which allows the processing by common techniques of the polymer industry.

Table 1. Composition of crude glycerol samples from different manufacturers (Source: [12])

	Glycerol [%]	H_2O [%]	Methanol [%]	NaCl [%]	K_2SO_4 [%]	pH	FA/Glyc*	FAE/Glyc**
ADM	80	10.8	<0.01	5.5	-	5.9	0	0
NEW	85	10.5	<0.01	5.0	-	6.4	0	0.07
BD	82	12.1	<0.50	4.2	-	6.8	0	0
EOP	85	9.2	0.03	-	0.8	4.1	1.11	0.52
Campa	90	6.6	0.50	-	1.6	6.0	0	0
TME	77	14.2	0.0001	-	6.6	5.3	0	0
PetroTech	88	5.3	1.70	-	1.0	4.5	0.04	0

ADM: ADM Hamburg AG; NEW: Natural Energy West GmbH, Marl; BD: Bio-Diesel Wittenberge GmbH; EOP: EOP Biodiesel GmbH, Falkenhagen; Campa: Campa Energy GmbH, Ochsenfurt; TME Methylesterwerke GmbH, Niederpöllnitz; PetroTech GmbH, Südlohn).
Analysis by BSAF Schwarzheide, *) ratio (fatty acid/glycerol) and **) ratio (fatty acid ester/ glycerol), both determined using FTIR spectroscopy.

In feed-batch fermentations, that might be used in the scale up of PHB production, the salts contained in crude glycerol accumulate in the fermentation broth, which may not only inhibit PHB synthesis but also biomass growth. In this case, the inhibitory effect, on the maximum specific growth rate, of NaCl was much stronger than in the case of K_2SO_4 [12].

Bormann *et al.* [17] reported that the bacterial strain *Cupriavidus necator* DSM 11348 can grow and produce PHB in a medium containing a complex nitrogen source such as protein hydrolysates (*i.e.* peptone or amino acids from plants -soya protein-, animals -casein, gelatin, meat- or microbial biomass), and glucose, glycerol or acetic acid as carbon source. This strain is a mutant of *Cupriavidus necator* DSM 531 (ATCC 17697) which is a glucose consuming strain that produces P(3HB-co-3HV) when cultivated together with propionic or other organic acids under aerobic conditions [18]. Continuous cultivation of *C. necator* DSM 11348 on glycerol as the sole carbon source, in 500 mL flask, 1.5 and 2.5 L reactor, obtained a maximun cell dry weight of 27 g L^{-1} and a PHB content of 65%. It was also founded out that PHB production by this recombinant strain is growth-associated [17].

Bormann *et al.* [13] used the same strain of *C. necator* DSM 11348 to produce polyhydroxybutyrate (PHB) using media which contained glycerol and casein hydrolysates as carbon and nitrogen sources. A cell dry mass of 32 g CDW L^{-1} (average) was achieved, containing 47% PHB, in a

casein peptone medium. When casamino-acids were used as nitrogen source, the maximum cell dry weight achieved was 27 g L^{-1} with a PHB content of 65% (w/w). The results presented above indicate that hydrolysates from crude protein sources could provide a cheap nutrient supplement for PHB production.

Nevertheless, fermentations carried out in media containing casein peptone and glucose, as nitrogen and carbon source respectively, showed higher PHB accumulation (80% PHB) than those using glycerol as carbon source [19]. Therefore, a further research on fermentation strategy with this bacterial strain and glycerol as carbon source is required.

Eggink *et al.* [5], developed and patented a method for generating a variant of the wild strain *Cupriavidus necator* H16, the glycerol-positive mutant of *C. necator* GE1 (DSM 7237), that is capable of producing PHB with a content of 80%, on a minimal medium containing only glycerol as source of carbon and energy. The wild strain H16 is not a glycerol consuming one, but its continuous cultivation, under the presence of glycerol as sole carbon source, and limiting quantities of nitrogen, oxygen, phosphorus, sulphur, potassium and magnesium, leads to the isolation, after 20 duplication times, of a glycerol consuming mutant able to produce PHB. The glycerol consuming mutant of H16 is suitable for fed-batch fermentations and a rapid growth can be achieve if the medium contains, in addition to glycerol, fatty acids, methylated fatty acids, ethanol, fructose, glucose or acetate. Moreover, simultaneous growth and PHB-production were achieve when the strain DSM 7237 was cultivated in continuous mode, on a medium comprising glycerol and without nutrient limitation during cultivation. A PHB content of 85% under nitrogen limiting conditions was reported, whereas without nutrient limitation a cell dry weight of 35 g L^{-1} with a PHB content of 80% was reached. This strain can also produce copolymers of P(3HB-*co*-3HV) by adding to the cultivation medium valerate and/or propionate during the accumulation phase.

Madden *et al.* [10] cultivated a *C. necator* strain, NCIMB 40529, in a two stage shake-flask culture where glucose and a completed medium (with salts) was used in the first stage and nitrogen limitation was applied in the second stage to promote polymer synthesis. During this second stage, in addition to glucose, a supplementary carbon source was added (including glycerol, 4- and 3- hydroxybutyric acid, lactic acid, acetic acid, poly-ethylene glycol, ethylene glycol, EG-monomethyl ether, 1,4- and 1,3-

propanediol, 1-propanol and 1-butanol). The inclusion of certain supplementary carbon sources resulted generally in a significant decrease in the number-average molecular mass (Mn) of the polymer produced, except for the addition of 3-hydroxybutyric acid and acetic acid. Particulary, the Mn of PHB produced from glycerol was substantially lower than for polymer produced from glucose. When media containing 75% of glycerol were used a cell dry weight of 84 g L^{-1} and a PHB content of 58% were reached, while on a medium containing 67% of glucose and 8% of glycerol media cell dry weight was increased up to 165 g L^{-1} with a PHB content of 69% [10].

Pereira *et al.* [20], studied also the use of *C. necator* strain NCIMB 40529 for the production of the copolymer P(3HB-co-3HV). By alternate substrate feeding of glucose and propionic acid cells containing 71%(w/w) of P(3HB-co-3HV) with a 3HV content of 7 mol% were produced.

2.2.2 Other agroindustrial and municipal Wastes

Manufacturers

Cupriavidus necator accumulates poly-3-hydroxybutyrate-co-3-hydroxyvalerate, as a source of carbon and energy, in the presence of propionate and valerate, and under limitation of an essential nutrient [4]. The fraction of 3-hydroxyvalerate in the copolymer, that will determine the degree of flexibility of the thermoplastic, can be regulated through the addition of different amounts of propionic acid in the medium. Propionic acid addition can not exceed 1.5 g L^{-1} thus *C. necator* growth might be inhibited.

Marangoni *et al.* [4] studied two feeding strategies (pH regulation and pulses) of propionic acid in the accumulation of P(3HB-co-3HV) by *C. necator* mutant DSM 545, using whey (lactose content 50 g L^{-1}) as the main substrate, obtained during the manufacture of cheese, together with inverted sugar. Propionic acid was not detected in the analyses, wich indicates that the whole acid was consumed inmediatly after addition by different time-pulses. 19 g of total biomass were obtained (4.5 g L^{-1}) from which 3.93 g were the accumulated polymer with a 3HV content of 37% (1.46 g). The yield of propionic acid in 3-hydroxyvalerate was 0.40 g g^{-1}. The productivity in polymer of this culture reached a value of 0.08 g L^{-1} h^{-1} during the whole culture, raising to 0.17 g L-1 h-1 during the production phase. For the monomer units of 3HV, the maximum value obtained in productivity was 0.014 g L^{-1} h^{-1}. This results suggests the possibility of the existence of precursors for 3HV in whey.

In a previous work, Marangoni *et al.* studied the influence of culture conditions (nitrogen / carbon sources and temperature) on the growth of the same strain *Cupriavidus necator* (mutant DSM 545) in stirred flasks using some low-cost sources: hydrolyzed lactose, inverted sugar and corn steep liquor. The best specific growth rate was obtained when inverted sugar was utilized as the substrate. Two different phases in the assimilation of the carbon source were observed when hydrolyzed lactose was present, suggesting the assimilation first of glucose and then of galactose. To confirm the growth of Cupriavidus necator using galactose as the only carbon source, experiments were carried out and the results showed that this bacterium is able to grow in the presence of this sugar at a growth rate of

0.13 h^{-1} [21].

A large amount of organic solids are discarded from food production and consumption and may be used as carbonaceous raw materials for production of PHAs. Nevertheless, organic solid wastes are usually in complex form that cannot be directly digested and utilized by PHA-producing microbes. Hydrolysis and acidogenesis are the first steps to convert biodegradable solids into short-chain volatile fatty acids that can be further utilized by PHA-producing bacteria.

Food scraps were collected by Du and co-workers [22], from a canteen on a campus and slurred with water to 18% dry solids. The organic matter was decomposed by natural microbes under anaerobic conditions and about 60% solid was converted to fermentative. The four major acids were acetic, propionic, butyric, and lactic acids at concentrations of 6, 2, 27, and 33 g L-1, respectively. Integrating acidogenesis and PHA polymerization, however, posing a big technical challenge. The high acid concentration, because of high metabolic activity of acidogenic microbes, would inhibit the activity of PHA-producing cells. Du and co-workers developed a new strategy based on molecular diffusion that was demonstrated to integrate two microbial subsystems. A membrane barrier kept the two populations separated in different physiological conditions, but let molecules of fatty acids diffuse from acidogenesis to PHA polymerization. An enriched culture of *C. necator* (ATCC 17699/ H16) was successfully maintained, giving a high PHA content in cell mass that was the same as that obtained from pure glucose and organic acids fermentation. The dry cell mass concentration and PHA content reached 22.7 g L^{-1} and 72.6%, respectively. The PHA was a copolymer of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) with 2.8 mole % of hydroxyvalerate [22].

Sugars from biomass derivatives

Using sugars as inexpensive carbon sources, PHB production with high productivity has been achieved in an optimized fermentation process [2]. In spite of such many efforts, PHB production from sugars has an unsolvable disadvantage on a low PHA yield from carbon substrate. The yield of P(3HB) production from glucose is ranging within 0.30–0.40 g of P(3HB) per g-glucose used. In face to the further reduction on the production cost, it is necessary to alter the carbon source from sugar to other

inexpensive one, which allows bacteria to produce PHA with a high yield.

The green biomass is a convenient source of green grass juice as a primary product from bio-refinery processes. Alternative inexpensive complex nitrogen and phosphate sources from agriculture, green grass juice (GGJ) and silage juice (SJ), were added, by Koller and co-workers [23], to cultivation medium in order to investigate their impact on growth of the strain *Cupriavidus necator* (DSM 545) on Glucose solution (50% w/w). The influence of these additives was directly compared with cultivations on defined minimal mineral medium (M) as well as on the same medium supplemented with more expensive complex additives: corn steep liquor (CSL) and casamino acids (CA). The specific composition of each media is reported in Table 2. It turned out that the supplementation with most complex additives results in shortening of lag-phases of bacterial growth and in higher end-concentrations of residual biomass compared with M-medium. Finally, higher volumetric productivities for PHB were achieved. The effect of the inexpensive additive SJ on volumetric productivity was similar to the result for the expensive CA (0.653 vs. 0.619 g L^{-1} h^{-1}). The same was found for the biomass concentration (7.00 vs. 7.44 g L^{-1} respectively).

The best results were obtained for media supplemented with SJ or CA (77.3 and 84.2 % 3-PHB/CDW (%w/w) respectively). The highest specific growth rate was achieved in medium supplemented with CSL (0.19 h-1). The 3-PHB content in biomass differed only slightly and fluctuated from 84.2 (CSL) to 76.7 %w/w (M-medium), values which are typical for this strain. Thus, the PHB content in the biomass was not influenced by the type of complex substrate added.

Table 2. Composition of green grass juice (GGJ), silage juice (SJ), casamino acids (CA) and corn steep liquor (CSL). (Source: [23]).

	GGJ	SJ	CA	CSL
pH value of liquid additive	5.89-5.95	4.26-4.32	6.2	4.12
Dry matter of liquid additive [g L^{-1}]	44.2	19.55	19.55	19.55
Protein content [g L^{-1}]	7.2	6.50	13.9[2]	17
Lactic acid [g L^{-1}]	10.3	57.25	-	7
Acetic acid [g L^{-1}]	0.80	29.80	-	-
Propionic acid [g L^{-1}]	0.00	8.40	-	-
NH_4^+ [g L^{-1}]	0.12	0.38	0.60	0.19
Glucose [g L^{-1}]	3.43	32.50	-	20[20]
Fructose [g L^{-1}]	5.69	39.25	-	-
Lactose [g L^{-1}]	1.29	5.38	-	-
Arabinose [g L^{-1}]	0.93	3.05	-	-
Xylose [g L^{-1}]	0.39	0.00	-	-
Ash/dry matter [%]	27.4	26.20	19.4	16

Forest biomass represents an enormous reservoir of renewable carbon-rich material, which has the potential to be utilized as a feedstock for the production of a wide variety of industrial and commodity products ranging from paper, lumber, and platform chemicals to a variety of fuels and advanced materials, including biodegradable polymers. Globally, approximately 80 billion tons of woody biomass is generated per year, with the production of total plant matter estimated at roughly 180 billion tons annually [24].

Cellulosic biomass is composed of (%): cellulose (42.9), xylan (25.2), arabinan (1.4), acid soluble lignine (6.6), acid insoluble lignine (19.0) and ash (4.9) [25].

Yu and co-workers, reported that sugarcane bagasse's pretreatment in dilute acid solution, under moderately severe conditions, breaks down the protective structure of lignin and hemicellulose, and exposes cellulosic fibers for enzymatic saccharification releasing sugars (mono- and oligosaccharides), volatile organic acids, furfurals and aromatic compounds, such as 4-hydroxybenzoic acid and 1,2-dihydroxybenzene, also called "catechol" or acid soluble lignin. An inhibitory effect of hydrolysates on microbial activity was observed, but it could be effectively relieved by

using a large inoculum, a diluted hydrolysate solution, and a tolerant strain, or a combination of the three. Individual organic substances were first tested, as the sole carbon source in a mineral solution for their utilization and mineralization by *C. necator*[2]. It exhibited good growth under aerobic conditions on short chain organic acids including acetic, lactic, propionic, butyric and valeric acids. It also showed high metabolic activity on glucose and fructose, but poor activity on xylose and arabinose. The strain is lack of hydrolysis enzymes for utilization of oligosaccharides. Except for propanol on which the cells grew very well, other alcohols such as ethanol and butanol were not good substrates. The strain showed good activity on phenol and benzoic acid, two representatives of the potential aromatic inhibitors generated in biomass pretreatment.

Although xylose and other pentoses are not good substrates of *C. necator*, their derivative furfural is a good substrate. Since furfural is an aldehyde (furan-2-carboxaldehyde) derived mainly from xylose, *C. necator* may also be able to use other poor substrate sugars such as arabinose after they are converted to aldehydes.

PHA biosynthesis, according to Yu and Stahl, is more sensitive to the inhibition of hydrolysates than the cell growth. Hydrolysates were, therefore, removed and utilized more favorably by cell growth than through PHA biosynthesis. A high PHA content of 65% w/w could be achieved with a high carbon to nitrogen ratio (C/N $\geq$ 20). P(3HB) was the predominant biopolyester formed on the hydrolysates, but in the presence of short chain organic acids with odd carbon numbers, such as propionic and valeric acids, a copolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), is formed. *C. necator* can also produce other PHA copolymers on hydrolysates such as levulinic acid, a precursor of 4-hydroxyvalerate (4HV) [25].

Xylose is the most abundant sugar in the hemicellulose of hardwoods and crop residues, and the efficient production of PHA from xylose reduces production cost. Some bacteria can produce PHA from xylose, however, high production rates and yields have not been achieved. A lactic acid bacterium *Lactococcus lactis* IO-1, is capable of efficiently fermenting xylose into L-lactic acid and acetic acid at a high production rate. Fermentative production of poly-3-hydroxybutyrate from a mixture of Lactic acid and Acetic acid by *Cupriavidus necator* (DSM 11348) was

[2] Strain not reported.

investigated by Tsuge and co-workers [26].

For fed-batch culture with high cell density, it is necessary to control the concentration of these organic acids in the culture medium below the inhibitory level for cell growth. A novel feeding method was developed by Tsuge and co-workers [26] using the rate of increase of the pH of the culture medium as an indicator for feed control. The pH-increasing rate, represented secondary information regarding substrate consumption by cells. When the pH-increasing rate decreased to 5% of the maximum increasing rate, acidic substrate solution was fed into the fermentor until the pH was reduced to 7.00. Using this feeding strategy, the cell dry weight concentration and PHA content obtained in 42 h were 75.0 g L^{-1} and 73.1% (w/w), respectively, resulting in a high P(3HB) productivity of 1.30 g L^{-1} h^{-1}. The consumption rate of acetate was significantly lower than that of L-lactate: specific consumption rates were 2.88 10^{-3} mol g^{-1} $cells^{-1}h^{-1}$ for acetate, and 6.32 10^{-3} mol g^{-1} $cells^{-1}h^{-1}$ for L-lactate. It appears that the maintenance of the organic acid concentration at a low level in the early cultivation phase is a key factor for successful fermentation. The cells consumed mainly L-lactate in the culture medium, and consumed hardly any acetate. Therefore, acetate accumulated in culture medium resulting in the inhibition of cell growth.

The influence of levulinic acid (LA) on the production of copolyester consisting of 3-hydroxybutyrate and 3-hydroxyvalerate by *Cupriavidus necator* (KHB-8862) was investigated by Chung and co-workers [27]. Addition of LA into the culture medium greatly increased the molar fraction of 3HV in the copolyester, indicating that LA can be utilized as a precursor of 3HV. In shake flask culture, the 3HV content in the copolyester increased from 7 to 75 mol% by adding 0.5 to 4.0 g L^{-1} LA to the medium containing fructose syrup as a main carbon source. A maximal copolyester concentration of 3.6 g L^{-1} (69% CDW) was achieved with a 3HV content of 40 mol% in a fermentor culture containing 4.0 g L^{-1} of LA. When LA was added repeatedly into a fermentor culture to maintain its concentration at a low level, the copolyester content and the 3HV yield from LA reached up to 85% of dry cell weight and 5.0 g g^{-1}, respectively, which were significantly higher than those when the same concentration of the LA was supplied all at once. The present results indicated that LA is more effective than propionate or valerate as a cosubstrate for the production of

copolyesters with varying molar fractions of 3HV by *C. necator*.

The use of levulinic acid (derivated from forest-based feedstocks) as a cosubstrate in PHA fermentations has also been shown to exhibit growth and PHA enhancing effects in shake-Flask cultures of *W. Eutropha* KHB-8862, with 2% fructose as primary carbon source and 0.4 % levulinic acid: 66 % PHA (w/w), with a P(HB-HV) yield of 2,0 g L^{-1} were achieved by keenan and co-workers [24].

A process for the large-scale production of 4-hydroxyvalerate (4HV)-containing biopolyesters with a new monomer composition was developed by means of high-cell-density cultivation applying recombinant strains of *Pseudomonas putida* and *Cupriavidus necator* (H16 PHB-4), harboring the PHA-biosynthesis genes phaC and phaE of *Thiocapsa pfennigii*, by Gorenglo and co-workers [28]. PHA content of 60.3 (% CDW) were obtained by 50 ml Erlenmeyer flask cultivation process using levulinic acid for the accumulation of 4HV-containing polyesters. Besides 4HV the polyester contained (see Table 3) significant amounts of both 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV).

Table 3. Composition of PHAs accumulated by recombinant strains of *C. necator* HF39 after growth on levulinic acid, (Source: [28])

Strain	PHA Content (% CDM)	Composition of PHA (mol %)			
		3HB	3HV	4HV	$3HH_x$
Cupriavidus necator					
HF 39	66.2	46.4	51.5	2.1	n.d.
H16 PHB-4 (pHP1014::E156)	60.3	73.6	22.6	3.8	n.d.
H16 PHB-4 (pHP1014::B28)	57.5	85.5	13.0	1.5	n.d.

3HB: 3-hydroxybutyric acid; 3HV: 3-hydroxyvaleric acid; 4HV: 4-hydroxyvaleric acid; 3HHx: 3-hydroxyhexanoic acid.

2.3 PHAs Production from Oils

Plant oils or their derived fatty acids are reasonable carbon sources for microbial PHA production since they are inexpensive renewable carbon sources (about 0.3 US$/kg plant oil). In addition, the theoretical yield coefficient of PHA from plant oil and fatty acid (0.65–0.98 g-PHA/g-butyric acid) is, since they compose a much higher number of carbon atoms per weight, considerably higher than that from glucose (0.32–0.48 g-PHA/g-glucose).

However, the relatively low growth rate of available PHA-producing bacteria seems to be one major problem when plant oils or fatty acids are used as carbon sources. Even if a bacterium that possesses the high cell growth rate on plant oils or fatty acids is employed for PHA production, the PHA content in the dry cells is relatively low [2]. Nevertheless, some authors have studied PHA production, using the bacterium *Cupriavidus necator* and different sorts of oil, with relatively successfully results.

Ikarashi and Kimura studied the effective production of the copolymer P(3HB-co-3HV) with ca. 10 mol% 3HV units from the mixture of plant oils and levulinic acid as renewable resources by a one-step batch cultivation of *Cupriavidus necator* (strain not reported). As a result, when used the concentration of 6 g L^{-1} of safflower oil and 4 g L^{-1} of levulinic acid, the maximum values of P(3HB-co-3HV) yield and content, in dry cell

weight, reached 5.7 g L^{-1} and 69 wt % respectively at 36h. At this time, the productivity of P(3HB-co-3HV) was 0.16 g L^{-1} h^{-1}, which was about three times higher than that of P(3HB) on l% (w/v) fructose by a one-step batch cultivation of *C. necator*, and 3HV mol fraction was obtained at about 8 mol % [29].

Fukui and Doi also investigated the ability of *Cupriavidus necator* to utilize plant oils. The wild-type strain H16 (ATCC 17699) was cultivated at 30 °C in a nitrogen-limited mineral salt medium containing 1.0 % olive oil. Both, cell growth and PHA accumulation of H16, clearly occurred in the presence of olive oil. The cells reached the stationary growth phase after 12 h cultivation, and then rapidly accumulated P(3HB) homopolymer. After 72 h cultivation, 4.3 g L^{-1} dried cell mass, containing 79% (w/w) P(3HB) homopolymer as a percentage of its cell dry weight, was obtained. The P(3HB) productivity on olive oil was calculated to be 0.077 g L^{-1} h^{-1} (2.7 g L^{-1} for 35 h), which was higher than the 0.056 g L^{-1} h^{-1} (2.0 g L^{-1} for 36 h) obtained on 1% (w/v) fructose in a one-step batch cultivation of the same strain [30].

Table 4 summarizes the results of PHA production by *A. eutrophus* H16 from olive oil, corn oil, palm oil, or oleic acid as a carbon substrate. The wild-type strain H16 grew well on these renewable carbon resources (3.6±4.3 g dry cells L^{-1}), and effectively accumulated P(3HB) homopolymer with a high content of 79-82% (w/w).

Table 4. Production of polyhydroxyalkanoates (PHA) from various plant oils or oleic acid (1.0%) by *A. Eutrophus* H16 for 72 h at 30 °C, (Source: [30])

A. eutrophus strain	Carbon source	Cell dry weight (g L^{-1})	PHA content (wt %)	Composition (mol%)	
				3HB	3HHx
H16	Olive oil	4.3	79	100	0
	Corn oil	3.6	81	100	0
	Palm oil	4.1	79	100	0
	Oleic acid	4.1	82	100	0

Kek and co-workers, used palm oil by-products such as palm acid oil (PAO) and palm kernel acid oil (PKAO), as the sole carbon source, to produce PHB from the same strain, *Cupriavidus necator* H16 [31]. It was

found that PKAO is superior with respect to higher amount of P(3HB) accumulation in 48 h (85 wt% of the dry cell mass), and that the nitrogen concentration in the culture medium is very crucial in promoting the biosynthesis of P(3HB). The highest P(3HB) yield (3.1 g L^{-1}) was initiated at 4 mmol L^{-1} of urea. Less than 0.4 g L^{-1} of P(3HB) was obtained when 30.0 mmol L^{-1} or higher urea concentrations were used.

Kahar *et al.* [2], determined the favorable concentration of soybean oil for cell growth in the same strain (*A.eutrophus* H16). A high specific growth rate could be obtained when the concentration of soybean oil in culture broth remained at around 20 g L^{-1}. The cell concentration must be high at the beginning of PHA accumulation phase so as to obtain the highest PHA concentration. Since enough nitrogen sources may be required to obtain a high cell concentration and in fact that nitrogen limitation could not increase the cultivation performance when plant oils were used, it was not employed, but phosphorus limitation occurred after 48 h and resulted in high PHA accumulation. After 96 h cultivation, 126 g L^{-1} of dry cells weight, containing 76% (w/w) of P(3HB) homopolymer, was obtained. The yield of P(3HB) was as high as 0.76 g g^{-1}-soybean oil used. Figure 1 shows the evolution of the most important parameters during this experiment with the time. It's very important to notice the production of lipase enzymes by the cells during the fermentation process.

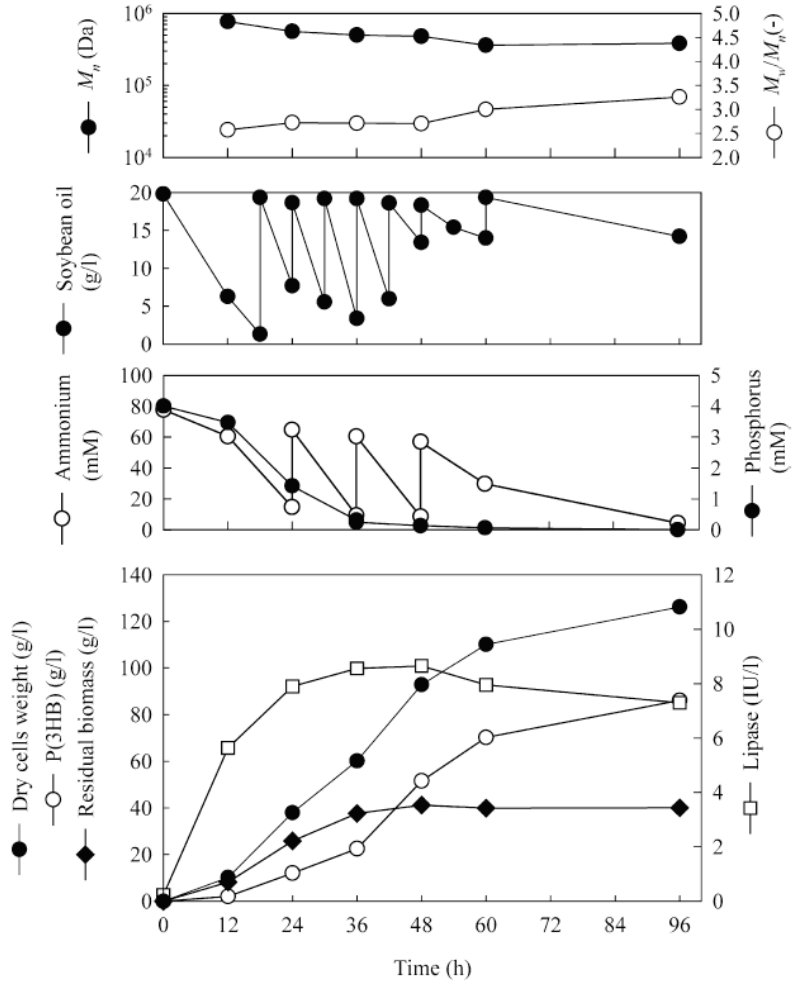

Figure 1. Production of P(3HB) homopolymer by *C. necator* H16 from soybean oil (10 L fermentor, 30 ºC) (Source: [2])

The biosynthesis of PHAs from waste edible oils and tallow by *Cupriavidus necator* (strain not reported) was investigated by Taguchi *et al*. [32]. Waste plant oils as well as waste tallow were assimilated and successfully converted to PHA with relatively high yield by the bacterial fermentation. The waste plant oils usually gave PHB, while waste tallow gave p(3HB-co- 3HV). The ratio of 3-hydroxyvalerate (3HV) unit in the copolyester was controlled by the addition of sodium propionate to the

cultivation medium. The ratio of PHA accumulated was up to 80% of the cell dry weight.

Saeed *et al*., studied the use of saponified soybean, vernonia, and "spent" frying oils, for the biosynthesis of PHAs by *Cupriavidus necator* (strain not reported). Analytical results revealed that the PHAs produced from saponified vernonia and soybean oils were copolymers of hydroxybutyrate and hydroxyvalerate (1% HV units), whereas the saponified "spent" frying oil produced only poly(β-hydroxybutyrate) homopolymer [33].

Loo *et al*., tested four types of palm oil products for the synthesis of P(3HB-co-3HHx) by *W. Eutropha* mutant PHB_4/pBBREE32d13 (DSM 541): palm kernel oil, palm olein, crude palm oil and palm acid oil. This recombinant strain incorporated 3HHx to form a copolymer of 95 mol % 3HB and 5 mol % 3HHx. The molar fractions of 3HHx remained constant regardless of the type and source of palm oil used [34]. The PHA content of the cells was up to 87% (w/w) when palm kernel oil was used as substrate. Palm olein, crude palm oil and palm acid oil produced lesser amounts of PHA contents. These differences in PHA production could be because palm kernel oil and palm oil differ greatly in both, fatty acid composition and characteristics. Palm kernel oil is rich in saturated fatty acids such as C12:0 and C14:0 while palm oil is rich in unsaturated fatty acids such as C18:1, C18:2 and trace quantities of C18:3. Recently, it was reported that *W. Eutropha* cells utilize C18:3 poorly compared to C16:0, C18:1 and C18:2. The concentration of palm kernel oil affected cell biomass and PHA synthesis. Cell biomass and PHA content were highest when 5 g L^{-1} palm kernel oil was used (4.3 g L^{-1} and 87% (w/w) respectively). They decreased by higher/lower concentrations of the oil.

Although palm kernel oil is an excellent carbon source for PHA production, is more expensive than other palm oil products. Byproducts of the palm oil industry such as palm acid oil that is rich in free fatty acids are especially interesting as a cheap carbon source for PHA production.

Fukui and Doi [30], demonstrated that, although cell growth rate was slightly lower than that of the wild-type strain (H16), the recombinant strain DSM 541 also grew well on olive oil and synthesized PHA during its stationary phase. A yield of 3.5 g L^{-1} dry cells and 76% (w/w) PHA were

obtained after 72 h cultivation.

Table 5 summarizes the results of PHA production by *A. eutrophus* DSM 541 from olive oil, corn oil, palm oil, or oleic acid as carbon substrates. P(3HB-co-3HHx) copolymer could be produced by this recombinant strain with a high PHA content of from 76-81% (w/w). Thus, 3.5-3.6 g dry cells L^{-1}, containing approximately 80% (w/w) P(3HB-co-3HHx), could be obtained, and the 3HHx composition was constant and within 4-5 mol%, whatever the structure of the triglycerides fed. The results indicate that plant oils are good carbon sources to produce P(3HB-co-3HHx) copolymer consisting of a low mole fraction of 3HHx units within cells of the recombinant strain DSM 541 of A. eutrophus.

Table 5. Production of PHAs from various plant oils or oleic acid (1.0%) by *A. eutrophus* mutant DSM 541, for 72 h at 30 °C (Source: [30])

A. eutrophus strain	Carbon source	Cell dry weight (g L^{-1})	PHA content (wt%)	Composition (mol%)	
				3 HB	3HHx
PHB$^-$ 4/pJRDEE32d13 (DSM 541)	Olive oil	3.5	76	96	4
	Corn oil	3.6	77	95	5
	Palm oil	3.6	81	96	4
	Oleic acid	2.2	70	96	4

The time profile of PHA production using soybean oil as a sole carbon source by the same recombinant strain DSM 541, for Kahar and co-workers [2] is shown in Figure 2. Similar to the wild strain H16, PHA was rapidly accumulated within the cells after phosphorous limitation at 48 h cultivation. As a result, 138 g L^{-1} of dry cells weight and 74% (w/w) of PHA content were obtained after 96 h cultivation. The yield of PHA was similar to the strain H16 as high as 0.72 g per g-soybean oil used. Gas Chromatograph (GC) analysis revealed that P(3HB-co-3HHx) copolymer was accumulated in the cells grown on soybean oil, and that 5 mol% 3HHx was incorporated into polyester after 96 h cultivation. Mole fraction of 3HHx in the copolyester produced by recombinant strain DSM 541 from soybean oil decreased from 10 to 5 mol% with time. Thus, the 3HHx fraction in copolymer was dependent on the cultivation time and the cultivation strategy. Lipase enzyme production was also achieved by this

recombinant strain, as well as happened by the wild strain H16.

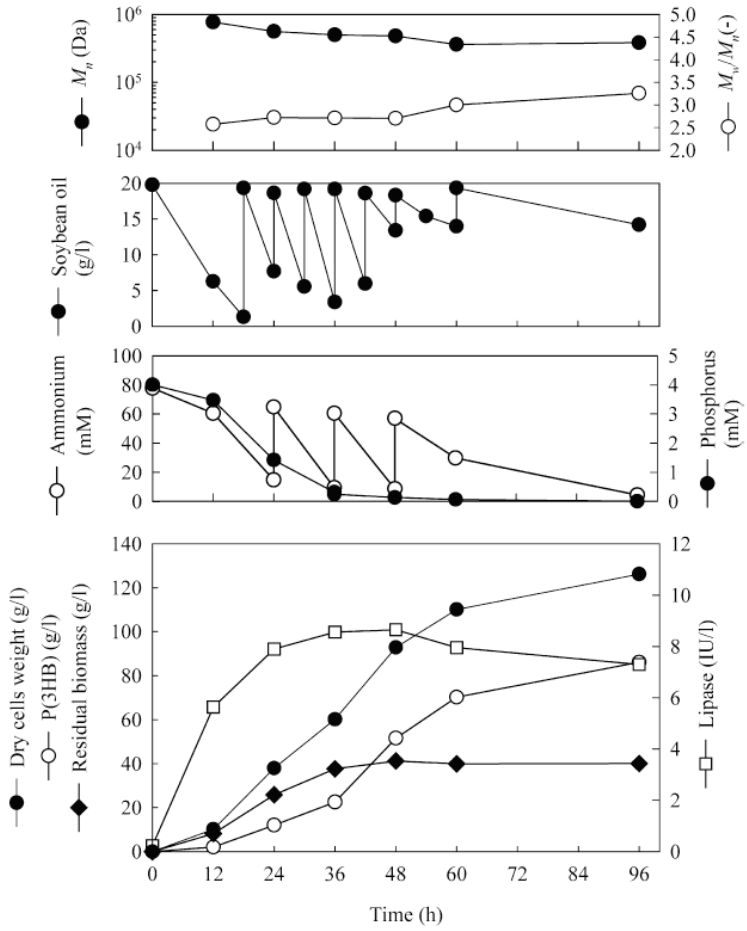

Figure 2. Production of P(3HB-co-3HHx) copolymer by *C. necator* DSM 541 from soybean oil (10 L fermentor, 30 ºC) (Source: [2])

A new strategy for bacterial PHA production by recombinant *Cupriavidus necator* DSM 541 harboring mutated PHA synthase genes (phaCAc) from *Aeromonas caviae* was investigated by Tsuge and co-workers. This strain, harboring wild-type phaCAc gene, produced a PHA copolymer consisting of P(3HB-co-3HHx) with 3.5 mol% of 3HHx fraction

from soybean oil. But when the mutants of phaCAc gene were applied to this production system, 3HHx fraction in copolymers was varied in the range of 0–5.1 mol-% [35].

Tsuge , Kikkawa *et al*., also demonstrated that the use of polyhydroxyalkanoate (PHA) synthase (PhaC) mutants having an amino acid substitutions is able to vary the copolymer composition of P(3HB-co-3HHx) synthesized from soybean oil by the recombinant bacteria [36].

2.4 PHA production from Organic Acids

Organic acids (butyrate, lactate, propionate and acetate) obtained from agricultural waste materials, industrial wastes, food wastes after anaerobic digestion, etc. Might be various of the potential feedstocks that could be used as carbon sources to produce short-chain-length polyhydroxyalkanoates (scl-PHAs).

Since acids are inhibitory to cell growth of microorganisms, a relative low initial concentration of acids in the media or slow feeding at a controlled speed is required.

Yan *et al*. [37], investigated the formation of scl-PHAs during the dual-nutrient-limitation[3] (carbon and nitrogen sources) zone by *Cupriavidus necator* ATTC 17699 / H16, with mixed organic acids and ammonium sulphate used as carbon and nitrogen source, respectively. When the feeding rate of carbon sources was low, feeding was not sufficient for cell growth. At the same time, if excess carbon sources were fed, the higher concentration of residual acids would prevent bacterial growth. Only when the carbon and nitrogen sources were fed at appropriate rates, could the length of dual-nutrient-limitation zone be as long as possible, since most of the nutrients fed were almost all consumed. Moreover, yield of scl-PHAs

[3] Dual-nutrient-limitation means to limit two nutrient factors (usually are carbon and nitrogen sources) simultaneously by controlling the feeding rates of these two kinds of nutrients.

would increase with the length increase of the dual-nutrient-limitation zone, since the limitation of one or two nutrients would lead to the scl-PHAs production. Two dual-nutrient-limitation methods were applied to feed the cultures: feed mixed acids to the fermentation culture, which initially only contained nitrogen sources and no carbon sources (DCW 8 g L^{-1} and scl-PHAs 3.72 g L^{-1}) or feed ammonium sulphate to the fermentation culture, which initially contained only carbon sources and no nitrogen source (46% lower than the first method).

Biosynthesis of scl-PHAs under unbalanced growth condition of either carbon or nitrogen source limitation, both depend on the excess of acetyl-CoA, which could enter the scl-PHAs biosynthesis pathway as the precursor of the HB or HV component of the polymer [37].

The effects of propionic acid feeding strategy on production of P(3HB-co-3HV) were studied by Du *et al.* [38], with *Cupriavidus necator* H16. Flask culture revealed that the time and concentration of propionic acid addition had significant effects on cell growth, copolymer synthesis, and HV fraction in it. In fed-batch culture, a low ratio of propionic acid to glucose (P/G) led to high dry cell weight, P(3HB-co-3HV) content and productivity, but low HV unit fraction. A high P/G ratio led to, on the other hand, to high HV unit fraction but low copolymer content and productivity. The specific P(3HB-co-3HV) synthetic rate and the specific HV synthetic rate declined in fed-batch cultures, which deteriorated with high P/G feeding due to the inhibitory effect of propionic acid accumulated in the culture broth. An optimal feeding strategy to control the propionic acid accumulation was developed, in witch the propionic acid feeding rate was reduced with time. Cell dry mass reached 52.1 g L−1, copolymer concentration and content 40.8 g L−1 and 78.3% respectively, HV fraction in copolymer 16.2 mol% and P(3HB-co-3HV) productivity was 0.74 g L−1 h−1.

Eggink *et al.* [39] reported an analysis of the potential use of long-chain fatty acids (LCFAs) as nutrients for PHB fermentations. It was found that the PHB yield of *A. eutrophus* (strain not reported) on oleic acid, as sole carbon source, is twice as high as the yield with carbohydrates. The use of LCFAs entails specific problems in fermentations, such as a high oxygen requirement and insufficient mass transfer because of the system, which is composed of two liquid phases. If the oleic acid concentration is maintained at 0.5% (w/ vol), 50 g L^{-1} of CDW comprising 65% PHB can be obtained. It

was also shown that poly(3-hydroxybutyrate-co-3-hydroxy-valerate) is synthesized when a mixture of oleic acid and nonanoic acid is supplied as feedstock.

Fukui and Doi used the *C. necator* mutant strain DSM 541 for their studies and reported that this recombinant strain, PHB-4/pJRDEE32d13, had a high ability to accumulate P(3HB-co-3HHx) on medium-chain-length alkanoic acids, and that 96% (w/w) of the P(3HB-co-3HHx) content within dry cells was achieved when this strain was cultivated on octanoate as the sole carbon source [40]

2.5 PHAs Production from Amino Acids

The biosynthesis of polyesters from different L-amino acids was investigated for wild-type *Cupriavidus necator* H16 by Kimura et al [41]. The accumulation and degradation of the polyesters was found to occur when C. necator was cultivated on nitrogen-poor medium containing 1% (w/v) of each L-amino acid as the sole carbon source by two-step batch fermentation (see Table 6).

L-valine was best among different L-amino acids used for the polyester productivity (polyester content up to 36 wt % of CDW, 90 mol% of (3HB) and 10 mol% of (3HV) in a two-step fermentation.

As shown in Table 6, polyester productivities from most of the L-amino acids used as a carbon source were higher at a culture time of 12 h rather than 24 h, and the essential amino acids, such as L-valine, L-phenylalanine, L-leucine, L-isoleucine, and L-methionine, among different L-amino acids, were comparatively effective for polyester accumulation.

Table 6. Biosynthesis of Polyesters from Different L-Amino Acids (1%, w/v) for 12 h and 24 h at pH 7.2 and 30 C by *C. necator* under Two-Step Fermentation (Source: [41])

L_Amino acid	Cultivation time	Dry cell weight	Polyester content[a]	Composition / mol%[b]	
10 g dm^{-3}	h	g dm^{-3}	wt %	3HB	3HV
Val	12	4.19	23.75	90	10
	24	5.35	32.02	92	8
Phe	12	5.94	22.10	100	0
	24	7.55	14.90	100	0
Leu	12	8.00	16.12	100	0
	24	8.18	8.11	100	0
Ile	12	7.63	10.13	98	2
	24	6.37	5.32	97	3
Met	12	2.59	6.83	97	3
	24	2.47	3.81	99	1
Lys	12	2.17	1.90	100	0
	24	2.07	2.52	100	0
Trp	12	4.13	2.33	100	0
	24	6.52	0.11	-	-
Thr	12	4.35	2.90	99	1
	24	5.02	0.01	-	-
Tyr	12	5.91	7.92	100	0
	24	5.84	1.97	100	0
Gly	12	1.83	2.13	99	1
	24	2.56	0.93	-	-
Ser	12	4.37	2.47	100	0
	24	3.52	0.17	-	-
Ala	12	4.81	0.44	100	0
	24	4.20	0.19	-	-
Cys	12	10.20	0.42	100	0
	24	9.65	0.45	99	1
Pro	12	4.66	2.84	100	0
	24	6.32	0.11		
Gln	12	5.61	0.16	-	-
	24	4.47	0.00	-	-
Asn	12	4.46	0.00	-	-
	24	4.15	0.00	-	-
Glu	12	5.20	3.46	99	1
	24	4.69	0.09	-	-
Asp	12	4.46	0.00	-	-
	24	4.84	0.06	-	-
Arg	12	2.22	1.53	100	0
	24	2.40	1.00	100	0
His	12	5.50	0.29	99	1
	24	5.04	0.00	-	-

a) Polyester content in dry cell weights. B) Determined by ^{1}HNMR

Bormann and co-workers [19], reported PHB accumulation and productivities, for the *Cupriavidus necator* strain DSM 11348, using glucose and casein hydrolysates as carbon sources. The biopolymers synthesized were almost pure PHB (PHV content was less than 0.2%).

Different initial concentrations of glucose in the seed cultures had no evident effects on PHB accumulation. The highest yields of PHB were achieved in media supplemented with 30 g peptone L^{-1}(52.2 g L^{-1} of PHB).

However, the absence of trace elements or the use of casamino acids, instead of peptone, resulted in only moderate PHB yields (28.3 and 25.3 g L^{-1} respectively). During the scaling up of the fermentation to the 300 L-pilot plant, PHB concentrations achieved the level of 38.4 g L^{-1} of PHB (0.98 g L^{-1} h^{-1}).

The main conclusion of this experiments was that protein hydrolysates consisting of amino acids and/or oligopeptides might be used as sources of nitrogen at concentrations of about 30 g L^{-1} by *C. necator* DSM 11348 instead of lower concentrations.

2.6 PHAs Production from Toxic Compounds

Even though toxic substrates have been considered unsuitable for PHB synthesis, a single-stage continuous process for producing PHB from toxic substrates using microorganisms was developed by Maskow and Babel [42], with the aim of developing large-scale biotechnological process.

Batch processes are unsuitable for this purpose because high stationary concentrations impair and intoxicate the cells. In carbon substrate limited chemostats the steady-state concentrations of carbon sources are usually low and as a result PHB accumulation does not occur.

C. necator DSM 4058/JMP 134, was grown continuously on phenol as sole source of carbon and energy. In an isothermal heat flux calorimeter with power compensation. The maximum heat flux during continuous growth and the maximum yield of PHB versus the substrate consumption rate were found to coincide. This suggests the possibility of controlling the conversion of a growth-inhibiting substrate into PHB and maximizing the process efficiency. The observed correlation occurred irrespective of the substrates investigated (phenol or sodium benzoate), the PHB-producing strain (*Cupriavidus necator* JMP 134 and *Variovorax paradoxus* JMP 116), or the type of limitation imposed. The maximum PHB yields obtained comprised up to 50% of cell dry mass.

Simon and co-workers[43], reported the relationship between the levels of intracellular NADH, as well as NADPH and cellular fluorescence, for the same strain of *R.eutropha* DSM 4058. The data were obtained from aerobic cultures of the strain growing chemostatically on phenol, phenol + sodium formate and fructose, as well as from aerobic/anaerobic transitions and substrate pulse experiments. The results obtained corroborate the capacity of this strain to grow on toxic substrates.

Also Volova *et al.* [3], studied the effect of carbon supply on polyhydroxyalkanoate synthesis by *C. necator* H 16 and B 5786. Synthesis of multicomponent PHA composed of short- and long-chain monomers (C4–C8) under mixotrophic conditions (CO2 + alkanoic acids as cosubstrates) was carried out. The PHA composition was shown to be dependent on the cosubstrate type:

- ✓ In the presence of odd fatty acids, four- and five-component polymers were synthesized; hydroxybutyrate, hydroxyvalerate, and hydroxyheptanoate were the major monomers, while hydroxyhexanoate and hydroxyoctanoate were minor.

- ✓ In the presence of even fatty acids, PHA contained not only the corresponding molecules (hydroxyhexanoate and hydroxyoctanoate), but also hydroxyvalerate; synthesis of four-component PHA which contain mainly hydroxybutyrate and hydroxyhexanoate (up to 18 mol %) is therefore possible.

Under autotrophic growth conditions, nitrogen limitation and a C1 substrate (CO_2), the biomass and the polymer yield of both strains were practically identical: after 48-h cultivation of strains H16 and B5786, the biomass yield was 5.8 and 6.1 g L^{-1}; final polymer concentrations were 63.0 and 61.4%, respectively. The polymer synthesized by both strains consisted mostly of HB (96.6–99.2 mol %); HV (0.6–2.8 mol %) and HH (0.2–0.7 mol %) were detected as minor components.

Introduction of valerate (C5) into 12-h cultures (biomass, 2.7–2.8 g l-1; polymer content, 22%) resulted in an increased biomass yield (to 4.1–4.8 g L^{-1}) and polymer yield (to 48.9–63.8%) for both strains. The polymer

content in the cells by the end of the experiment exceeded 90%. Both strains synthesized four- and five-component polymers.

When grown on mixtures of CO_2 and enanthic acid (C7), bacteria synthesized polymers containing mostly HV and HB. The introduction of Hhep was insignificant (less than 2 mol %), and that of HH and HO was low and irregular. The composition of the polymer samples did not differ from the composition of the corresponding biomass samples after methanolysis and it varied with the duration of bacterial cultivation. The polymer yield was comparable to that in the above experiment with valerate (over 90%).

In the course of growth on the mixtures of CO_2 and salts of even fatty acids, multicomponent PHA of a different composition were formed. When hexanoate was introduced into the medium, both strains synthesized PHA with HB and long-chain HH as the major components. HO and HV were also detected in the polymer. By the end of the experiment, the polymer content in the cells increased to 70%. The observed increase in the HO fraction may indicate formation of the monomer precursors not only by β-oxidation of fatty acids, but by elongation of their acyl chains. Growth and polymer synthesis resumed at lower octanoate concentrations. By the end of the experiment, PHA concentration in the cells was 70–77% and the biomass yield was 7.8–8.7 g L^{-1}. HV content was significantly higher, Hhep was present, and HO incorporation was decreased.

Under organotrophic growth conditions on fructose, the polymer yield was higher than in the autotrophic mode (over 90% of the dry biomass for both strains). Addition of valerate and hexanoate had no effect on this value. By the end of the experiment, the polymer content was 88 and 92% for strains B5786 and H16, respectively. Octanoate inhibited polymer biosynthesis, and PHA content in the cells did not exceed 60%. When grown heterotrophically on a mixed substrate (fructose + fatty acid), both strains synthesized a three-component PHA containing HB (over 97%) with minor inclusions of HV and HH. The mode with valerate addition was exceptional in that a three-component PHA was synthesized with up to 26% content of hydroxyvalerate; HH was detected as a minor component [3].

In Table 7 a resume chart of *Cupriavidus necator* PHAs production from different waste streams is shown.

Table 7: Cupriavidus necator PHAs production chart

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
DSM 428 ATCC 17699 / H16 / Stanier 337 / NICB 10442	PHB & PHB-PHV	36% of PHA max	32.02 PHA (wt %) 5.35 DCM[4]	- 2 steps batch fermentation; - Cell growth and accumulation were simultaneous.	L-amino acids: • L-Valine • Others	[41]
DSM 541 H16 PHB-4 Mutant carrying *Aeromonas caviae* PHA synthase gene	P(3HB-co-3HHx)	87% (w/w) of the cell dry weight as P(3HB-co-3HHx) 5% of HHx	4.3 g L^{-1}	n.d.	Palm kernel oil, palm olein, crude palm oil and palm acid oil	[34]
H16 ATCC 17699	PHB	79% (w/w) P3HB, DCM	4.3 g L^{-1} DCW	1 step batch cultivation	Olive oil Corn oil, Palm oil, Oleic acid.	[30]
DSM 541 PHB-4 Mutant	P(3HB-3HHx), 4 mol% 3HHx	76% (w/w) of PHA DCM	3.5 g L^{-1} DCM			
H16 ATCC 17699	PHB	72-76 % (w/w) PHB DCM	DCM 118-126 g L^{-1}	- 0.72-0.76 g PHA / g Soybean oil - 10 L fermentor	Soybean oil & MSM[5]: (g L^{-1} in water): KH_2PO_4, 1.2;$Na_2HPO_4.12H_2O$, 11; NH_4Cl, 16	[2]
DSM 541 PHB-4 Mutant	P(3HB-3HHx), 4-5 mol% 3HHx	71-74% (w/w) Copol. DCM	DCM 128-138 g L^{-1}			

[4] DCM: Dry cell mass;
[5] MSM: Mineral Salts Medium

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
H16 PHB-4 Mutant	4HV, 3HB, 3HV, 3HHx, 3HO	Oct./Lev.: 52% (w/w)	(Cell density) Oct./Lev.: 20 g L^{-1}	2-stage-fed-batch 25 L fermentor	Octanoic acid/ Glucose and Gluconic acid (gp); and levulinic acid (ap)[6].	[28]
H16 ATCC 17699	scl-PHAs	3.72 g PHA L^{-1}	DCM 8 g L^{-1}	5 L fermentor; 3L initial vol. And 10% inoculum. Carbon and nitrogen source were fed by two different peristaltic pumps	Mixed organic acids as carbon source: butyric, propionic, acetic & lactic acid, (3:3:1:1); $(NH_4)_2SO_4$ as nitrogen source.	[37]
H16 ATCC 17699	P(3HB-co-3HV) 2.8 mol % HV	DCM = 22.7 g L^{-1}	72.6 % PHA of DCM	2 Reactors coupled by a organic acids permeable membrane.	Food scraps	[22]
n.d. Direct adaption and evolution of the strain	P(3HB-3HV) – 4HV	60.2 wt %	10.7 g L^{-1} (Cell density)	Flask	Sugarcane Bagase[7]	[25]

[6] gp: Growth phase; ap: Accumulation phase.

[7] Sugarcane Bagage: cellulose, xylan, arabinan, acid soluble lignin, acid insoluble lignin and others, ash / Valerate, propanol, butyrate, benzoate, phenol, lactate, glucose, furfural (der. Xylose); propionate, acetate, butanol, citrate, catechol, etanol and octonate

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
DSM 7237 Mutant, by continuous cultivation from H16	PHB > 99% PHV < 1%	85%PHB from DCM	n.d.	Fed-batch Nutrient Limit (50 mL cultures)	Glycerol from biodiesel industry	[5]
		80%	35 g cell L^{-1}	Continuous fed-batch cultivation, no nutrient limit (2 L fermentor)		
DSM 545	P(3HB-co-4HB) P(3HB-4HB-3HV)	mol%: 11.4-21.5 4HB 24.8-43.6 4HB & 5.6-9.8 3HV	n.d.	n.d.	Crude glycerol from biodiesel industry. Gamma-butyrolactone & propionic acid.	[11]
DSM 545	PHB	1.1 g L^{-1} h^{-1} PHB	50 % PHB of DCM	2 L fermentor, fed-batch	Crude glycerol from the biodiesel industry	[14]
DSM 545 Mutant	P(3HB-co-3HV)	0.40g/g propionic ac to 3HV. 37% of the pol. Was 3HV	0.98 g L^{-1} of P3Hbco3HV. 3.93 g	4 L fermentor	Whey and inverted sugar	[4]
DSM 545 Mutant	P(3HB-co-3HV) 16.2% mol HV	0.74 g PHA L^{-1} h^{-1}	DCM 52.1 g L^{-1} 78.3% PHA 40.8 g PHA L^{-1}	Fed-batch. Propionic acid feeding rate was reduced with time	Propionic acid and Glucose	[38]

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
DSM 545 Mutant	PHB	3PHB (g L^{-1} h^{-1}) GGJ: 0.283 **SJ: 0.653**	%PHB/DCM (w/w) GGJ: 77.2; SJ: 77.3.	2 L bioreactor equipped with three axial propeller stirrers.	Green grass juice (GGJ) and silage juice (SJ) as additional complex substrates for Glucose (50% w/w).	[23]
TISTR 1095	PHB	25.32 g PHB L^{-1}	46.96 $g_{biomass}$ L^{-1} 53.92% CDM	20-L fermentor	Crude glycerol from biodiesel industry	[16]
DSM 11348 ATCC 17697 Mutant of DSM 531	PHB	1.2 g PHB L^{-1}.h^{-1}	80% DCM <0.2 PHV	2.5 L fermentor (2 L working volume)	Casein Peptone & Casamino Acids (Glucose and Casein hydrolysates)	[19]
		0.98 g PHB L^{-1}.h^{-1}	78 % PHB (w/w)	300 L fermentor		
DSM 11348 ATCC 17697 Mutant of DSM 531	PHB	65% PHB from DCM	27 g L^{-1}	Continuous cultivation: 500 mL Flask, 1.5 L reactor and 2.5 L reactor.	Glycerol and protein hydrolysates[8]. Glucose and acetic acid.	[17]
DSM 11348 ATCC 17697 Mutant of DSM 531	PHB	Casein peptone: 47% PHB Casamino acids: 65 % PHB	Casein peptone: 32 DCM L^{-1} Casamino acids: 27 DCM L^{-1}	2.5 L bioreactor (batch)	Glycerol and casein hidrolysates	[13]

[8] Protein Hydrolysates: peptone or amino acids from plants (soya-protein); animals (casein, gelatin, meat) or microbial biomass.

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
DSM 11348 ATCC 17697 Mutant of DSM 531	PHB	1.30 g L^{-1} h^{-1}	75 g PHA L^{-1} 73.1% (w/w)	2-stages fed-batch culture: cell growth & P(3HB) accumulation.	Lactic and acetic acids (from xylose, transformed by *Lactococcus lactis* IO-1).	[26]
NCIMB 40529	P3HB	Glycerol (75%): 58% PHB Glyc + Gluc: 69% PHB	Glycerol (75%): 84 g L^{-1} biomass Glyc + Gluc: 165 g L^{-1} biomass	High density 1.5 L fermentor, fed batch culture.	Glycerol and glucose.	[10]
DSM 4058 JMP 134	PHB	n.d.	57.1 g L^{-1} PHB		Raw glycerol from biodiesel production	[15]
DSM 4058 JMP 134	PHB	n.d.	50% CDM	2-stages cultivation with NH4 limitation in accumulation stage	Fructose, phenol, sodium benzoate (carbon source) and mineral medium	[42]
DSM 4058 JMP 134	n.d.	n.d.	n.d.	n.d.	Phenol; phenol + sodium formate and fructose	[43]
DSM 4058 JMP 134	n.d.	n.d.	n.d.	n.d.	Phenol and 2,4-dichlorophenoxyacetate	[44]
DSM 4058 JMP 134	n.d.	n.d.	n.d.	n.d.	Phenol	[45]

Strain Number	Produces	Yield [g/g]	Content [g L^{-1}]	Fermentation Mode/phases	Raw Material	References
DSM 4058 JMP 134	PHB	Crude glycerol: 65%	50 g L^{-1}	Fed batch (2L fermentor)	Crude glycerol, glucose and fructose. (Biodiesel-salts contamination study)	[12]
H16	PHAs: HB, HV, HH, HO, HHep	(PHAs %) Autotrophic: CO_2: 63.0 % CO_2+C5/C7: >90 % CO_2+C6/C8: 70% Organotrophic: 90%	Autotrophic: CO_2: 5.8 g L^{-1}; CO_2+C8: 7.8 g L^{-1}	Batch culture; 1 L flasks	Autotrophic conditions: H_2-CO_2; Heterotrophic conditions: fructose; Cosubstrate: Salts of fatty acids (valerate, hexanoate, heptanoate, and octanoate); 1st stage: N limit; 2nd stage: N-free medium.	[3]
B5786 Fast-growing variant of Z1		(PHAs %) Autotrophic: CO_2: 61.4 % CO_2+C5/C7: >90% CO_2+C6/C8: 70-77% Organotrophic: 90%	Autotrophic: CO_2: 6.1 g L^{-1}; CO_2+C8: 8.7 g L^{-1};			
KHB-8862	P(3HB-co-3HV)	66% PHA (w/w)	2 g L^{-1}	Shake-Flask	Forest Biomass (Levulinic Acid)	[24]

2.7 References

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Chapter 3

Production of fermentation feedstock from Jerusalem artichoke tubers and its potential for polyhydroxybutyrate synthesis

3.1 Introduction

Jerusalem artichoke (*Helianthus tuberosus*) (JA) is a perennial herbaceous plant of the Asteraceae family. It is mainly cultivated for its tuber across the temperate zone. JA can be cultivated in environments, ranging from saline and alkaline to dry conditions, unsuitable for cultivation of food crops such as wheat, rice and potato. Furthermore, a significant advantage of JA cultivation is the high production yields achieved (40 – 70 tonnes/ha for JA tubers). The tubers of JA contain mainly the fructose-rich polysaccharide inulin at 70 – 80% (w/w) of the total dry weight [1, 2]. The water content of JA tubers is in the range of 75 – 85% [2]. Inulin contains fructose unit chains with length in the range of 3 – 50 linked by β-(2 → 1) D-fructosylfructose bonds that are usually terminated by a single glucose unit linked by an α-D-glucopyranosyl bond [3]. Inulin is enzymatically hydrolysed to fructose and glucose by the combined action of inulinase and invertase [4]. Apart from inulin, JA tubers also contain free reducing sugars, proteins and minerals [5]. Its suitability for cultivation, high biomass yields per hectare, favourable composition and ability to grow under extreme conditions make JA an appropriate renewable crop for producing fructose-rich hydrolysates that can be used as fermentation media. It could be considered as an alternative to glucose-rich crops and biomass that contain starch and cellulose.

JA could be also employed in food-related applications and it has been evaluated for the production of fructose syrup, inulo-oligosaccharides and inulin that has many health benefits. Inulin and its derivatives are on a constantly increasing demand by the food industry [3]. Although chicory is the main crop utilized as raw material for industrial production of inulin, JA could be also used for inulin extraction. The utilisation of JA for food and non-food applications, coupled with its cultivation under conditions unsuitable for cultivation of food crops, offers a significant advantage for industrial implementation due to the flexibility provided to suit the needs of different markets. Therefore, the selection of the most suitable application or combined market alternatives for JA depends on the profitability and market/societal needs for each specific region.

JA can be characterised as an energy crop, but its cultivation imposes important disadvantages that could outweigh its potential as fermentation feedstock [6]. Although JA cultivation is easy, its harvest and storage imposes certain difficulties. JA harvesting is a labour intensive process because after mechanical collection the tubers cannot be easily separated from crown tissue and soil. In addition, the tubers are easily spoiled during storage. These are also important disadvantages that have prevented the widespread utilisation of JA as a source of inulin for food related applications [7]. A solution to this problem could be given by storing JA tubers in the field as they are preserved in a good condition as long as the soil is not too wet [6]. The ability to cultivate JA under desert conditions and frost could outweigh the problems with collection and storage.

There is an imminent need to evaluate various renewable resources (e.g. agricultural crops, non-food crops, residues and waste) as feedstock for the production of chemicals, biopolymers and biofuels via fermentation or green chemical conversion routes. Each region or country should evaluate different renewable resources depending on sustainability issues. JA has been used in previous studies for fermentative production of 2,3-butanediol [8], ethanol [9], single cell protein [1], microbial lipids [10, 11], butanol [12] and lactic acid [13]. In some studies, mixed cultures in simultaneous saccharification and fermentation experiments have been employed for the production of extracellular products (e.g. 2,3-butanediol, lactic acid) from JA tubers [8, 13]. JA extracts have been employed for the production of intracellular products, such as microbial lipids [10, 11]. JA hydrolysis and

fermentation could be achieved either simultaneously or sequentially. The production of intracellular metabolic products prevents the utilisation of the simultaneous process because remaining solids that are not hydrolysed prevent the separation of intracellular products from microbial mass. The simultaneous process is industrially feasible in the case of extracellular metabolic products.

Previous studies utilised JA only as a source of carbon neglecting the presence of other nutrients (e.g. protein, minerals) that are essential in the formulation of nutrient-complete fermentation media. In this study, JA was evaluated not only as a renewable resource for the production of carbon sources (i.e. fructose and glucose) but also for the generation of sources of nitrogen and phosphorus.

The fungal strain *Aspergillus awamori* 2B. 361 U2/1 has been employed extensively at the SATAKE Centre for Grain Process Engineering (SCGPE), in the development of wheat-based biorefineries for the production of succinic acid, bioethanol and polyhydroxybutyrate (PHB) [14-16]. Previous studies have shown that it is a prolific producer of glucoamylase and to a lesser extent protease and phosphatase [17, 18]. This study demonstrates that this strain of *A. awamori* can also produce significant quantities of inulinase and invertase that could be employed in the hydrolysis of inulin. The extent of inulin hydrolysis achieved in this study is among the highest reported in the literature using a single fungal strain.

To evaluate the efficiency of the medium derived from JA, preliminary shake flask fermentations were carried out with *Cupriavidus necator* for the production of polyhydroxybutyrate (PHB). PHB belongs to the family of polyhydroxyalkanoates (PHAs) that are considered as candidates to replace petroleum-derived synthetic plastics due to their structural diversity, biodegradability, biocompatibility and the fact that they can be produced from renewable carbon sources via fermentation [19]. Biosynthesis of PHAs by *C. necator* is carried out as secondary anabolic activity products where cellular growth occurs in the first growth stage that takes place without nutrient limitation (balanced growth phase) and PHAs are accumulated as intracellular storage compounds in the second stage under limiting conditions of a specific nutrient (e.g. N, P) and abundant availability of a carbon source. Recent research on PHB production focus

on the substitution of purified carbon sources (e.g. purified glucose, sucrose and vegetable oils) and commercial nutrient supplements by renewable resources such as wheat, grass and silage residues, whey, molasses, meat and bone meal, various sources of triacylglycerols, lignocellulosic wastes and crude glycerol [20-22]. Some research efforts have concentrated on the utilisation of pure fructose for the production of PHB [23, 24]. However, there is no published record of the utilisation of crude agricultural resources that contain fructose for the production of PHB.

It should be stressed that this study focuses on the presentation of a conceptual bioprocess investigating the potential to produce nutrient-rich fermentation media from JA. The potential of *A. awamori* as microbial factory for JA meal hydrolysis was considered. Preliminary shake flask fermentations were carried out to evaluate JA hydrolysates as fermentation media for PHB production by *C. necator*. Future studies will focus on bioprocess design to assess the commercial potential of this bioprocess.

3.2 Material and methods

3.2.1 Microorganisms

Fungal fermentations were carried out using the strain *Aspergillus awamori* 2B. 361 U2/1. *A. awamori* spores were stored dry in sand at 4°C. Prior to experimental work, spores were purified, sporulated and stored in slopes containing a solid medium consisted of 50 g L^{-1} lyophilized JA meal and 20 g L^{-1} agar. The inoculum employed in SSF contained approximately 106 spores per g of JA on a wet basis (wb).

Submerged bacterial fermentations for PHB production were conducted with *Cupriavidus necator* DSM 4058. Lyophilized bacterial cells were reactivated at 30°C for 24 h in a growth medium containing 10 g L^{-1} fructose, 10 g L^{-1} yeast extract, and 5 g L^{-1} peptone. Bacterial cultures were stored at 4°C in slopes containing the previous growth medium and 20 g L^{-1} agar. Fermentation inocula were prepared by transferring bacteria cells using a wire loop into a shake flask containing 50 mL of the growth medium that were cultivated at 30°C for 20 h.

3.2.2 Jerusalem artichoke tubers

JA tubers were directly purchased from Grenoble (Isère Province,

France). The tubers were washed, cut and lyophilized for 24 h. JA meal was produced by grinding dried JA pieces with a Universal Grinding Mill. JA meal was used as the sole nutrient source for enzyme production by SSF and as the sole substrate for the production of hydrolysates that were subsequently employed for PHB production. The composition of JA tubers is presented in Table 1:

Table 1. Composition of Jerusalem artichoke tubers

Component	Content (%, w/w) *
Total dry matter (wet basis)	22.8 ± 0.81
Total carbohydrate (dry basis)	77.5 ± 2.32
Reducing sugars (dry basis)	3.4 ± 0.13
Inulin (dry basis)	74.1 ± 1.76
Kjeldahl nitrogen (dry basis)	1.4 ± 0.35
Protein (6.25 × Kjeldahl nitrogen)	8.75 ± 0.49
Phosphorus (dry basis)	0.31 ± 0.08

* Data is expressed as mean ± 1 standard error

3.2.3 Solid state fermentations

Prior to each SSF, cultures of *A. awamori* were sporulated on solid medium, consisting of 5% (w/v) JA meal and 2% (w/v) agar, in 20 mL test tubes for 5 days. Subsequently, an aqueous spore suspension was formed by adding 10 mL of sterile tap water with two drops of Tween 80 (0.01% v/v) to each test tube containing *A. awamori* spores. Spore suspensions were used as inocula for SSF, which were carried out in 250 mL shake flasks containing 5 g of dried JA meal as the sole nutrient source. Shake flasks were incubated at 30°C and natural pH. The main objective of the SSF was the production of crude enzyme-rich extracts for subsequent enzymatic

hydrolysis of JA meal.

3.2.4 Production of medium for bacterial fermentation

Prior to the production of medium for bacterial fermentation by enzymatic hydrolysis, inulin extraction was carried out in 1 L Duran bottles containing 40 g L^{-1} of JA dried meal. The conditions used for inulin extraction were taken from Lingyun et al. [7]. A JA aqueous suspension consisting of 11 mL of water per g of lyophilised JA meal was heated to 77°C for 25 min and agitated using a magnetic stirrer.

Enzymatic hydrolysis was carried out by mixing fermented solids from SSF with an aqueous suspension of JA meal that was previously processed for inulin extraction. The total volume of the reaction mixture was 200 mL. The fermented solids mixed with JA meal aqueous suspensions were produced at different SSF times. This means that varying quantities of JA macromolecules were still unconsumed when fermentations were stopped at different times. Especially at early fermentation times, significant quantities of JA macromolecules should be still intact during enzymatic hydrolysis. For this reason the calculation of fructose production yield and inulin hydrolysis yield was based on the total amount of inulin that was present in the total JA meal that was used in both SSF (5 g) and aqueous JA meal suspensions (40 g L^{-1} in 200 mL reaction mixture). Thus, a theoretical maximum reducing sugar equivalent of 53 g L^{-1} can be produced from a total JA meal concentration of 65 g L^{-1} (an inulin to reducing sugar conversion factor of 1.1 and an inulin content of 74.1%, see Table 1, have been assumed).

The pH of the solution during hydrolysis was kept natural. Hydrolytic reactions were carried out in a water bath at 55°C, with constant agitation by a magnetic stirrer. The temperature employed during hydrolytic reactions was based on optimum values reported in the literature for fungal strains belonging to the genus of Aspergillus [25-27]. Hydrolytic reactions were carried out for 48 h.

At the end of the hydrolysis period, remaining solids were separated by vacuum filtration (Whatman No. 1). The fructose- and nutrient-rich

solution was filter sterilized using a 0.2 μm filter unit (POLYCAP 36 AS, Fisher). The pH of the medium was adjusted to the optimal value (6.8) for *C. necator* growth with 10 M KOH.

3.2.5 Bacterial fermentations

Three sets of bacterial fermentations were carried out in 250 mL Erlenmeyer flasks on a 200 rpm rotary shaker (ZHWY-211C Series Floor Model Incubator, PR China) at 30°C and an initial pH of 6.8. Fermentation medium of 40 mL and inoculum of 1 mL were used in each flask. The first set of shake flask fermentations was carried out with commercial sources of nutrient supplements (i.e. 10 g L^{-1} yeast extract and 5 g L^{-1} peptone) employing a constant free amino nitrogen (FAN) concentration (around 600 mg L^{-1}) and different fructose concentrations (10, 20 and 30 g L^{-1}). The other two sets of shake flask fermentations were carried out with JA hydrolysates as fermentation medium. This experiment was carried out in order to compare the efficiency of commercial sources of nutrients with JA hydrolysates on bacterial growth and PHB accumulation.

It was observed that the nutrient concentration in JA hydrolysates was not adequate to promote sufficient microbial growth and PHB formation. For this reason, different amounts of yeast extract were added to the medium in order to enhance the nutrient content of the JA hydrolysates. As a result, the second and third sets of shake flask fermentations were carried out using two initial fructose concentrations (14 and 28 g L^{-1}) and five individual FAN concentrations in the range of 100 – 900 mg L^{-1}.

3.2.6 Analytical methods

Fructose, glucose and sucrose concentrations were analyzed by HPLC (Waters 600E) with an Aminex HPX–87H (300 mm × 7.8 mm, Bio Rad, CA) column coupled to a differential refractometer (RI waters 410). Operating conditions were as follows: sample volume 40 μL; mobile phase 0.005 M H_2SO_4; flow rate 0.6 mL min^{-1}; column temperature 65°C. Before injection, samples were diluted to appropriate concentration with deionized water and filtered through a 0.45 μm membrane filter.

Total carbohydrates were analysed with the modified colorimetric method reported by Mullin et al. [28] using pure inulin (Sigma Aldrich Co.) as standard. Reducing sugars concentration was measured by the dinitrosalicylic acid (DNS) method [29] using fructose as standard. The inulin content was calculated as the difference between total carbohydrate and reducing sugars [3, 7]. FAN concentration was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention [30]. Phosphorus and Kjeldahl nitrogen (KN) in lyophilised JA meal were determined via digestion of solid samples with a DK_6 Digester (Velp Scientifica, Italy) using concentrated H_2SO_4 and 35% H_2O_2. Phosphorus content in the digest and in any liquid sample was measured using the method described by Herbert et al. [31]. KN content was measured using a Kjeltek^{TM} 8,100 distillation Unit (Foss, Denmark). The moisture content in JA meal was analyzed by drying three samples of approximately 1 g pre-weighed JA meal at 105°C until constant weight.

During enzymatic hydrolysis, samples (5 mL) were taken at random intervals. They were centrifuged at 3,000×g for 10 min to separate the supernatant from unhydrolysed solids. The supernatant from hydrolytic reactions was employed for the analysis of fructose, glucose, sucrose, reducing sugars, total carbohydrates, FAN and phosphorous.

Samples (2 mL) from bacterial fermentations were also taken at random intervals. Each sample was centrifuged at 3,000×g for 10 min and the sediment was washed with distilled water and centrifuged twice. The solids were re-suspended in acetone and transferred into 14 mL universal bottles. The total dry weight was measured by drying the solids at 50°C and cooling in a desiccator until constant weight. Residual bacterial cell concentration (non-PHB bacterial mass) in each sample was calculated by substracting the PHB concentration measured by GC analysis from the total dry weight. The supernatant from bacterial fermentations was used for the analysis of fructose, glucose, FAN and phosphorus.

PHB was measured by the chromatographic method proposed by Riis and Mai [32] using benzoic acid as internal standard. A gas chromatographic analyzer Fisons 8060 equipped with a Flame Ionization Detector (FID) and a Chrompack column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was used for PHB analysis. Helium was used as

carrier gas at a flow rate of 2 mL min^{-1}. The injector and detector temperatures were 230°C and the initial temperature was 120°C.

3.2.7 Enzyme activities

The quantification of inulinase activity was based on the hydrolysis of 25 g L^{-1} pure inulin for the production of fructose in 200 mM phosphate buffer at pH 6 and 55°C within 15 min. After each sample was taken, enzymatic hydrolysis was terminated via boiling at 100°C for 10 min. One unit (U) of inulinase activity was based on the amount of enzyme required for the release of 1 mg fructose in 1 min under the reaction conditions mentioned above.

Invertase activity was assayed as the production of glucose (or fructose) during hydrolysis of 25 g L^{-1} sucrose in 200 mM phosphate buffer at pH 6 and 55°C within 10 min. One unit (U) of invertase activity was defined as the amount of enzyme required for the release of 1 µg glucose in 1 min under the reaction conditions mentioned above.

Protease activity was assayed as the production of FAN at 55°C in 30 min using 15 g L^{-1} casein as substrate in 200 mM phosphate buffer. One unit (U) of proteolytic activity was defined as the amount of enzyme required to release 1 µg FAN within 1 min under the reaction conditions mentioned above.

3.3 Results and discussion

3.3.1 Enzymatic hydrolysis of Jerusalem artichoke meal

The development of a JA-based bioprocess is dependent on the efficient enzymatic hydrolysis of macromolecules contained in JA. The production of a nutrient-rich feedstock from JA would require the utilisation of inulinase to hydrolyse inulin into fructose and sucrose, invertase to hydrolyse sucrose into glucose and fructose and protease to hydrolyse protein into directly assimilable amino acids and peptides. The production of these enzymes could be efficiently achieved via SSF eliminating the need to purchase commercial enzymes that increase significantly processing costs. JA meal was employed as substrate in SSF with *A. awamori* because inulin is the most suitable substrate for inulinase production [33].

The fungal strain of *A. awamori* employed in this study has never been evaluated for its ability to hydrolyse JA meal. It has been extensively studied for the production of wheat hydrolysates due to its ability to produce amylolytic (mainly glucoamylase) and proteolytic enzymes[17, 18]. It should be stressed that this study was focused on illustrating the efficiency of this *A. awamori* strain in the production of JA meal hydrolysates that could be subsequently employed as fermentation media. Figure 1 presents kinetics of inulinase, invertase and protease activities produced during SSF of *A. awamori*. The activities of all enzymes increased significantly up to 48

h SSF. After this point, enzyme activities either reached a plateau (invertase) or increased slightly (inulinase and protease).

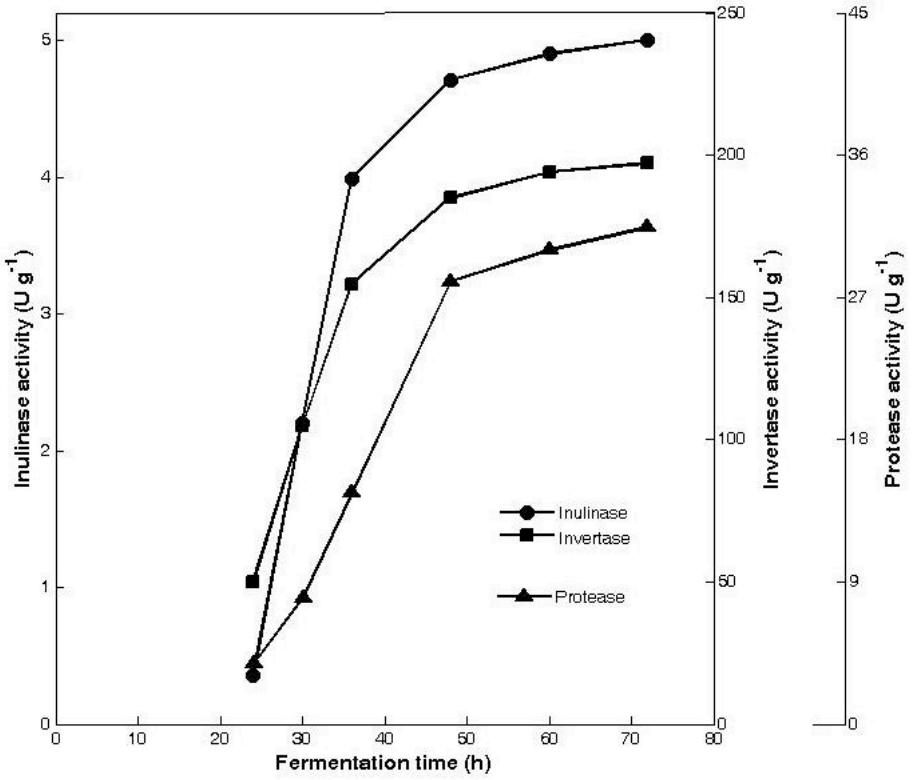

Figure 1. Inulinase, invertase and protease production during SSF *of A. awamori* on JA meal

In the first set of SSF experiments, the main target was the identification of the optimum duration of SSF that resulted in the production of JA hydrolysates containing the highest amount of nutrients. The efficiency of hydrolysis was evaluated through the determination of inulin and protein hydrolysis products. Fermented solids taken at five different SSF times (24, 36, 48, 60 and 72 h) were employed in hydrolytic reactions of JA meal. It is important to mention that both SSF experiments and enzymatic hydrolysis experiments were carried out two to three times in order to verify the validity of the results.

The lyophilised JA powder used in these experiments will not be employed in industrial applications as drying the wet tuber will contribute a significant expenditure. Processing of tubers or roots with similar moisture contents is not an uncommon practise in either food or fermentation industry. For instance, sugar beet has a moisture and sugar content of 75% and 19%, respectively. Prior to the extraction of sugar from wet sugar beet roots, they are mechanically sliced into thin strips. A similar process could be followed as an initial step in the case of JA tubers. Thus, production of thin strips could be followed by boiling of JA strips to release inulin. The suspension will be finally enzymatically hydrolysed by adding the solids from solid state fermentation that will contain all necessary enzymes.

Table 2 presents the results considering product formation (i.e. fructose, glucose, sucrose, FAN and phosphorus) and overall conversion yields (i.e. fructose and inulin) at the end of each hydrolytic reaction using aqueous suspensions of SSF solids taken at five individual SSF times. The concentrations and yields presented in Table 2 were taken at the end of each hydrolytic reaction that lasted up to 48 h. Fructose production yield and inulin hydrolysis yield were calculated by taking into consideration the total JA meal used during both SSF and hydrolysis (initial JA meal concentration of 65 g/L on a dry basis). It should be stressed that each hydrolytic reaction presented in Table 2 was carried out in parallel with a hydrolytic reaction using only SSF solids so as to evaluate the nutrients released from JA macromolecules that were not consumed during SSF by fungal cells. It was observed that hydrolytic reactions with SSF solids taken at fermentation times higher than 48 h resulted in significantly lower fructose and FAN production.

Table 2. Evaluation of hydrolysis performance using crude enzymes produced at different SSF times

SSF time (h)	Maximum concentration					Maximum yield	
	Sugars (g L^{-1}) [1]			Nutrients (mg L^{-1}) [1]		Fructose production	Inulin hydrolysis
	Fructose	Glucose	Sucrose	FAN	Phosphorus	$Y_{F/S}$ (g g^{-1}) [2]	$Y_{FG/S}$ (g g^{-1}) [3]
24	21.4 ± 1.3	2.1 ± 0.1	0.6 ± 0.1	111 ± 8.5	66.8 ± 4.5	0.4	0.44
36	25.7 ± 0.9	4 ± 0.2	0.75 ± 0.1	117.3 ± 6.4	84.8 ± 5.6	0.49	0.56
48	32.6 ± 2.3	4.7 ± 0.1	1 ± 0.1	129.8 ± 7.9	101.6 ± 9.1	0.62	0.7
60	27.5 ± 1.7	3.7 ± 0.3	0.6 ± 0.1	100.15 ± 9.2	92 ± 7.2	0.52	0.59
72	26.9 ± 0.4	3.6 ± 0.5	1.6 ± 0.2	100.6 ± 4.3	90.85 ± 6.9	0.51	0.58

[1] Data is expressed as mean ± 1 standard error.

[2] YF/S = Ft / TRS, where Ft is the final fructose concentration achieved (the initial fructose concentration has already been subtracted from these values) and TRS is the total reducing sugar equivalent (53 g L-1) from the JA meal (65 g L-1) used in both SSF and hydrolytic reactions

[3] YFG/S = FGt / TRS, where FGt is the final fructose and glucose concentrations achieved (the initial fructose and glucose concentrations have already been subtracted from these values) and TRS is the total reducing sugar equivalent (53 g L-1) from the JA meal (65 g L-1) used in both SSF and hydrolytic reactions

The highest concentration of fructose, glucose, FAN and phosphorus, as well as the highest degree of inulin hydrolysis, was observed at 48 h of SSF duration. Figure 1 showed that all enzyme activities increased significantly during SSF up to 48 h. Although enzyme activities were still high after 48 h SSF, hydrolytic reactions using SSF solids after 60 and 72 h resulted in lower nutrient concentrations (Table 2). As presented in Table 2, using crude enzymes produced at 48 h SSF resulted in a fructose production yield of 0.62 g g^{-1} and an inulin hydrolysis yield of 0.7 g g^{-1}. The highest conversion of total Kjeldahl nitrogen to FAN concentration was 11.5%. The maximum phosphorus released as related to the initial phosphorus in JA meal was 50.9%. The nitrogen (FAN) and phosphorus conversion yields were calculated as related to Kjeldahl nitrogen and phosphorus present in the total JA meal used in both SSF and hydrolytic reactions.

In the case that crude enzymes produced at 48 h SSF were used, approximately 14 g L^{-1} of solid concentration remained unhydrolysed. As compared to the total weight of JA used in SSF and hydrolysis, the total dry weight reduction achieved was approximately 78.5%. The solid residue contained 35.7% inulin and 29% protein. This means that 10.4% and 71.2% of inulin and protein remained unhydrolysed.

Figure 2 presents the concentration profile of fructose, glucose, sucrose, FAN and phosphorus during JA hydrolysis using SSF solids after 48 h fermentation. The production of fructose during hydrolysis indicates the existence of inulinase that hydrolyse inulin into fructose and sucrose. The reduction of sucrose concentration and the increase of glucose concentration during hydrolysis are two factors directly associated to the presence of invertase activity that hydrolyse sucrose into glucose and fructose. This means that *A. awamori* produces both inulinase and invertase.

Figure 2 shows that inulin hydrolysis and phosphorus release were terminated at approximately 24 h, while protein hydrolysis continued at a very low rate. The increase of phosphorus content should be attributed to fungal autolysis that occurs simultaneously with JA hydrolysis. This has been identified by Koutinas et al. [34] that presented optimisation of *A. awamori* autolysis where a nutrient-rich solution containing 1.6 g L^{-1} free amino nitrogen, 5.3 g L^{-1} total nitrogen and 0.5 g L^{-1} phosphorus was

produced.

A similar autolytic process of *A. awamori* takes place during JA hydrolysis presented in this work. The different prosphorus concentration achieved during hydrolytic experiments carried out using enzymes produced at different SSF times should be attributed to the varying ability of fungal biomass to autolyse at different SSF times or the different growth stages of the fungus during SSF.

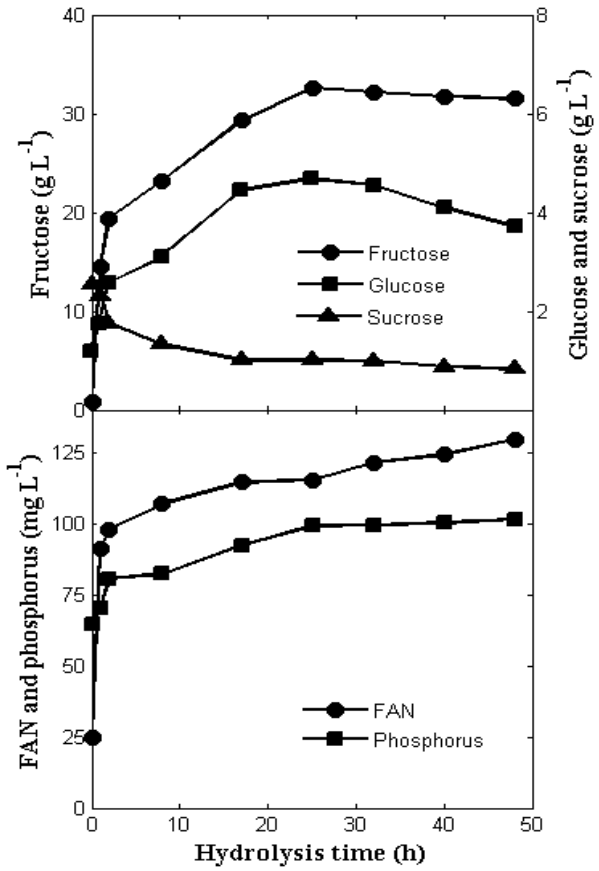

Figure 2. Concentration profiles of sugars and nutrients during hydrolysis of JA meal carried out with solids taken from 48 h solid state fermentation

Various fungal (e.g. *A. niger*) and yeast strains (e.g. *Kluyveromyces marxianus*) have the ability to produce high quantities of inulinase [13, 25, 26, 35, 36]. Mixed cultures of fungal and yeast strains have been employed for the production of crude inulinase and invertase that could be used for efficient JA hydrolysis [35, 37]. Öngen-Baysal and Sukan [37] reported that enzymatic hydrolysis experiments of JA meal carried out at 50°C resulted in the hydrolysis of inulin at 34.7%, 62.6% and 87.9% in the case that crude enzymes from *A. niger* A42, *K. marxianus* NCYC 587 and a mixed culture of the two strains were used, respectively. Sirisansaneeyakul et al. [35] also reported that mixed crude enzymes produced by *A. niger* TISTR 3570 and *C. guilliermondii* TISTR 5844 proved superior to individual crude inulinases in hydrolysing inulin to fructose. There are limited studies on the utilisation of single cultures of *A. niger* for the production of enzymes that resulted in satisfactory inulin hydrolysis that could be subsequently employed as fermentation medium, as in the case of lactic acid production reported by Ge et al. [13].

The highest inulin conversion yield (0.7 g g^{-1}) achieved in this study is significantly higher than the respective yields achieved by single cultures of *A. niger* employed in the studies mentioned above [35, 37]. Such a high inulin conversion yield is usually achieved when mixed cultures are used that contain sufficient quantities of both inulinase and invertase. Further optimisation of enzyme production by this strain of *A. awamori* is likely to increase production yields. Future studies should also focus on the production and characterisation of inulinase and invertase produced by this strain of *A. awamori*.

Another important advantage of JA hydrolysates is the fact that they contain complex sources of nitrogen such as amino acids and peptides, which provide not only nitrogen but also an additional carbon source. Previous studies have demonstrated that amino acids have the ability to increase fermentation yields, enhance productivities and reduce reaction times [16, 38].

A statistical analysis on the experimental results of enzymes production via SSF and JA hydrolysis showed that all experimental data have a good reproducibility. For both cases, standard error values between 1 and 4 % of the reported value were obtained. These results coincide with values reported in Table 2, where standard errors show a similar trend. This

indicates that SSF and hydrolysis experiments are statistically valid.

3.3.2 Bacterial fermentations for PHB production

Table 3 presents the initial fructose and FAN concentrations as well as the final fermentation time, total dry weight, PHB concentration, residual biomass concentration and PHB content during three sets of shake flask fermentations carried out with commercial sources of nutrients (i.e fructose, yeast extract and peptone) and JA hydrolysates supplemented with yeast extract. In the case that a commercial fermentation medium was used, 3 shake flask fermentations were carried out with a constant FAN concentration of 600 mg L^{-1} and three different fructose concentrations (10, 20 and 30 g L^{-1}). The total dry weight, PHB concentration and residual microbial biomass concentration were increased gradually with fructose concentration. A maximum PHB concentration of 4.4 g L^{-1} was achieved when 30 g L^{-1} fructose concentration was used. However, the highest PHB content (42.9%) was reached when an initial fructose concentration of 20 g L^{-1} was used.

The other two sets of shake flask fermentations were carried out with mixed JA hydrolysates (Table 2). Fructose and FAN concentrations in the mixture of JA hydrolysates were 28 g L^{-1} and 120 mg L^{-1}, respectively. In the second set of shake flask fermentations, the original JA hydrolysate was diluted twice to reach a fructose concentration of 14 g L^{-1}. Due to the low FAN concentration, shake flask fermentations were supplemented with yeast extract to reach the initial FAN concentration presented in Table 3. Total dry weight and residual biomass concentration were gradually increased with increasing FAN concentration indicating that higher nutrient concentrations promote microbial growth. The PHB concentration was increased up to a FAN concentration of 490 mg L^{-1} and was gradually decreased at higher FAN concentrations. Higher FAN concentrations may facilitate microbial growth because PHB accumulation in *C. necator* is usually triggered under nutrient limiting conditions. It was also observed that PHB accumulation occurred during bacterial growth. This most probably could be attributed to oxygen limitation that cannot be avoided during shake flask fermentations.

Table 3. Effect of different fructose and FAN concentration on C. necator growth and PHB production during shake flask fermentations using commercial sources of nutrients and JA hydrolysates

Initial fructose ($g\ L^{-1}$)	Initial FAN ($mg\ L^{-1}$)	Fermentation duration (h)	Total dry weight ($g\ L^{-1}$)	PHB concentration ($g\ L^{-1}$)	Residual microbial biomass ($g\ L^{-1}$)	PHB content (%)
Commercial fermentation medium (fructose, 10 g L^{-1} yeast extract, 5 g L^{-1} peptone)						
10	600	19	5.2	1.7	3.5	32.7
20	600	40	9.8	4.2	5.6	42.9
30	600	48	11.5	4.4	7.1	38.3
JA hydrolysate supplemented with yeast extract						
14	100	32	5.3	1.5	3.8	28.3
14	290	34	7.1	3	4.1	42.2
14	490	50	8.3	3.8	4.5	45.8
14	560	50	8.2	3.1	5.1	37.8
14	900	50	8.5	1.5	7	17.6
28	120[a]	56	5.4	1.4	4	25.9
28	340	56	7.5	3.2	4.3	42.7
28	430	56	7.7	4	3.7	51.9
28	540	54	8.5	1.9	6.6	22.4
28	800	49	9.2	1.4	7.8	15.2

[a] No yeast extract supplementation

In the third set of shake flask fermentations, the original mixture of JA hydrolysates was used only in the first shake flask experiment where the FAN concentration was 120 mg L^{-1}. In the other four shake flask fermentations, JA hydrolysates were supplemented with yeast extract in order to increase the initial FAN concentration. PHB concentration and content were increased up to 430 mg L^{-1} and then decreased at higher FAN concentrations. The highest PHB concentration (4 g L^{-1}) was achieved when fructose and FAN concentrations of 28 g L^{-1} and 430 mg L^{-1} were used.

Koutinas et al. [39] reported PHB production in shake flask fermentations using *C. necator* NCIMB 11599 and mixtures of wheat hydrolysates and fungal extracts as fermentation media. The PHB produced with 16.5 g L^{-1} glucose concentrations and 450 mg L-*1* FAN concentration was 4.9 g L^{-1}. In addition, current studies by our research group is focused on PHA production using *C. necator* DSM 545 and crude glycerol mixed with rapeseed cake hydrolysates (see chapter 4). Shake flask fermentations resulted in lower than 4.5 g L^{-1} PHA concentration when glycerol and FAN concentrations of approximately 16 g L^{-1} and 450 mg L^{-1} were used (results not published yet). This means that the results reported in this study when a JA hydrolysate of 14 g L^{-1} fructose concentration was employed are comparable to both wheat-based fermentation media and media derived from by-products of biodiesel production plants. However, at glucose and glycerol concentrations higher than 20 g L^{-1}, the PHB concentrations produced in previous studies were higher than the PHB concentration achieved in this study using JA hydrolysates with 28 g L^{-1} initial fructose concentration.

Figure 3 presents kinetics of total dry weight, PHB concentration and fructose concentration during shake flask fermentations carried out with a medium containing commercial sources of nutrients (20 g L^{-1} fructose and 600 mg L^{-1} FAN) and two JA hydrolysates (14 g L^{-1} and 28 g L^{-1} fructose concentration and 490 mg L^{-1} and 430 mg L^{-1} FAN concentrations, respectively). Fructose was consumed at a higher rate when commercial medium was used. When a JA hydrolysate of 28 g L^{-1} fructose concentration was used, fructose was not entirely consumed when microbial growth and PHB production stopped after 56 h fermentation. In the experiment with JA hydrolysate of 14 g L^{-1} fructose concentration, fructose was completely consumed at approximately 50 h fermentation. This

indicates that JA hydrolysate does not provide a sufficient source of nutrients to support microbial growth and PHB production. This could be also indicated by the trend of total dry weight and PHB concentration. In the case of the commercial medium both profiles are increasing linearly until complete fructose consumption, whereas in the case of JA hydrolysates the linear trend changes at approximately 30 h fermentation. The linear trend also indicates oxygen limitation during shake flask fermentations.

In Figure 3, total dry weight represents the summation of microbial mass and intracellular PHB. Total dry weight increases constantly during fermentation because of either microbial growth or PHB accumulation. It should be stressed that the highest PHB concentration produced by using either commercial media or JA hydrolysates were similar. This indicates that optimisation of JA-based fermentation media should be employed so as to improve PHB fermentations. Future experiments should also focus on fermentations using JA hydrolysates in bioreactor experiments in order to control aeration and pH that will lead to concrete conclusion on the effect of JA hydrolysates on PHB accumulation by *C. necator*.

Future studies focused on further optimisation of JA meal hydrolysis to increase the concentration of fructose (and glucose) in the final hydrolysate will be necessary in order to develop fed-batch fermentations in a bioreactor. In addition, the results presented in Table 2 indicate that the FAN and other nutrient content of JA hydrolysates are too low to support sufficient bacterial growth for high levels of PHB accumulation. This problem could be solved either by enhancing protein hydrolysis in JA meal or by using a crude protein-rich industrial supplement. The first option has been explored by exploiting in the hydrolysis of JA a fungal strain (namely *Aspergillus oryzae*) that has been characterised as a prominent protease producer [18, 40]. Protein hydrolysis was not, however, improved significantly (results not presented). It should be stressed that the low protein content in JA meal will not provide a sufficient amount of nitrogen for subsequent PHB fermentations. For this reason, future studies should focus on the utilisation of crude protein-rich industrial by-products as raw material for the production of nitrogen-rich as well as other nutrient-rich fermentation supplements. Oilseed residues from biodiesel production plants (e.g. sunflower, soybean and rapeseed) could be regarded as ideal renewable resources for the production of such crude

nutrient supplements [41, 42]. Oilseed residues are particularly rich in protein and minerals including phosphorus, thus offering high biotechnological potential. Rapeseed hydrolysates with up to 2061.2 mg L^{-1} FAN and 304 mg L^{-1} inorganic phosphorus could be produced via a novel SSF-based bioprocess [42].

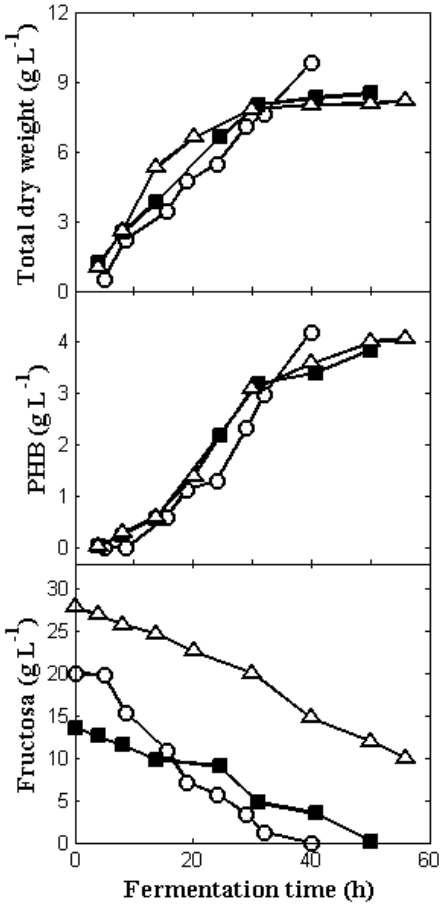

Figure 3. Concentration profiles of total dry weight, PHB and fructose during shake flask fermentation of C. necator using a medium with commercial sources of nutrients and two JA hydrolysates. Symbols: (○), Commercial medium with 20 g L^{-1} fructose and 600 mg L^{-1} FAN; (■), JA hydrolysate with 14 g L^{-1} fructose and 490 mg L^{-1} FAN; (Δ), JA hydrolysates with 28 g L^{-1} fructose and 430 mg L^{-1} FAN

3.3.3 Potential of JA for PHB production

The economic perspective of PHB production from JA cannot be estimated with accuracy because the whole bioprocess has not been optimized yet. At this stage, it is more meaningful to present the potential of PHB production from JA as compared to other agricultural crops. Table 4 presents the prospects of JA as a renewable feedstock for PHB production in comparison to corn, wheat and sugarbeet. The crop production yield per hectare, carbohydrate content and market price for JA were taken from literature-cited publications [43, 44]

Table 4 shows that potential PHB production per hectare of cultivated land is higher in the case of JA provided that optimum yields are achieved. Furthermore, as calculated in several publications investigating the potential of JA utilisation for bioethanol production as compared to other roots and tubers, the bioethanol yield was much higher. For instance, Mays et al. [6] identified JA as the second best crop among various carbohydrate producing crops (sweet potato, sweet sorghum, potato, sugar beet and fobber beet) regarding bioethanol production yield per hectare of cultivated land. This means that the potential of JA utilisation for PHB production should be investigated further.

The price of crude JA powder was found only in the case of China as $180 per tn, which is lower than the respective price of glucose [45]. This price will be lower in the case that the crude JA tuber is used. Table 4 shows that this market price is comparable to wheat and corn and higher than sugar beet. Cheng et al. [45] claimed that JA is a drought-resisting crop that can be cultivated in desertified land for soil improvement and water conservation. In addition, JA has low requirements for fertilizer, pesticides and water supply. For this purpose, JA is more resistant crop than wheat, corn and sugar beet in desert conditions and frost. This is an important advantage of JA utilization for chemical and biomaterial production.

Table 4. PHB production yield and market price for wheat, corn, sugarbeet and JA

Crop	Country	Crop production yield [1] (tn/ha)	Carbohydrate content (g/g) [2]	Carbohydrate yield (tn/ha)	Potential PHB production (tn/ha) [3]	Market Price ($/tn) [4]
Wheat	World	3	0.65	2	1	-
	USA	3.12		2	1.1	215
	China	4.75		3.1	1.6	-
	Argentina	3.41		2.2	1.2	144
Corn	World	5.21	0.6	3.1	1.7	-
	USA	9.60		5.8	3	148
	China	5.46		3.3	1.7	-
	Argentina	7.81		4.7	2.5	117
Sugarbeet	World	48.86	0.19	9.3	4.8	-
	USA	61.88	0.145	9	4.7	73.5
	China	42.45	0.19	8.1	4.2	-
JA	Spain [43]	40 – 70	0.15 – 0.18	6 – 12.6	3.2 – 6.7	-
	[44]	90	0.04 – 0.15	3.6 – 13.5	1.9 – 7.2	180

[1] The crop production yield for wheat, corn and sugarbeet were taken from FAO for 2010 (http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor)
[2] Based on average carbohydrate content (wheat, corn, JA) or recovery rate achieved (only for sugarbeet in USA)
[3] Based on a glucose to PHB conversion yield of 0.48 g g-1. An inulin, starch and sucrose to sugar conversion yield of 1.1 has been used
[4] The market price for wheat and corn were taken from FAO Statistical Yearbook 2010. The market price for sugarbeet were taken from USDA (source: "Agricultural Prices,"Agricultural Statistics Board, NASS, USDA) for 2010/11. The market price for JA refers to JA powder and was given by Cheng et al. [45].

3.4 Conclusions

A fungal strain of *A. awamori* to produce enzyme consortia via solid state fermentation (SSF) could efficiently produce a JA hydrolysate rich mainly in fructose with lower concentrations of glucose, FAN and phosphorus. Shake flask fermentations, with *Cupriavidus necator* DSM 4058, using JA hydrolysate as fermentation medium showed that PHB production is feasible. However, enrichment of JA hydrolysates with nutrient-rich supplements (e.g. oilseed meal hydrolysates) should be carried out in bioreactor applications.

It should be stressed that industrial implementation of PHB production has not been established yet due to the high cost of conventional raw materials and processing stages.

The utilization of non-food crops or industrial waste strains could assist in the commercialization of PHB production. JA cultivation for chemical and biopolymer production could be achieved in desertified land where water shortage is unavoidable and irrigation is not effectively achieved.

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Chapter 4

Valorization of by-products from biodiesel industrial plants for microbial production of polyhydroxyalkanoates

4.1 Introduction

Compared to other biofuels produced from renewable resources, biodiesel generation is an efficient process and more environmentally benign as compared to fossil fuels. In 2009, biodiesel production in EU reached an annual capacity of 9,046 10^3 tonnes per year, which corresponds to an annual increase of 16.6 %, while in 2010 biodiesel production in EU rised to 9,570 10^3 tonnes per year [1]. Although many biodiesel production plants stopped operation in the last few years, production capacity may increase further due to the high versatility in raw material that can be employed in biodiesel production. Biodiesel can be produced from nearly all types of oilseeds, both edible such as rapeseed, soybean or palm and non-edible such as jatropha, animal fat and waste cooking oil. Soybean is the most popular raw material for biodiesel production in the world, whereas rapeseed is the most popular in Europe. Biodiesel could be also produced from non-conventional renewable raw materials such as single cell oil (or microbial oil) produced by yeast, fungi and algae [2].

Most conventional and novel processes for biodiesel production generate significant quantities of by-products. For instance, the utilization of oilseeds as raw materials lead to the production of a protein-rich oilseed meals that remains after oil extraction and a crude glycerol stream that is produced from oil transesterification. The current biodiesel production

plants from oilseeds should be restructured so as to integrate technologies that will valorize oilseed meals and crude glycerol into various products including chemicals, biodegradable polymers, value-added ingredients (e.g. extracts with antioxidant properties), food and feed. Crude glycerol is nowadays regarded as a platform chemical that could be converted into many different chemicals through either chemical synthesis or fermentation [3].

Polyhydroxyalkanoates (PHAs) is a family of biodegradable polymers produced as intracellular energy-reserve granules during fermentation by more than 300 microorganisms including *Cupriavidus necator*. The microbial production of PHAs by *C. necator* is mainly based on the limitation of a nutritional element such as N, P, Mg, K, O, or S, while at the same time there is an abundant source of carbon. PHAs could be used as substitutes for petroleum-derived polymers in various commercial applications such as food packaging, biocomposite production, adhesives, medical purposes, agriculture and flushable hygiene products [4]. Polyhydroxybutyrate (PHB), the most well-known member of the PHA family, is a homopolymer of 3-hydroxybutyric acid units. Apart from PHB, research has also focused on the production of different copolymers such as P(3HB-co-3HV), a copolymer of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) units at various proportions [5, 6].

PHAs could be produced by various commercial carbon sources such as carbohydrates (e.g. glucose, fructose, sucrose, lactose), methanol, alkanes (i.e. hexane to dodecane), vegetable oils and short and long chain fatty acids (e.g. butyrate upwards) [4, 7, 8]. One of the major problems that hinder industrial PHA production is the cost of the carbon source and fermentation media formulation in general. For this reason, recent research initiatives aim at the utilization of agricultural or industrial waste and byproduct streams for PHA production [8]. Crude glycerol has already been tested as fermentation feedstock for the production of PHB using *C. necator* DSM 545 [9, 10]. Cavalhiero *et al* [11] reported the production of poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) using *C. necator* DSM 545 cultivated on crude glycerol and 3HV/4HB precursors (i.e. γ-butyrolactone and propionic acid). These studies focused on the utilization of inorganic chemicals as sources of nutrients.

Oilseed meal that is also generated as by-product from oilseed-

based biodiesel production plants could be converted into a nutrient-rich hydrolysate that could be used as fermentation media supplement. Wang *et al.* [12] reported the production of hydrolysates from rapessed meal that contained up to 2061.2 mg L^{-1} free amino nitrogen (FAN), 304 mg L^{-1} inorganic phosphorus (IP), and 1.8 g L^{-1} glucose by employing crude enzyme-rich extracts from solid state fermentations of *Aspergillus oryzae*. Crude hydrolysates or commercial formulations rich in amino acids and peptides could be used as nutrient supplements in microbial bioconversions for PHB production [13-15]. The utilization of wheat-derived hydrolysates enriched in glucose and various sources of nutrients (e.g. amino acids, peptides, phosphorus) led to the production of 162.8 g L^{-1} PHB [15]. Utilizing specific or mixtures of amino acids may enhance PHB production by various microorganisms [14, 16, 17]. On the other hand, the utilization of specific amino acids may lead to the production of P(3HB-co-3HV) [18].

This study explored the potential production of PHAs, including P(3HB-co-3HV), with *C. necator* DSM 545 using rapeseed meal hydrolysates and crude glycerol as nutrient supplements and carbon sources, respectively. PHA property analysis verified the potential to produce complex PHAs from rapeseed hydrolysates. This work focus on the potential of rapeseed meal hydrolysates as nutrient supplements for the production of complex PHAs, instead of the maximization of PHA production.

4.2 Materials and methods

4.2.1 Microorganisms

An industrial strain of *Aspergillus oryzae* isolated from the company Amoy Food Ltd. (Hong Kong), kindly provided by Professor Colin Webb (Satake Centre for Grain Process Engineering, School of Chemical Engineering and Analytical Science, University of Manchester, UK) was utilized in solid state fermentations to produce crude enzymes essential for rapeseed meal hydrolysis. The isolation and purification of this fungal strain is reported by Wang *et al.* [19]. Fungal spores were maintained at 4 °C in slopes containing solid medium of 30 g L^{-1} rapeseed meal, 15 g L^{-1} wheat bran and 20 g L^{-1} agar.

Submerged fermentations for PHA production were carried out with *Cupriavidus necator* DSM 545, a glucose consuming mutant of *C. necator* DSM 529 as it was listed by the DSMZ culture collection. The original bacterial strain was not efficient in the production of PHAs using glycerol as carbon source. To adapt this bacterial strain for glycerol consumption, successive cultivations in shake flasks at gradually increasing glycerol concentrations (5 – 25 g L^{-1}) were carried out. After successive cultivations, *C. necator* could consume glycerol at significantly improved rates. Bacterial stock cultures were stored at 4 °C in Petri dishes containing 10 g L^{-1} glycerol, 10 g L^{-1} yeast extract, 5 g L^{-1} peptone and 20 g L^{-1} agar. Inocula

for shake flask and bioreactor fermentations were prepared by transferring bacteria cells using a wire loop into liquid medium containing 10 g L^{-1} glycerol, 10 g L^{-1} yeast extract and 5 g L^{-1} peptone.

4.2.2 Raw materials used as fermentation media

Crude glycerol was kindly provided by the biodiesel producer ADM Industries (Germany) and it contains glycerol (81%), water (10-12%), potasium salts (5-6%), free fatty acids (1%) and methanol (<1%). Rapeseed meal (*Brassica napus*) was a by-product of biodiesel production that contained 9.43 % moisture content, 57.9 mg g^{-1} (on a dry basis, db) total Kjeldahl nitrogen (TKN), 36.18 % protein (db, 6.25 × TKN) and 7.9 % ash content.

4.2.3 Solid state fermentation

Solid state fermentations (SSF) of rapeseed meal were carried out in 250 mL Erlenmeyer shake flasks at 30 °C. Each shake flask contained 5 g of rapesead meal with a moisture content of 65 % (w/w, db). All shake flasks were sterilized at 121 °C for 20 min prior to SSF.

A suspension of *A. oryzae* spores was used as fermentation inocula. To increase the concentration of fungal spores, *A. oryzae* was cultivated on a solid medium in 250 mL shake flasks. Sterile tap water (10 mL) was aseptically transferred in a slope containing the fungal spores and then a spore suspension was formed by scrapping the surface of the slope with a wire loop. Subsequently, 1 mL of this spore suspension was transferred on solid medium (30 g L^{-1} rapeseed meal, 15 g L^{-1} wheat bran and 20 g L^{-1} agar) in a 250 mL shake flask. These flasks were incubated for 5 days or until complete sporulation on the surface of the shake flask. The inoculum for SSF was formulated by adding 50 mL of sterilized tap water and a few glass beads (4 mm diameter) in each shake flask containing fungal spores, followed by vigorous shaking until a fungal suspension was formed. The spore concentration employed was approximately 2×106 spores mL^{-1}.

To produce a nutrient-rich supplement for subsequent PHA

fermentations, solids were collected after 44 h of SSF and used as an abundant source of crude enzymes (e.g. proteolytic enzymes) for the hydrolysis of rapeseed meal macromolecules (mainly proteins) and the release of important nutrients (e.g. phosphorus) from rapeseed meal.

4.2.4 Production of rapeseed meal hydrolysate

Rapeseed meal hydrolysates were produced in 1 L Duran bottles by mixing remaining solids from SSF with 50 g L^{-1} (db) of fresh rapeseed meal suspension in tap water. Duran bottles were placed in a water bath operated at 55 °C and agitated using magnetic stirrers. At the end of the reaction (approximately 24 h), remaining solids were removed by vacuum filtration (Whatman No 2). Rapeseed meal hydrolysates were filter sterilized using a 0.2 μm filter unit (PolycapTM AS, Whatman Ltd.). The pH of rapeseed meal hydrolysates was adjusted to the optimum pH range (6.7 – 6.9) for *C. necator* growth with 5 M NaOH.

4.2.5 Bacterial bioconversions in shake flasks

Bacterial fermentations in 250 mL Erlenmeyer shake flasks (50 mL broth volume) were carried out in an orbital shaker (ZHWY-211C Series Floor Model Incubator, PR China) at 180 rpm, 30 ºC and initial pH in the range of 6.7 – 6.9. The pH of the mixture was re-adjusted manually with 5 M NaOH in the optimum range at regular intervals during shake flask fermentations. An inoculum of 1 mL was used for each flask. Two sets of batch shake flask fermentations were carried out to investigate the effect of FAN and salt contamination on microbial growth and PHA formation. Shake flask fermentations were also carried out on fed-batch mode to identify the effect of the addition of rapeseed meal hydrolysates during fermentation. Glycerol and FAN concentrations were analyzed in each flask immediately after inoculation in order to measure initial concentrations. The measured concentrations of glucose and FAN in each flask correspond to ±3% of the respective values reported in each experiment (rounded to the closest number). Each shake flask fermentation was repeated three times so as to identify accurately the measurements taken.

The first set of shake flask fermentations was used to evaluate the effect of different initial FAN concentrations on microbial growth and PHA production. Five shake flasks were prepared with fermentation media that contained rapeseed meal hydrolysates with initial FAN concentrations in the range of 170 and 460 mg L^{-1} and crude glycerol with initial concentration of 25 g L^{-1}. Two shake flask fermentations were supplemented with mineral medium and a sterile stock solution of trace elements (1 mL) as was reported by Kim *et al.* [20]. Another two shake flask fermentations were carried out at the best FAN concentration (460 mg L^{-1}) with initial glycerol concentrations of 9 and 16 g L^{-1}. Glycerol and FAN concentrations higher than 500 mg L^{-1} are not reported because preliminary shake flask fermentations at such conditions were incomplete and resulted in significantly reduced PHA accumulation.

Crude glycerol generated from biodiesel production processes was contaminated by sodium or potassium salts resulting from the catalyst (NaOH or KOH) employed in the traditional transesterification process. One of the aims of this study was to identify the effect of salt concentration on PHA production and bacterial growth. Thus, in the second set of batch shake flask fermentations, media were prepared with initial crude glycerol concentration of 25 g L^{-1}, an initial FAN concentration of 400 mg L^{-1} and different initial concentrations of NaCl or K_2SO_4 (2, 6, 10, 15, 20, 25 g L^{-1}).

Shake flask fermentations were also carried out in fed-batch mode to identify the effect of rapeseed meal hydrolysate addition. Two fed-batch fermentations were conducted in shake flasks with the same initial glycerol (21 g L^{-1}) and FAN (400 mg L^{-1}) concentrations. A third shake flask fermentation was carried out in batch mode with exactly the same medium. A trace element solution of 1 mL was employed in all shake flasks. The initial volume in all shake flasks was 40 mL. The first two shake flasks were fed with the same crude glycerol solution and a concentrated rapeseed meal hydrolysate solution. The initial rapeseed meal hydrolysate was concentrated 6 times with a rotary evaporator (Buchi rotavapor R-114) in order to increase FAN concentration. In this way, a low volume of rapeseed meal hydrolysate was added to each flask and the fermentation was not influenced by media dilution. Rapeseed meal hydrolysate and crude glycerol were added at random intervals.

4.2.6 Bacterial bioconversions in a bioreactor

Batch bacterial bioconversions were carried out in a 3 L bioreactor (Bioflo 110, New Brunswick Scientific, USA) at 30 ºC and a pH value in the range of 6.7 – 6.9. The bioreactor was sterilized at 121 °C for 20 min. The pH was controlled by adding 10 M NaOH and 10 % H_2SO_4 solutions. Air sparging was carried out at a rate of 1 vvm. The dissolved oxygen (DO) in the broth was maintained at values higher than 40% of saturation by varying automatically the agitation speed in the range of 300 – 1000 rpm. An antifoam solution (Dow Corning; BDH) was added manually to break the formation of foam whenever it was formed. Fermentation media were pumped aseptically into the bioreactor at the beginning of each fermentation.

4.2.7 PHA Extraction

PHA extraction was carried out by the dispersion-extraction method reported by Hahn *et al.* [21]. Cells were harvested by centrifugation at 3000 g for 10 min. Subsequently, 30 mL of chloroform and 30 mL of sodium hypochlorite (30 %, v/v in water) were used per gram of dried cell mass. This mixture was treated at 30 ºC for 90 min in an orbital shaker at 150 rpm. The mixture was centrifuged at 3000 g for 15 min that led to the formation of three dinstinctive phases. The upper phase was hypochlorite solution, the middle phase contained non-PHA cell materials and undisrupted cells, and the bottom phase was chloroform containing PHA. The upper phase was carefully removed with a pipette and the chloroform phase was obtained by filtration. Then, PHA was recovered by precipitation using 10 volumes of ice-cold methanol.

4.2.8 Nuclear magnetic resonance (NMR)

NMR spectra were recorded in a BRUCKER 400 NMR spectrometer at 125 MHz (13C-NMR) using deuterated chloroform ($CDCl_3$) as solvent. For each analysis, 10 mg of biopolymer sample and 1 mL of solvent were employed.

4.2.9 Differential scanning calorimetry (DSC)

Thermal properties of PHAs were determined by DSC using a PERKIN-ELMER PYRIS 1 calorimeter calibrated with indium and n-heptane standards. Samples (around 10 mg) were heated from 25 ºC to 190 ºC, cooled to 25 ºC and then heated again to 190 ºC at a rate of 10 ºC min^{-1}. Runs for determining glass transition temperatures (Tg) were carried out between -50 ºC to 50 ºC by using nitrogen as cooler agent and helium as purge gas.

4.2.10 Thermogravimetric analysis (TGA)

Thermal stability of samples was determined with a thermogravimetric analyzer (PERKIN ELMER II). Samples were heated from 30 to 500 °C at a constant heating rate of 10 °C min^{-1} under a nitrogen atmosphere.

4.2.11 Analytical methods

Samples from bacterial fermentations were taken at random intervals. Each sample was centrifuged at 3000 g for 10 min (Hettich Universal 320-R, Germany). The supernatant was used to determine glycerol and FAN concentrations. The solid sediment was washed with distilled water and centrifuged. The solids were subsequently re-suspended in acetone and transferred into 14 mL McCartney universal bottles. Total dry weight (TDW) was measured by drying the solids at 50 ºC and cooling down in a desiccator untill constant weight. Residual microbial biomass (RMB) concentration in each sample was determined by substracting PHA concentration measured by GC analysis from TDW.

Glycerol concentration was determined by high performance liquid chromatography (HPLC, Waters 600E) equipped with an Aminex HPX–87H (300 mm × 7.8 mm, Bio Rad, CA) column coupled to a differential refractometer (RI waters 410). Operating conditions were as follows: sample volume 40 μL; mobile phase 0.005 M H_2SO_4; flow rate 0.6 mL/min; column temperature 65 °C. Before injection, samples were diluted to

appropriate concentration with deionized water and filtered through a 0.45 μm membrane filter.

Free amino nitrogen (FAN) concentration in liquid samples was determined by the ninhydrin colorimetric method promulgated in the European Brewery Convention [22]. Total Kjeldahl nitrogen (TKN) concentration was measured using a KjeltekTM 8100 distillation Unit (Foss, Denmark). Ash content was determined following the AACC Approved Method 08-01.

PHA concentration was determined by the chromatographic method proposed by Riis and Mai [23] using benzoic acid as internal standard. A gas chromatographic analyzer (Fisons 8060) equipped with a Flame Ionization Detector (FID) and a Chrompack column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific) was used for PHA concentration measurements. Helium was used as carrier gas at a flow rate of 2 mL min^{-1}. The injector and detector temperatures were 230 °C and the initial temperature was 120 °C. The appearence of each PHA monomer (propyl esters of 3HB and 3HV) in the chromatogram was confirmed based on the retention time of respective monomers from commercial PHB and P(3HB-co-3HV) standards (Sigma).

4.3 Results and discussion

4.3.1 Shake flask fermentations

Five shake flask fermentations (Table 1) were carried out using crude glycerol and rapeseed meal hydrolysates to obtain varying initial FAN concentrations (170, 240, 320, 400 and 460 mg L^{-1}) and 25 g L^{-1} initial glycerol concentration. All results presented in Table 1 were determined when glycerol was completely consumed or stopped. It is obvious that rapeseed meal hydrolysates can be used as nutrient supplements to achieve microbial growth followed by P(3HB-co-3HV) intracellular accumulation. Total dry weight, RMB and PHA concentration and content were increased when initial FAN concentrations up to 460 mg L^{-1} were used. Since glycerol concentration is constant, these results show that rapeseed hydrolysate provide carbon sources (e.g. amino acids) for both bacterial growth and PHA accumulation. The highest total dry weight (15.1 g L^{-1}), PHA concentration (7 g L^{-1}) and PHA content (46.3%) were achieved at an initial FAN concentration of 460 mg L^{-1}. FAN concentrations lower than approximately 250 mg L^{-1} resulted in incomplete glycerol consumption indicating that rapeseed meal hydrolysates with higher FAN concentrations is required. It should be stressed that such crude hydrolysates do not only provide sources of nitrogen but also other nutrients such as phosphorus, vitamins and trace elements.

Table 1. Shake flask fermentations of *C. necator* using media derived from by-products of biodiesel production

Initial glycerol (g L^{-1})	Initial FAN (mg L^{-1})	T_F [a] (h)	Total dry weight (g L^{-1})	Residual microbial biomass (g L^{-1})	PHA (g L^{-1})	3HB (%)	3HV (%)	PHA content (%)
Rapeseed meal hydrolysate								
25	170	64	5.1	3.7	1.4	92	8	27.4
25	240	62	5.3	3.9	1.4	92.5	7.5	27.4
25	320	60	7.4	5.2	2.2	95.9	4.1	29.7
25	400	74	10.1	5.9	4.2	96.9	3.1	41.5
25	460	74	15.1	8.1	7	97.2	2.8	46.3
16	460	48	12.5	7.3	5.2	96.8	3.2	41.6
9	460	28	8.2	5.1	3.1	96.5	3.5	37.8
Rapessed meal hydrolysate supplemented with trace elements								
25	400	74	11.4	5.5	5.9	96.4	3.6	51.8
Rapessed meal hydrolysate supplemented with mineral medium								
25	400	57	13.7	7.9	5.8	98.5	1.5	42.6

[a] Final fermentation time

Table 1 shows that rapeseed cake hydrolysates and crude glycerol could lead to of P(3HB-co-3HV) without addition of precursors for the production of 3HV monomer. The final 3HV content produced in each fermentation carried out at constant glycerol concentration (25 g L^{-1}) is reduced with increasing FAN concentration. In addition, 3HV concentration decreases during fermentation (Figure 1). For instance, at initial FAN concentration of 170 mg L^{-1} the 3HV content at 10 h and 64 h fermentation were 12.3 % and 8 %, respectively. When the initial FAN concentration was 400 mg L^{-1} the 3HV content at 10 h and 74 h fermentation were 9.2 % and 3.1 %, respectively. Glycerol and most amino acids lead to 3HB production, while only certain amino acids lead to 3HV production. Thus, the gradual consumption of amino acids that lead to 3HV production results in the reduction of 3HV content during fermentation.

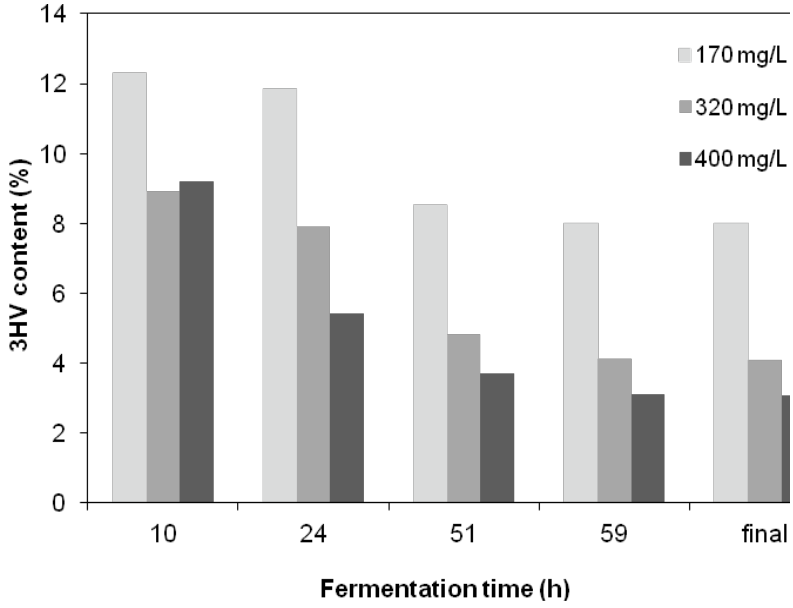

Figure 1. 3HV content during shake flask fermentations carried out with 170, 320 and 400 mg L^{-1} inital FAN concentration

As it is shown in Table 1, two shake flask fermentations were carried out at the best FAN concentration (460 mg L^{-1}) and lower initial

glycerol concentrations (9 and 16 g L^{-1}). Decreasing glycerol concentrations resulted in a reduction of total dry weight, residual microbial biomass and P(3HB-co-3HV) concentration and content. However, the 3HV content was slightly increased probably due to the increased ratio of 3HV (i.e. specific amino acids) to 3HB (mainly glycerol) precursors. Higher PHA production was achieved when pure glycerol was used in shake flask fermentations (results not presented). At initial pure glycerol and FAN concentrations of 9 – 25 g L^{-1} and 300 – 470 mg L^{-1}, the highest P(3HB-co-3HV) concentration and content achieved were 9.1 g L^{-1} and 65 %, respectively. Cavalheiro *et al*. [9] also reported improved PHB production in bioreactor experiments when pure glycerol was used.

Table 1 presents two shake flask fermentations that were supplemented with a trace element solution and a mineral medium, respectively. Initial glycerol and FAN concentration in those fermentations were 25 g L^{-1} and 400 mg L^{-1}, respectively. The addition of trace elements or mineral medium led to an increase in total dry weight and P(3HB-co-3HV) concentration as compared to shake flask fermentations carried out with only rapeseed meal hydrolysates at an initial FAN concentration of 400 mg L^{-1}. Ongoing research is focussed at increasing the release of nutrients from oilseed cakes aiming at the production of nutrient-rich supplements for fermentation processes.

Figure 2 presents kinetic profiles of glycerol and FAN consumption as well as total dry weight (TDW) and P(3HB-co-3HV) production during fermentation with initial glycerol and FAN concentration of 25 g L^{-1} and 460 mg L^{-1}. It can be observed that FAN consumption coincided with PHA production. However, it should be taken into consideration that during shake flask fermentations the dissolved oxygen concentration should be below the critical point for exponential microbial growth. Thus, oxygen could be the limiting factor that leads to PHA accumulation. When wheat-based media were used for PHB production using *C. necator* NCIMB 11599, PHB accumulation mainly occurred after the exhaustion of FAN [3, 15]. In all shake flask fermentations with rapeseed hydrolysates, PHA production stopped when FAN was completely consumed regardless the fact that glycerol was still present in the broth. It was also observed that *C. necator* DSM 545 could produce copolymers containing 3HV monomers when it was cultivated in rapeseed hydrolysates. These observations

indicate significant variations in the mechanism of PHA production when crude hydrolysates generated from different renewable resources are employed. It has been reported that amino acids (maybe even short peptides) and other micronutrients (e.g. phosphorus) may influence PHA accumulation and copolymer production [5, 24, 25].

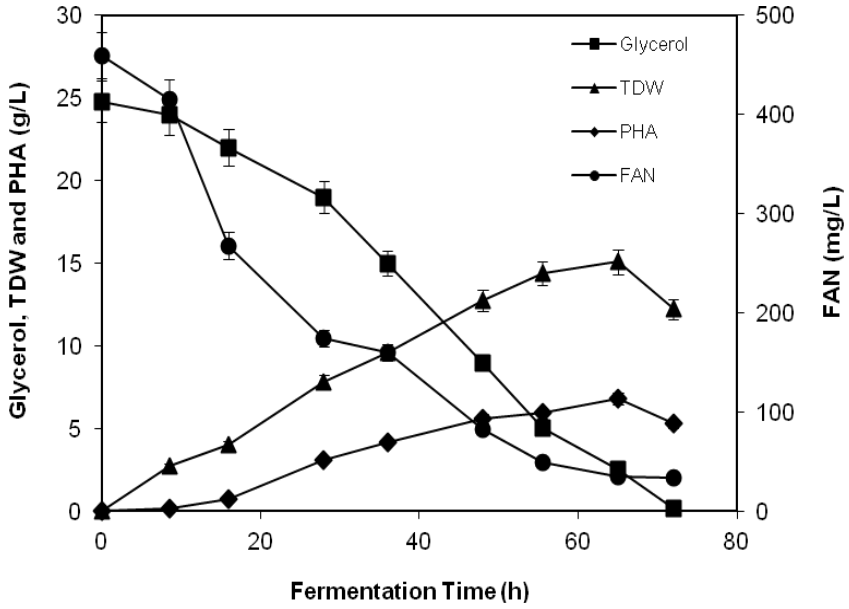

Figure 2. Glycerol, TDW, PHA and FAN concentrations during shake flask fermentation carried out with initial glycerol and FAN concentrations of 25 g L^{-1} and 460 mg L^{-1}

Figure 3 presents concentration profiles of glycerol, FAN, total dry weight and PHA during shake flask fermentations carried out on fed-batch (Figure 3a) or batch (Figure 3b) mode using the same initial glycerol (21 g L^{-1}) and FAN (400 mg L^{-1}) concentrations. Rapeseed meal hydrolysate was added at 48 h, while crude glycerol was added at 58 h and 72.5 h. The profiles of TDW and PHA are similar until 58 h. From this point, PHA production continued only in the case of fed-batch fermentation until glycerol was completely consumed. The highest PHA concentration (10.9 g L^{-1}) and content (55.6 %) were achieved in fed-batch fermentation. These

values are 49.5 % and 18.7 % higher than values of PHA concentration (5.5 g L^{-1}) and content (45.2 %) achieved in batch fermentation. The addition of rapeseed meal hydrolysates was necessary because PHA production ceased when FAN was depleted even though glycerol was still present in the broth. In both cases presented in Figure 3, the 3HV was 9.8 % and 3.3 % at 10 h and 58 h, respectively. The 3HV content remained constant after 58 h in fed-batch fermentation.

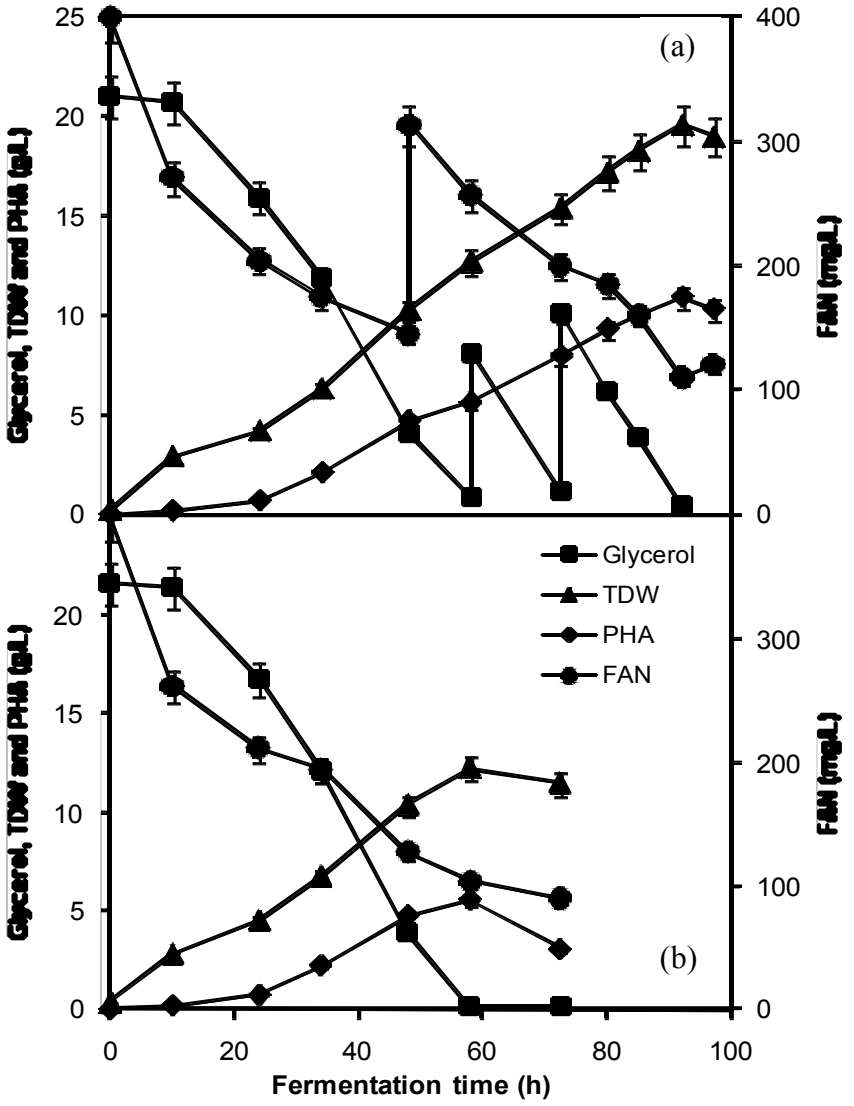

Figure 3. Glycerol, TDW, PHA and FAN concentrations during shake flask fermentations carried out on fed-batch (a) or batch (b) mode

4.3.2 Effect of salt concentration during shake flask fermentations

Two series of shake flask fermentations were carried out to study the inhibitory effect of salts (K_2SO_4 and NaCl) present in crude glycerol that will gradually accumulate in the broth during fed-batch fermentation. All fermentations were conducted with initial FAN and crude glycerol concentrations of 400 mg L^{-1} and 25 g L^{-1}, respectively. In the case of K_2SO_4 addition (Figure 4a) at the beginning of the fermentation, bacterial growth and PHA production was not significantly affected up to 20 g L^{-1} K_2SO_4 concentration. When initial K_2SO_4 concentrations of 2 to 20 g L^{-1} were used, glycerol was totally utilized within 40 h. TDW varied between 11.8 and 10.2 g L^{-1} and PHA production between 4 and 3.1 g L^{-1} (PHA contents of 34.7 % to 30.4 %). However, in shake flask fermentations supplemented with a K2SO4 concentration of 25 g L^{-1}, TDW and PHA content were reduced to 6.6 g L^{-1} and 0.7 g L^{-1}, respectively (PHA content of 10.6 %). The presence of K_2SO_4 concentrations higher than 20 g L^{-1} led to significant inhibition of both bacterial growth and PHA accumulation.

In the case of NaCl addition, the inhibitory effect was observed at lower concentrations (Figure 4b). A NaCl concentration of 2 g L^{-1} resulted in 10.4 g L^{-1} CDW and 3.7 g L^{-1} PHA concentration (PHA content of 35.6 %). However, at higher NaCl concentrations TDW and PHA concentration were reduced significantly indicating an important drawback of NaCl containing crude glycerol as feedstock for industrial PHA production. Even at a NaCl concentration of 6 g L^{-1}, TDW and PHA concentration were reduced to 6.5 g L^{-1} and 1.4 g L^{-1}, respectively. The reduction of cell growth and PHA synthesis continued, as the initial concentration of NaCl was increased. The inhibitory effect of NaCl concentrations higher than 6 g L^{-1} was also observed regarding glycerol and FAN utilization. The results of this study are similar to those reported by Mothes *et al.* [10] regarding the effect of different salts on PHB fermentation using *C. necator* DSM 4058.

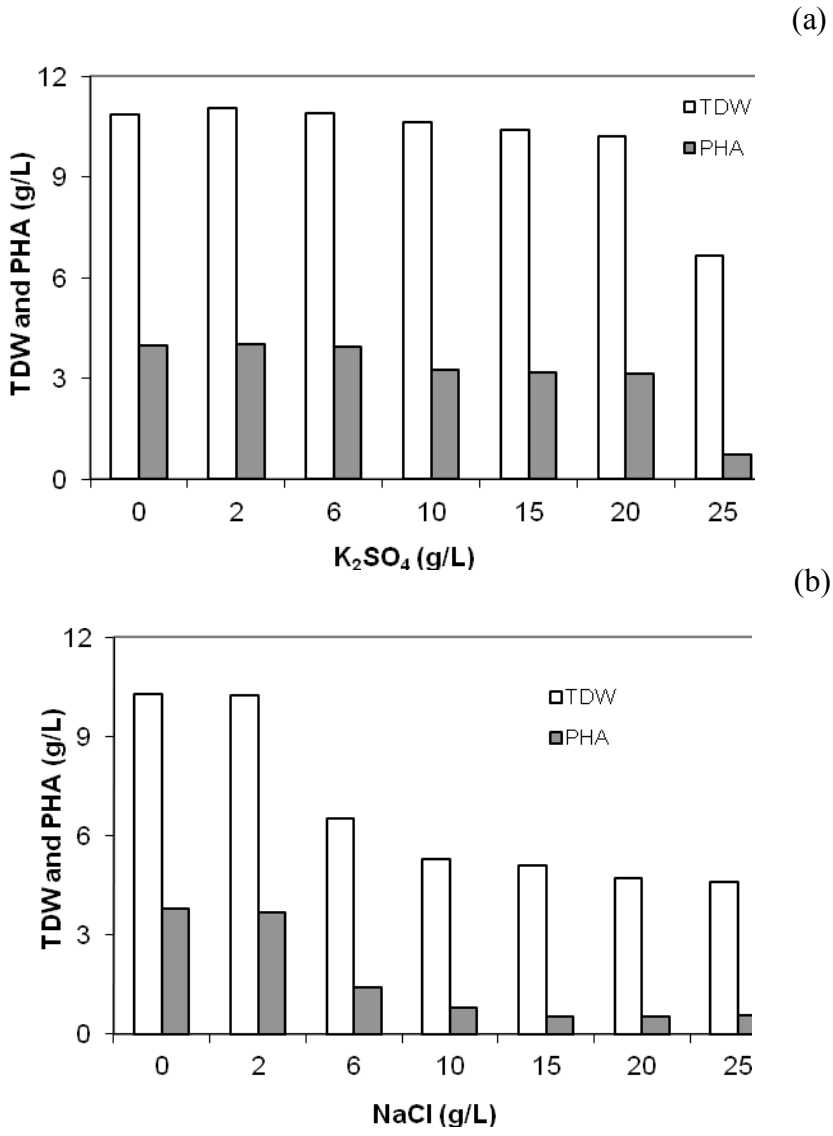

Figure 4. Effect of K_2SO_4 (a) and NaCl (b) concentration on TDW and PHA production during shake flask fermentations

4.3.3 Chemical structure and thermo-physical properties of PHAs

Preliminary fermentations in a bioreactor were carried out to isolate PHA samples for physicochemical analysis and optimization of bioreactor experiments will be the focus of a future study. 13C-NMR, DSC and TGA techniques were employed to identify the chemical structure and the principal thermal properties of PHAs produced in bioreactor fermentations. It should be mentioned that besides the P(3HB-co-3HV) copolymer, bioreactor experiments produced a complex PHA that contained 3HB, 3HV, a long-chain monomer and a five-carbon linear monomer. Due to the complexity of this PHA, a complete analysis of its structure and properties is currently under development. A more detailed analysis will be published in a subsequent study.

Figure 5 presents the chemical displacements of P(3HB-co-3HV) samples into the 13C-NMR spectrum corresponding to the carbons of the PHA chain. The chemical shift signals obtained are straightforward with the peak assigments for hydroxybutyrate (3HB) and hydroxyvalerate (3HV) units. The major peaks at 19.67, 40.99 and 67.54 ppm are due to chemical signals of methyl (1), methylene (3), and methine (2) groups, respectively, in the 3-hydroxybutyrate unit (3HB). Additional peaks located at 9.31, 26.59, 38.74, and 71.73 ppm are assigned to chemical displacements of the C–H stretching vibrations of groups CH_3 (5), CH_2 (6 and 8), and CH (7), respectively, in the 3-hydroxyvalerate unit (3HV). These signals are in agreement with chemical displacements reported by other authors for P(3HB-co-3HV) copolymers [26, 27]. 13C-NMR spectrum confirmed that *C. necator* was able to produce P(3HB-co-3HV) copolymers by employing a bioprocessing strategy based on the utilization of rapeseed meal hydrolysates and crude glycerol generated from industrial biodiesel production processes.

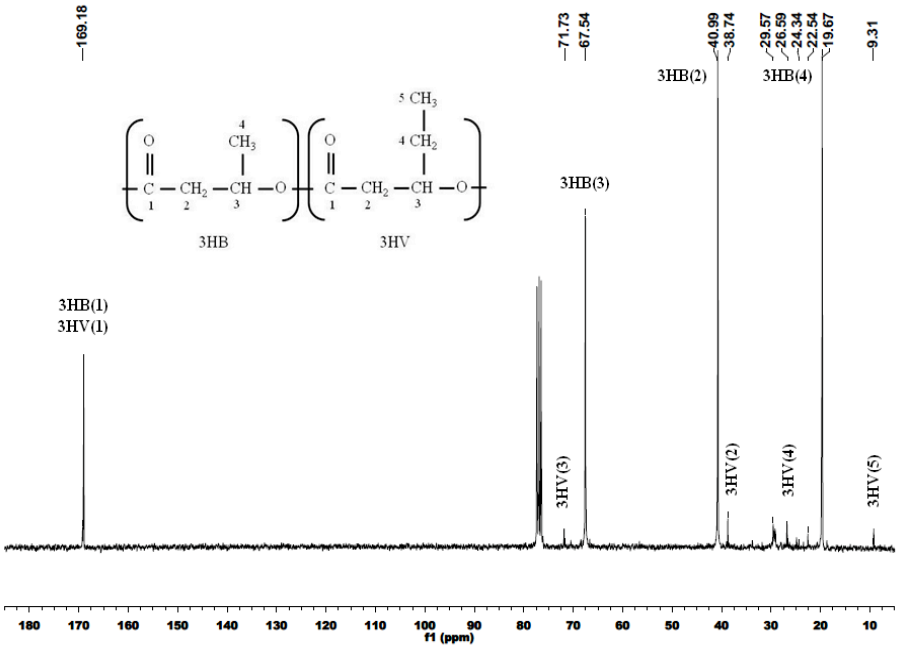

Figure 5. Proton-noise-decoupled ^{13}C-NMR spectrum at 125 MHz of P(3HB-*co*-3HV) produced from rapeseed meal hydrolysates and crude glycerol

Thermal properties of P(3HB-co-3HV) and complex PHA samples were obtained by DSC and TGA techniques. Table 2 presents 3HV molar fraction, glass transition temperature (T_g), melting temperature (T_m), enthalpy of fusion (ΔH_m), percentage of crystallinity (% Xc) and degradation temperature (T_d(50%)) for P(3HB-co-3HV). The properties for the PHAs obtained in this study are similar to those reported in other publications for P(3HB-co-3HV) produced with different microorganisms. The 3HV molar fraction was calculated based on the proportionality between the 3HV fraction and the melting temperature which was reported by Bluhm *et al.* [26] for copolymer containing between 3 and 29 % of 3HV on a molar basis. This value was confirmed by calculating the area ratio of peaks for methyl groups (1 and 5) corresponding to the 3HB and 3HV units in the 13C-NMR spectrum (Figure 5). In addition, Table 2 presents the properties of the complex PHA produced by *C. necator* DSM545.

Table 2. Thermo-physical properties of P(3HB-*co*-3HV) and complex PHA produced during bioreactor fermentations and comparison with values reported by other authors

Polymer	3HV (mol%)	T_g (°C)	T_m (°C)	ΔH_m (J/g)	$\%X_c$	$T_{d(50\%)}$ (°C)	Reference
P(3HB-*co*-3HV)	12	-1.9	155	72.61	49	271	This work
P(3HB-*co*-3HV)	4.1	2.3	163	-	-	252	[28]
P(3HB-*co*-3HV)	8	-1.2	169	49.00	33	-	[5]
P(3HB-*co*-3HV)	12	-	153	66.40	45	-	[29]
P(3HB-*co*-3HV)	30	2.1	111	-	-	352	[30]
Complex PHA	-	-2.1	141	44.11	29	231	This work

Figure 6 presents the TGA curves for both PHA samples. It can be noted that the complex PHA has a lower degradation temperature than P(3HB-co-3HV). The degradation profile of complex PHA can be a result of crosslinking among polymeric side chains [31]. From this profile, two degradation stages can be oberved, one from 175 to 245 °C and the second until approximately 420 °C. This kind of two-stage thermal degradation has been also reported by other authors for medium-chain and long-chain-length PHAs [32, 33].

As it was mentioned before, Cavalheiro *et al*. [11] employed *C. necator* DSM545 for the production of P(3HB-co-4HB-co-3HV) terpolymers using crude glycerol and 4HB/3HV precursors (i.e. butyrolactone and propionic acid). In this study, P(3HB-co-3HV) and more complex PHAs were produced entirely from industrial by-product streams. This could be attributed to the presence of different amino acids in rapeseed hydrolysates. The protein of rapeseed meals contains predominantly glutamic acid, while other amino acids (e.g. threonine, methionine, isoleucine and valine) are present in significantly lower and more or less comparable quantities [34]. Steinbüchel and Lütke-Eversloh [24] reported that specific amino acids such as valine, isoleucine, threonine and methionine are precursors for 3HV synthesis because they are catabolized

via propionyl-CoA. In addition, Yoon *et al.* [35] have reported that small amounts of threonine, isoleucine and valine could increase the production of 3HV during fermentation with Alcaligenes sp. SH-69, which was identified as a microorganism capable of producing the copolymer P(3HB-co-3HV) from single carbon sources. Lee *et al*. [5] reported that 3HV monomer composition in P(3HB-co-3HV) produced by *C. necator* H16 can be regulated in the range of 0 – 23 mol % by changing culture parameters such as initial pH, nitrogen source and nitrogen concentration.

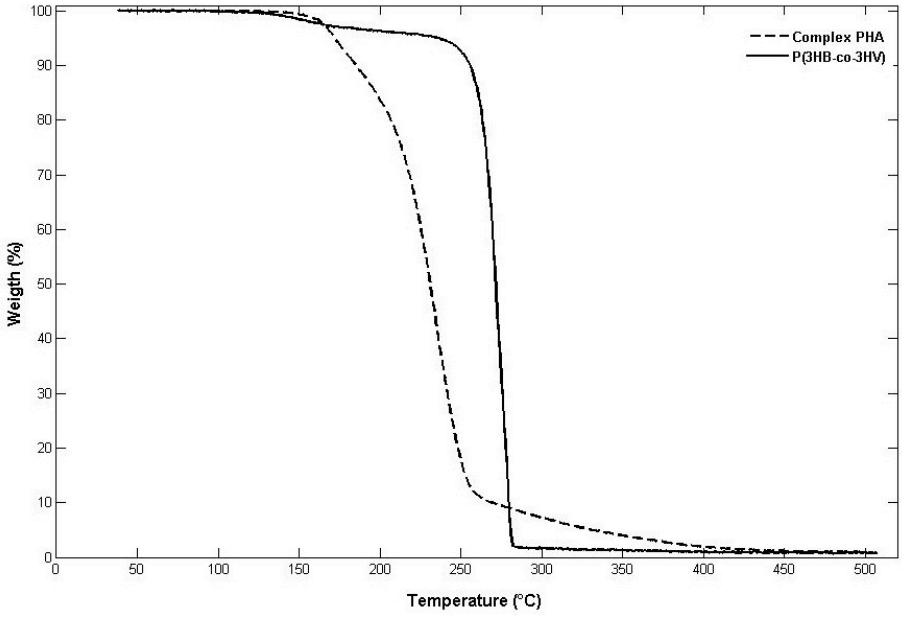

Figure 6. Thermogravimetric analysis (TGA) curves for P(3HB-*co*-3HV) copolymer and complex PHA produced from rapeseed meal hydrolysates and crude glycerol

Preliminary two-dimensional NMR studies involving heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectroscopy techniques indicated that a monomer with structure similar to glutamic acid has been produced (this will be presented in a future publication). In addition, NMR results suggest that the long-chain monomer found into the complex PHA structure could correspond to 3-hydroxynonanoate. A complete quantification and

characterization of the amino acids present in rapeseed hydrolysates is also necessary in order to identify the specific amino acids associated with the biosynthesis of these complex monomers.

4.4 Conclusions

This study shows the potential of rapeseed hydrolysates as nutrient supplements for the production of PHAs. The utilization of crude by-products from the biodiesel industry (rapeseed hydrolysates and crude glycerol), by *Cupriavidus necator* DSM 545, as the sole raw materials for PHA production could lead to the replacement of expensive commercial carbon sources, nutrient supplements and monomer precursors. This advantage could be coupled in this study with the production of PHA copolymers with better thermal and mechanical properties than PHB.

4.5 References

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Chapter 5

Valorization of confectionary industry wastes for the microbial production of polyxydroxyalkanoates

5.1 Introduction

The most promising alternative to petroleum-derived plastics is the production of biodegradable and durable polymers that can be produced from renewable resources [1, 2]. Polyhydroxyalkanoates (PHA) is one of the most promising families of biodegradable plastics that can be produced as intracellular energy-reserve granules via microbial fermentation of renewable resources.

PHAs have numerous potential applications including food packaging materials, biocomposite production, speciality biopolymers for medical applications, agriculture and flushable hygiene products [3]. They can be accumulated intracellularly by more than 300 microorganisms under unbalanced growth conditions.

Poly(3-hydroxybutyrate) is the most widely studied member of the PHA family and the first one that has been produced at industrial scale. It is a homopolymer of 3-hydroxybutyric acid units and can be produced by different bacterial species including *Cupriavidus necator* under the limitation of a nutritional element such as N, P, Mg, K, O, or S in the presence of excess carbon source.

The commercialization of PHB is currently hindered by the significant cost of the conventional carbon sources (e.g. glucose, sucrose,

vegetable oils) used during fermentation that account for up to 50 % of the total production cost. This means that research initiatives should focus on the development of technologies that utilize industrial or agricultural waste streams and by-products of negligible cost for the production of PHAs. Food processing waste streams are regarded as unique renewable feedstocks that could be used in future bioprocesses for the production of biopolymers. The utilization of food processing wastes will eliminate severe environmental burdens caused by the current disposal, treatment or recycling of food waste streams and petroleum-derived polymers. Food industry processing waste streams such as whey, potato processing waste, molasses and waste vegetable and plant oils are some examples that have been considered for the production of PHAs [2, 4, 5].

The main objective of this study was to investigate the microbial production of PHB from confectionary industry waste streams through the development of a three-stage bioprocess. The bioprocessing stages include:

1. Solid state fermentation (SSF) of *Aspergillus awamori* growing on bran-rich wheat milling by-products to produce amylolytic and proteolytic enzymes;

2. Enzymatic conversion of waste streams into a nutrient-rich fermentation feedstock;

3. PHB production via fermentation with *Cupriavidus necator* DSM 4058.

Initially, expired flour-based food for infants returned from the market and waste streams generated from the production line of flour-based food for infants (FBFI) were converted into a nutrient-rich fermentation feedstock. The macromolecules in FBFI (i.e. starch, protein) were hydrolyzed using crude enzymes (i.e. amylase, protease) produced via solid state fermentation of *Aspergillus awamori*. FBFI hydrolysates were tested as fermentation media in shake flask and bioreactor fermentations for the production of PHB. The feasibility of bioplastic production from flour-based waste streams has been demonstrated.

5.2 Materials and methods

5.2.1 Microorganisms

Solid state fermentations were carried out with an industrial strain of *Aspergillus awamori* 2B. 361 U2/1 (ABM Chemicals Ltd., Woodley, Cheshire, UK). Submerged fermentations for the production of PHB were carried out with *Cupriavidus necator* DSM 4058.

5.2.2 Raw material

The renewable resource used for the production of fermentation media was out-of-date commercial food for infants and processing waste from the production line of the same product. These streams will be called flour-based food for infants (FBFI) that mainly contains 84.8 % starch, 7.3 % protein and various minerals.

5.2.3 Solid state fermentation

Solid state fermentation of bran-rich wheat milling by-products (BWMB) was carried out in 250 mL shake flasks for the production of various enzymes. BWMB (5 g) were initially sterilized in shake flasks at

121°C for 30 min. The desired *A. awamori* spore concentration was formed in sterile distilled water for distributing the spores throughout the sterilized wheat bran. The flasks were placed into a 200 rpm shaker and incubated for 3 days at 30°C. The final moisture content was 65 % on a wet basis (wb).

5.2.4 FBFI hydrolysis

At the end of the SSF, the solids were transferred into a 1 L pre-sterilized Duran bottle that contained 200 mL of an aqueous suspension of FBFI. The pH was adjusted to 4.5. The reaction mixture was stirred with a magnetic stirrer in a water bath at 60°C for 24 h. The purpose of this experiment was the enzymatic hydrolysis of the starch and protein content in FBFI. Samples of 1 mL reaction mixture were taken at random intervals during hydrolysis and transferred to 2 mL Eppendorf tubes that contained 1 mL of trichloroacetic acid (5 %, w/v) to deactivate the enzymes. After the end of hydrolysis, the solids were separated by centrifugation (3000×g) from the crude hydrolysate that was used as fermentation medium for PHB production in both shake flasks and bioreactor experiments. The pH of the medium was adjusted to 6.6 to 6.8 and subsequently the liquid was filter sterilized using a 0.2 μm filtration unit. The hydrolysate was rich in glucose, sources of nitrogen (e.g. amino acids and peptides) and various other nutrients (e.g. phosphorous). In some cases that a higher Free Amino Nitrogen (FAN) concentration was required at the beginning of PHB fermentations, yeast extract was added into the hydrolysate prior to the filter sterilization stage.

5.2.5 PHB fermentation

FBFI hydrolysates were used in shake flasks and bioreactor fermentations for PHB production. A volume of 50 mL of FBFI hydrolysate was added in 250 mL presterilized shake flasks (121°C, 20 min) under aseptic conditions. A 16 h inoculum was prepared with 10 g L-1 glucose, 10 g L-1 yeast extract and 5 g L-1 peptone, and a volume of 1 mL was added to each flask under aseptic conditions. Shake flask fermentations were also carried out with commercial nutrient supplements including 10 g L-1 yeast extract and 5 g L-1 peptone. The flasks were placed in a shaker at 30 °C and agitation of 200 rpm. A whole shake flask was used each time as a sample

for the analysis of fermentation kinetics.

Batch fermentations were also carried out in a 3 L bioreactor (1.5 L working volume) using FBFI hydrolysate medium in three fermentations and commercial nutrient supplements in two fermentations. An inoculum of 10% (v/v) was used in all bioreactor fermentations. The pH and temperature were controlled at 6.7 - 6.9 and 30 °C throughout fermentation. Aeration rate was kept at 1 vvm. The agitation rate was set between 300 and 700 rpm to maintain dissolved oxygen (DO) above 30% of saturation.

5.2.6 Analytical methods

Glucose was analyzed with an enzymatic method (GLUCOSE PAP SL, ELITECK). FAN concentration was analyzed by the ninhydrin colorimetric method promulgated in the European Brewery Convention [6]. Phosphorus content in liquid samples was analyzed by the method published by Herbert *et al*. [7].

Samples (2 – 10 mL) from bacterial fermentations were taken at random intervals to measure total dry weight, PHB, glucose, FAN and phosphorus. Each sample was initially centrifuged at 3,000 × g for 10 min and the sediment was subsequently washed with distilled water and centrifuged. The supernatant was used for the analysis of glucose, FAN and phosphorous. The solids were re-suspended in acetone and transferred into universal bottles. Dry weight measurements were carried out by drying the solids at 50 ºC and cooling in a desiccator to constant weight. PHB was measured following the protocol proposed by Riis and Mai [8], employing gas chromatography (Fisons 8060) equipped with a Chrompack column (60 m × 0.25 mm).

5.2.7 PHB characterisation

PHB extraction was carried out by the methodology proposed by Hahn et al. [9]. Fourier transform infrared spectroscopy (FTIR) spectra were recorded with a NICOLET FTIR 520 spectrometer. Thermal properties were determined by differential scanning calorimetry (DSC) with a

PERKIN-ELMER PYRIS 1 calorimeter, calibrated with indium and n-heptane standards.

5.3 Results and discussion

5.3.1 Production of fermentation medium

Efficient starch hydrolysis can be achieved only in the case that the starch granules are gelatinized prior to enzymatic treatment. The waste streams used in this study have been processed at a temperature of more than 85 °C and therefore the starch granules have been gelatinized. Therefore, further gelatinization of starch prior to hydrolysis was not necessary. This is an additional processing advantage as a heat treatment stage can be avoided. For this reason, the temperature used during hydrolysis was 60 °C which is the optimum for glucoamylase produced by *A. awamori*. Each hydrolysis reaction was completed in 24 h.

Figure 1 shows the glucose and FAN production during enzymatic hydrolysis for each by-product concentration employed (40, 60, 80 g L-1 FBFI). The maximum glucose concentrations were 35.5 g L^{-1}, 53.2 g L^{-1} and 70.8 g L^{-1}, respectively. The starch to glucose conversion yield achieved was around 95% for all by-product concentrations employed. In the case of FAN, the maximum concentrations achieved were 166.5 mg L^{-1}, 212 mg L^{-1} and 237.65 mg L^{-1}, respectively. The FAN content was considered low for microbial growth and therefore supplementation of the medium with commercial nutrient supplements was necessary.

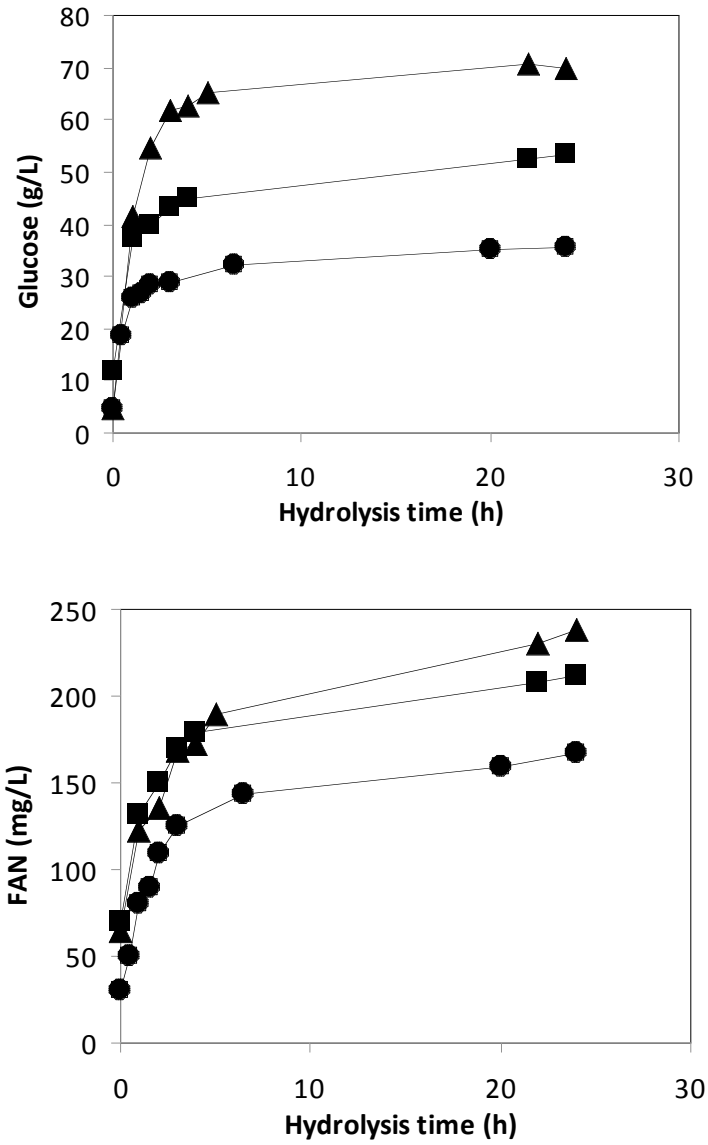

Figure 1. Glucose and FAN concentration as a function of enzymatic hydrolysis time. Symbols: (●) 40 g/L FBFI, (■) 60 g/L FBFI, and (▲) 80 g/L FBFI

5.3.2 Shake flask fermentations

After the enzymatic hydrolysis of the confectionary industry waste streams, the crude hydrolysate produced was utilized as fermentation medium in shake flask fermentations with *C. necator*. It should be stressed that the crude hydrolysate contains various nutrients that could be used for microbial growth. These nutrients have been released either from FBFI or solids that remained after the solid state fermentation process. The utilization of this nutrient-rich hydrolysate could lead to the reduction of commercial nutrient supplements that represent a significant cost to PHB production economics.

Table 1 presents the results from four shake flask fermentations with *C. necator* in FBFI hydrolysates that contained various initial glucose and FAN concentrations. Fermentation H1 was carried out without yeast extract supplementation, whereas various quantities of yeast extract were added in fermentations H2 – H4. Fermentation H1 showed that the hydrolysate could lead to the production of PHB. However, in bioreactor applications the FAN concentration should be increased in order to enhance microbial growth prior to PHB accumulation. The highest PHB concentration (5.95 g L^{-1}) was achieved in fermentation H4. Fermentation H3 shows that high initial FAN concentrations may lead to low PHB accumulation.

Table 1. Shake flask fermentations with FBFI hydrolysates.

Fermentation	Glucose (g L^{-1})	FAN (mg L^{-1})	Time (h)	TDW (g L^{-1})	PHB (g L^{-1})	PHB (%)
H1	34.4	171	50	10.05	3.86	38.4
H2	36.3	272.7	28	11.2	4.7	41.8
H3	33.3	656.4	15	19.6	1.1	5.6
H4	61.2	492.3	44	14.95	5.95	39.8

Shake flask fermentations were also carried out with commercial nutrient supplements (Table 2). The glucose concentration in C1 and C2 were closer to H1 and H2 respectively. Although FAN concentration was significantly lower in H1 and H2 as compared to C1 and C2 respectively, the total dry weight (TDW) and PHB concentration were at a similar level.

This probably occurred because FBFI hydrolysates were richer fermentation feedstocks as compared to the chosen commercial nutrient supplements. This comparison indicates that FBFI hydrolysates could be employed for PHB production.

Table 2. Shake flask fermentations with commercial nutrient supplements

Fermentation	Glucose (g L^{-1})	FAN (mg L^{-1})	Time (h)	TDW (g L^{-1})	PHB (g L^{-1})
C1	30	500	30	10.25	3.95
C2	40	500	50	10	4.64

5.3.3 Bioreactor fermentations

Three batch fermentations were conducted using the FBFI hydrolysate medium. These fermentations were supplemented with different quantities of yeast extract in order to enhance the FAN content in the fermentation broth. Batch fermentations were also carried out using commercial glucose (20-30 g L^{-1}) and commercial nutrient supplements, i.e. yeast extract (10 g L^{-1}) and peptone (5 g L^{-1}). The main target of these experiments was to compare commercial media with FBFI-based media for the production of PHB.

Table 3 presents the initial concentrations of glucose, FAN and phosphorus at the beginning of four fermentations carried out in the bioreactor. It also presents fermentation duration, total dry cell weight, final PHB concentration and PHB content in microbial cells.

Fermentations B1-B3 were carried out with FBFI hydrolysate under optimal operating conditions. In these fermentations, significant glucose and phosphorus concentrations were still present at the end of the fermentation, whereas FAN was almost completely consumed. This means that fermentations B1-B3 stopped due to nitrogen or other micronutrient limitations. It can be also deduced that higher FAN concentration led to higher total dry weight, PHB concentration, PHB content and residual biomass. However, fermentations B1-B3 stopped prematurely due most probably to insufficient nutrient content.

Fermentations were also carried out on commercial nutrient supplements (i.e. yeast extract, peptone) using optimal operating conditions as in the case of fermentations B1-B3, where PHB accumulation and microbial growth was significantly lower than fermentation B1. This is another indication that FBFI hydrolysates provide a richer feedstock for PHB production. Fermentation B4 was carried out under oxygen limitation and supplementation of commercial sources of nutrients.

Table 3. Fermentations in bioreactor

Fermentation	Glucose ($g\ L^{-1}$)	FAN ($mg\ L^{-1}$)	Phosphorus ($mg\ L^{-1}$)	Time (h)	TDW ($g\ L^{-1}$)	PHB ($g\ L^{-1}$)	PHB (%)
B1	28.4	457.9	267.4	8	13.8	1.55	11.2
B2	27.3	541.7	325.9	8	15.7	2.5	15.9
B3	31.4	656.2	328.2	8	18.7	3.2	16.9
B4	21.8	615.3	-	14	12.7	3.6	28.3

Figure 2 shows the consumption profiles of glucose, FAN and phosphorus as well as the production profiles of total dry cell weight, PHB and residual biomass in fermentation B3.

The glucose consumption stopped at around 8 h and the total concentration consumed was 21.3 $g\ L^{-1}$. As in the case of fermentations B1 and B2, glucose consumption, microbial growth and PHB accumulation stopped at the same time as the FAN concentration was depleted from the fermentation broth.

The final phosphorus concentration was around 123 $mg\ L^{-1}$. Thus, phosphorus depletion was not the reason that triggered the end of microbial growth and PHB accumulation. Consequently, this fermentation probably stopped as the fermentation broth run out of either nitrogen or other nutrients. The higher amount of FAN resulted in increased TDW, PHB and residual biomass concentration as compared to fermentations B1 and B2.

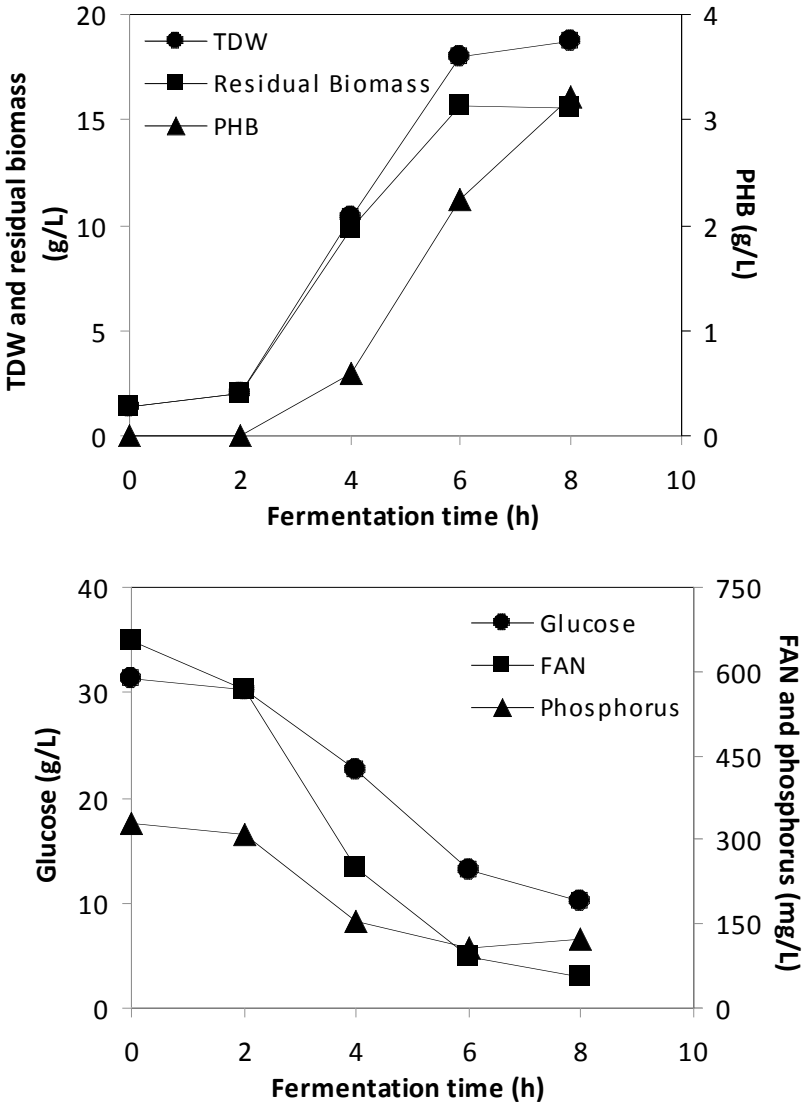

Figure 2. Profiles of glucose, FAN, phosphorus, TDW, residual biomass and PHB concentration during fermentation B3 with initial glucose and FAN concentrations of 31.4 g L^{-1} and 656.2 mg L^{-1} respectively

Fermentation B4 was carried out with commercial sources of nutrients. The major difference with all previous fermentations was the fact that it was carried out under oxygen limiting conditions after 6 h fermentation. This was achieved by controlling the DO concentration at 8-20% of saturation. Figure 3 presents the consumption profiles of glucose and FAN as well as the production profiles of total dry cell weight, PHB and residual biomass.

The initial glucose concentration was completely consumed at 14 h fermentation. However, there was still more than 250 mg L^{-1} of FAN concentration at the end of the fermentation. After the DO concentration dropped below 20%, oxygen limiting conditions were imposed to microbial cells and this led to the initiation of PHB accumulation.

Figure 3 shows that the FAN consumption rate was reduced. However, FAN was never the limiting nutrient during fermentation. The results obtained in fermentation B4 indicate that the presence of nutrients during PHB accumulation is necessary. In all previous fermentations, PHB production stopped because there was not enough amount of nutrients to sustain the maintenance of microbial cells.

The experiments showed that FBFI hydrolysates could be used for PHB production but further work is required in order to optimize media composition.

FTIR and DSC techniques were applied so as to confirm both chemical structure and thermal properties of the biopolymer produced. FTIR spectrum confirmed PHB structure because all characteristic bands present in the PHB molecule were identified (results not reported). Thermal analysis showed that FBFI hydrolysates promote the biosynthesis of a PHB biopolymer with a melting point, fusion enthalpy and crystallinity index of 169.1°C, 88 J/g and 58.9 %, respectively.

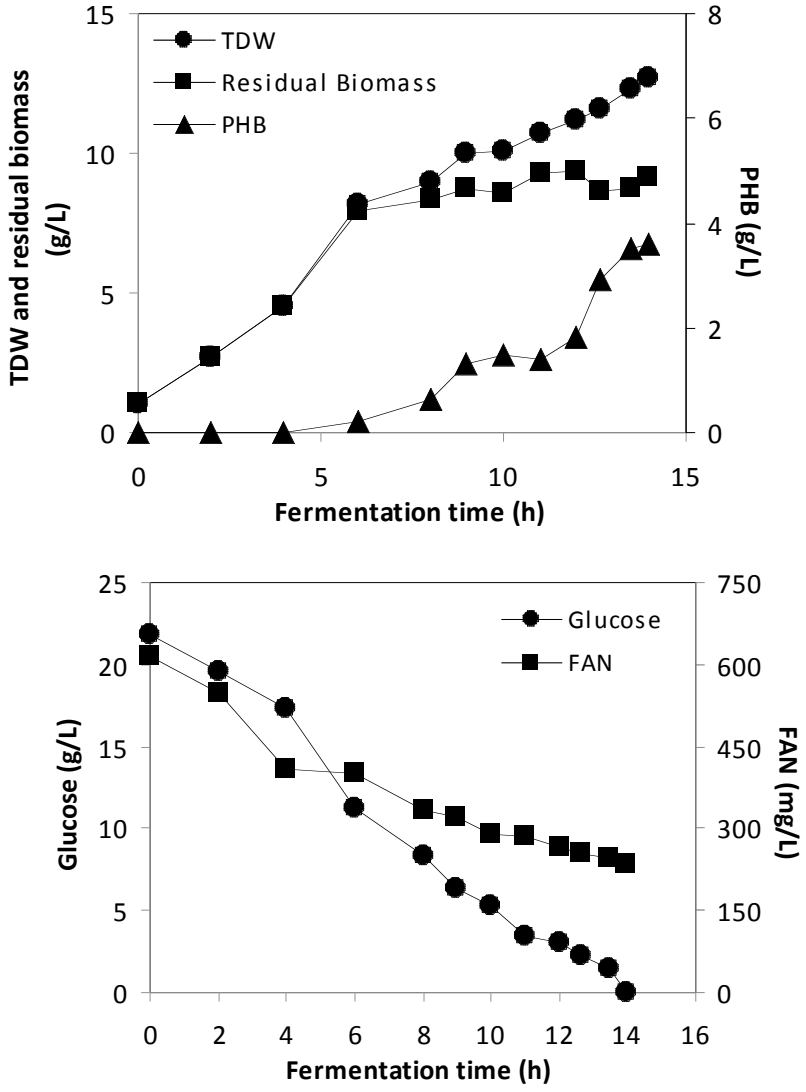

Figure 3. Profiles of glucose, FAN, TDW, residual biomass and PHB concentration during fermentation B4 with initial glucose and FAN concentrations of 21.8 g/L and 615.3 mg/L respectively

5.4 Conclusions

FBFI hydrolysates could be employed as fermentation media for PHB production. FBFI hydrolysates did not contain enough nitrogen. Nevertheless, shake flask experiments showed that the utilization of FBFI hydrolysate could yield higher PHB concentrations compared to the use of commercial nutrient supplements. Bioreactor experiments showed that further optimisation of media composition and operating conditions is necessary.

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Chapter 6

Design and techno-economic evaluation of microbial biopolymer production from food industry wastes and agricultural crops

6.1 Introduction

Nowadays, petroleum is the main raw material that is used for the production of plastics. The annual capacity of approximately 150 million tons of petroleum-derived plastics produced worldwide is currently disposed mainly in landfills or it is incinerated. In addition, approximately 135 thousand tons of plastics are disposed to the sea on an annual basis. Therefore, petroleum-derived plastic production will be eventually hindered by the depletion of petroleum and the inevitable environmental pollution that is caused by their disposal. The production of biodegradable plastics is the only solution to the replacement of petroleum-derived plastics.

Polyhydroxyalkanoates (PHA) is a family of biodegradable plastics that can be produced intracellularly by many microorganisms through fermentation of renewable resources. Three of the most important members of PHAs are poly-(3-hydroxybutyrate) (PHB), poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV and poly-(3-hydroxybutyrate-co-3-hydroxyhexanoate) P(3HB-co-3HHx).

However, the commercial production of these bioplastics is hindered by the high production cost that is mainly attributed to the fermentation feedstock used and the downstream separation methodology employed. The only way to reduce the cost of PHA production is to advance

research on the valorization of crude feedstocks such as food processing wastes and agricultural crops. This should be coupled with techno-economic evaluation in order to assess the potential of each process alternative. The utilization of waste and by-product streams in existing food plants will improve both PHA production economics and environmental problems caused by the current disposal methods of food processing wastes.

Chanprateep [1] has reported that the price of PHAs produced by current and potential large scale manufacturers is in the range of 1.5 – 5 €/kg. Several costing studies have been carried out in the last 15 years to evaluate the production cost of PHAs from various renewable resources including pure carbon sources, such as glucose and vegetable oils and industrial waste streams, such as whey and crude glycerol generated from biodiesel production plants [2-5]. The PHA production cost estimated in these studies varied in the range of 1.94 – 6.08 €/kg. The final cost is highly dependent on the production capacity of the plant, the renewable raw material, the fermentation strategy (e.g. selected microorganisms, conversion yield and productivity) and the downstream separation process (e.g. mechanical, chemical or enzymatic disruption of bacterial cells).

The main objective of this study was to evaluate specific processes for the microbial production of PHAs. Design and techno-economic evaluation has been employed so as to compare two process flowsheets based on different initial raw materials, microorganisms and downstream separation strategies.

All required information used in this study was taken from literature-cited publications [6-13]. The processing schemes that were evaluated utilize food processing wastes and by-products (i.e. whey) and wheat as raw materials.

The fermentation process used in each flow sheet was carried out by recombinant *Escherichia coli* and *Cupriavidus necator*, respectively. Specific recovery methodologies for each microorganism have been employed.

The process flowsheets were divided into the upstream processing stage, where the food waste stream was converted into a fermentation

feedstock, the fermentation stage and the downstream processing stage, where PHAs were separated and purified via enzymatic or chemical methods.

6.2 Materials and Methods

Costing studies were based on preliminary economic analysis (accuracy ± 30%) that were carried out for the estimation of the total capital investment and operating cost for two process flowsheets used for the production of PHAs.

The simulation of both process flowsheets was based on batch operation, 330 days/year and 24 h/day. An annual production capacity of 2000 t PHAs per year has been assumed in all process flowsheets.

The design and costing studies were carried out with the software SuperPro Designer (Intelligen, Inc. V.7.0). The cost of various equipments employed in fermentation processes was based on information provided by Blanch and Clark [14]. The calculation of the capital investment and operating cost was based on techniques provided by the software. The operating life of the plant and the annual depreciation of the initial investment were assumed as 12 years and 10% of the fixed capital investment.

The cost of whey that is used as raw material in process flowsheet 1 is considered as negligible because it is the main waste stream of the dairy industry. Wheat is utilized as raw material for the production of PHB in process flowsheet 2. Representative market prices for wheat, corn starch

and purified glucose could be assumed as 0.14 \$ kg^{-1}, 0.22 \$ kg^{-1} and 0.5 \$ kg^{-1}, respectively. The cost of wheat hydrolysate that was used in process flowsheet 2 was assumed as 0.4 \$ kg^{-1} based on data provided by Koutinas et al [9] and Arifeen et al. [13].

6.3 RESULTS & DISCUSSION

6.3.1 Process flowsheet 1

The first process (Figure 1) focuses on the utilization of whey for the production of PHB. The information required for the design of the fermentation stage was taken from Ahn *et al*. [6] and Choi and Lee [7].

Whey is the main waste stream generated from the dairy industry that contains approximately, on a w/v basis, 4.5% lactose, 0.8% protein and 1.0% salts [6]. Whey is initially treated via ultrafiltration to remove the protein content and subsequently concentrated via evaporation. In this way, a whey solution containing up to 280 g/L lactose could be produced. This could be used as a feeding solution during PHB accumulation in a fed-batch fermentation to achieve a high PHB concentration and yield. The whey stream is heat sterilized and used as fermentation medium.

The fermentation is carried out by a recombinant strain of *E. coli* CGSC 4401, harboring the plasmid pJC4 containing the *Alcaligenes latus* PHA biosynthesis genes. Ahn et al. [6] reported that under optimal operating conditions a total dry cell weight, PHB concentration and PHB productivity of 119.5 g L^{-1}, 96.2 g L^{-1} and 2.57 g $L^{-1}h^{-1}$ could be achieved. A lactose to PHB conversion yield of 0.33 g g^{-1} was used in the simulation as

was reported by Choi and Lee [7].

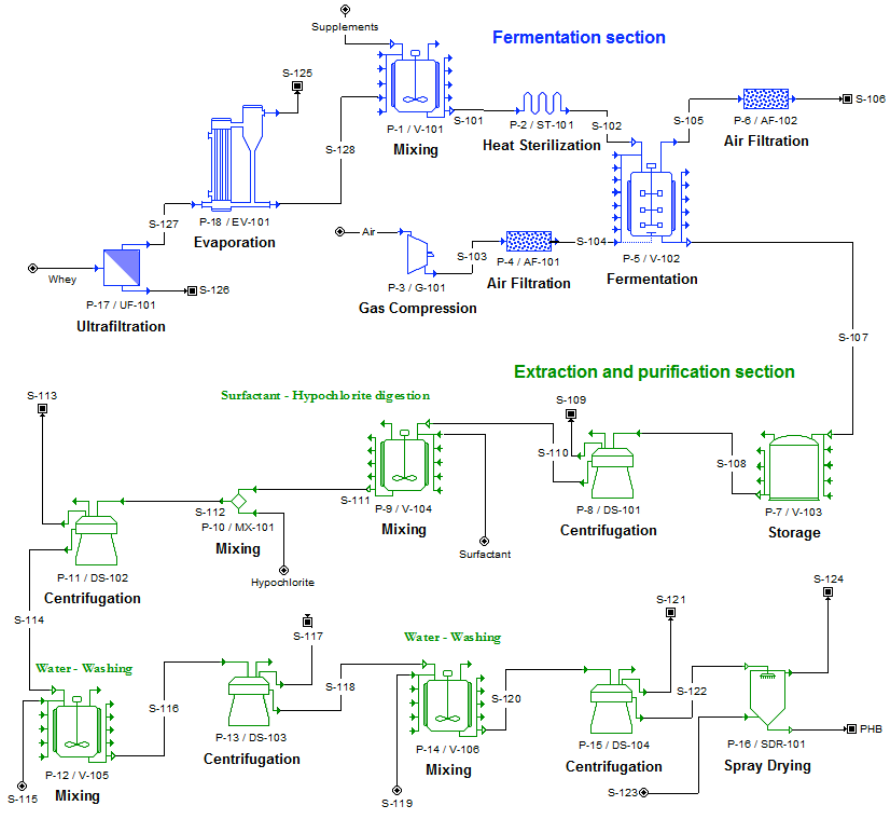

Figure 1. Process flowsheet based on whey utilization

The downstream separation strategy that has been employed in process flowsheet 1 for the purification of PHB from *E. coli* cells was based on a surfactant-hypochlorite digestion method. The process stages were taken from Choi and Lee [3, 8]. After the end of the fermentation, the bacterial cells are collected via centrifugation of the fermentation broth. Microbial cell lysis is carried out via combined surfactant-hypochlorite digestion. A surfactant solution of 1% (w/v) is added to the microbial biomass followed by hypochlorite digestion. This combined step results in

microbial lysis and separation of PHB from residual cell material. The aqueous solution containing the residual cell material is separated by centrifugation. Finally, PHB granules are purified via washing with water and drying via a spray-drying step.

6.3.2 Process flowsheet 2

The second process (Figure 2) focuses on the utilization of wheat for the production of PHB as the major product and gluten as a valuable by-product. The calculations were based on a wheat composition containing 68% starch, 15% gluten, 12% bran and 5% other components (on a dry basis).

Wheat is initially milled into whole wheat flour in a single stage using a hammer mill. The whole wheat flour is separated into two fractions. The first fraction (92% w/w) is used for gluten extraction (30% w/w of the total gluten content) and the generation of fermentation media that are used for PHB production. Gluten can be used as a high value by-product that can improve process economics. The amount of gluten that will be separated from whole wheat flour was decided by taking into consideration the amount of nitrogen required in the PHB fermentation stage.

The other fraction of whole wheat flour (8% w/w) is utilized as fermentation feedstock in a continuous fungal fermentation carried out by *Aspergillus awamori* for the production of amylolytic and proteolytic enzymes that are used for the hydrolysis of starch and gluten. After filtration of the fermentation broth, the filtrate is mixed with the whole wheat flour stream, from which the gluten has been partly removed, in order to initiate the hydrolysis of starch into glucose and protein into amino acids and peptides.

The solids from the fungal fermentation that are rich in fungal biomass are subjected to autolysis that generates an autolysate that is rich in various nutrients such as amino acids, peptides and phosphorus. This process has been described in several publications [9-11, 13]. Figure 2 does not present all these stages. The cost of wheat-derived feedstock production has been taken from Koutinas *et al*. [9] and Arifeen *et al*. [13].

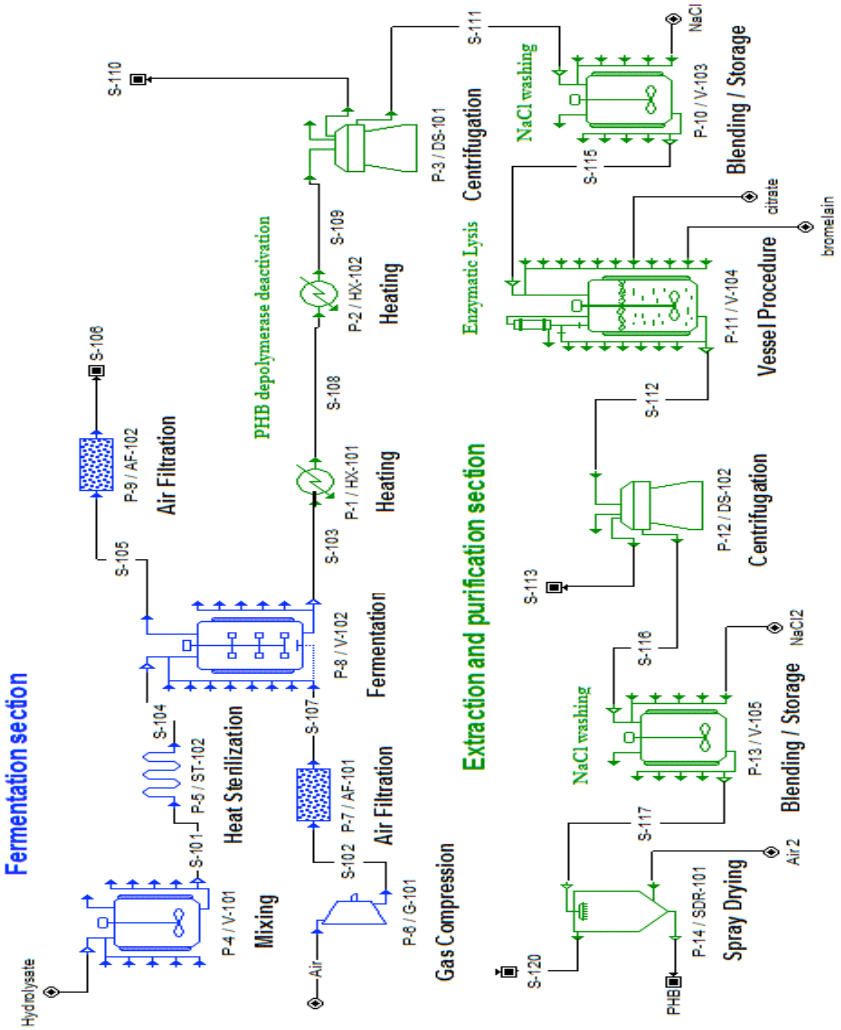

Figure 2. Process flowsheet based on wheat utilization

The hydrolysates and autolysates generated from flour hydrolysis and fungal cell autolysis respectively are used as fermentation media for the production of PHB via fermentation using *Cupriavidus necator* NCIMB

11599. Xu *et al*. [11] reported that fed-batch fermentations with wheat flour hydrolysates and fungal cell autolysates led to the production of 162.8 g L^{-1} PHB concentration and 93% (w/w) PHB content with a productivity of 0.9 g $L^{-1}h^{-1}$ and a glucose to PHB conversion yield of 0.47 g g^{-1}. These data have been used in this study to design the fermentation stage in process flowsheet 2.

The downstream separation strategy that has been employed in process flowsheet 2 for the purification of PHB from *C. necator* cells was based on enzymatic cell lysis using commercial enzymes (i.e. bromelain). The process has been developed by Kapritchkoff et al. [12].

Initially, the fermentation broth containing bacterial cells are treated at 85°C for 15 min and then at 95°C for 45 min in order to deactivate PHB depolymerase. The bacterial cells are subsequently separated via centrifugation and washed with a 0.85% (w/w) NaCl solution. Disruption of bacterial mass is achieved via enzymatic lysis at 50°C using a commercial bromelain formulation (2% w/w). The highest cell lysis is achieved at 10 h reaction, where 90% (w/w) of the total quantity of PHB has been recovered with a purity of 88.8%. Finally, the reaction solution is centrifuged to remove PHB granules, which are treated with a 0.85% (w/w) NaCL solution and dried using a spray drier.

6.3.3 Preliminary techno-economic analysis

Table 1 presents major costing data as were calculated for the production of PHAs based on the two process flowsheets presented above.

Table 1. PHA production cost from process flowsheets 1 and 2 based on an annual production of 2000 t

Process flowsheet	**1**	**2**
Biopolymer	PHB	PHB
Batches per year	197	48
Economic factors ($1,000)		
Equipment purchase cost	9,484	13,032
Working capital	355	401
Raw materials (per year)	2,088	3,183
Labour-dependent (per year)	707	477
Annual operating cost	6,841	5,185
Production cost ($/kg)	**3.42**	**2.58**

Based on an annual biopolymer production of 2000 t, the most efficient process in terms of the calculated production cost was process flowsheet 2 where wheat is used as raw material, *C. necator* NCIMB 11599 is used as the microorganism in the fermentation stage and enzymatic cell lysis is applied for the separation of PHB from residual cell material. This PHB production cost is within the range presented in the literature [1-5].

It should be stressed though that the production capacity employed in this study (2000 t) is lower than the one utilized in literature-cited publications [1-5].

This study indicates that in order to obtain a clear idea about the

commercial prospects of each PHA production process published in the literature, the cost-competitiveness of each process should be evaluated.

It should be stressed that wheat is an agricultural crop that is mainly used for food purposes and therefore there is a strong competition with the end-use proposed in this study. Therefore, further work is required in order to substitute wheat for flour-rich waste streams that are produced by various food industries (e.g. confectionary and cereal milling industries). Furthermore, costing studies should be combined with life cycle analysis studies so as to evaluate the environmental impact of proposed processes.

6.4 Conclusions

Two different process flowsheets have been evaluated for the production of PHB. The raw materials used were agricultural crops (i.e. wheat) and by-products or waste streams generated from food industry (i.e. whey).

The most efficient process in terms of the calculated production cost was process flowsheet 2 where wheat is used as raw material, *C. necator* NCIMB 11599 is used as the microorganism in the fermentation stage and enzymatic cell lysis is applied for the separation of PHB from residual cell material. Nevertheless, wheat is an agricultural crop used mainly with food purposes and the use of this agricultural products with other purposes, different than food markets, might create an end-use conflict.

The calculated cost per kg PHA produced from each process showed that preliminary costing studies should be carried out to evaluate the potential of experimentally developed processes. This is the only way to ensure that the most cost-competitive process will be commercialized. Moreover, this costing studies should be combined with life cycle analysis studies so as to evaluate the environmental impact of proposed processes.

6.5 References

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Chapter 7

Conclusions and Future Research Lines

7.1 Conclusions

The production of polyhydroxyalkanoates, a family of biodegradable polymers that can be produce intracellulary by numerous microorganisms, might be a potencial solution for the environmental problems caused by the masive utilization of petroleum derived plastics. Selecting a proper carbon source and an adequate bacterial strain can lead to a successful process for the efficient and economic competitive polyhydroxyalkanoates (PHAs) production.

In the present work, three different renewable sources (jerusalem artichoke, confectionary industry wastes and biodiesel by-products) were studied, in the laboratory scale, for the production of PHAs with the bacterium *Cupriavidus necator*.

In the first research study, a fungal strain of *A. awamori* was used to produce enzyme consortia via solid state fermentation (SSF) that could efficiently produce a jerusalem artichoke hydrolysate rich mainly in fructose with lower concentrations of glucose, FAN and phosphorus. Shake flask fermentations, with *Cupriavidus necator* DSM 4058, using JA hydrolysate as fermentation medium showed that PHB production is feasible. However, enrichment of JA hydrolysates with nutrient-rich supplements (e.g. oilseed meal hydrolysates) should be carried out in bioreactor applications. It should be stressed that industrial implementation of PHB production has not been established yet due to the high cost of conventional raw materials and

processing stages.

The utilization of non-food crops or industrial waste strains could assist in the commercialization of PHB production. JA cultivation for chemical and biopolymer production could be achieved in desertified land where water shortage is unavoidable and irrigation is not effectively achieved.

The second research study shows the potential of rapeseed hydrolysates as nutrient supplements for the production of PHAs. The utilization of crude by-products from the biodiesel industry (rapeseed hydrolysates and crude glycerol), by *Cupriavidus necator* DSM 545, as the sole raw materials for PHA production could lead to the replacement of expensive commercial carbon sources, nutrient supplements and monomer precursors. This advantage could be coupled in this study with the production of PHA copolymers with better thermal and mechanical properties than PHB.

Flour-based food for infants returned from the market and waste streams generated from its production line (FBFI) hydrolysates could be employed as fermentation media for PHB production. The third research study of this work showed that FBFI hydrolysates did not contain enough nitrogen for sufficient PHB accumulation by *Cupriavidus necator* DSM 4058. Nevertheless, shake flask experiments showed that the utilization of FBFI hydrolysate could yield higher PHB concentrations compared to the use of commercial nutrient supplements. Bioreactor experiments showed that further optimization of media composition and operating conditions is necessary for the production of cost-competitive and high-quality biopolymers.

Finally, two different process flowsheets have been economically evaluated for the production of PHB. The raw materials used were agricultural crops (i.e. wheat) and by-products or waste streams generated from food industry (i.e. whey).

The most efficient process in terms of the calculated production cost was process flowsheet 2 where wheat is used as raw material, *C. necator* NCIMB 11599 is used as the microorganism in the fermentation stage and

enzymatic cell lysis is applied for the separation of PHB from residual cell material. Nevertheless, wheat is an agricultural crop used mainly with food purposes and the use of this agricultural products with other purposes, different than food markets, might create an end-use conflict.

The calculated cost per kg PHA produced from each process showed that preliminary costing studies should be carried out to evaluate the potential of experimentally developed processes. This is the only way to ensure that the most cost-competitive process will be commercialized. Moreover, this costing studies should be combined with life cycle analysis studies so as to evaluate the environmental impact of proposed processes.

7.2 Future lines

Different future lines of research are defined in the present work.

Enrichment of jerusalem artichoke (JA) hydrolysates with nutrient-rich supplements (e.g. oilseed meal hydrolysates) should be carried out in bioreactor applications for succesfull PHB production, therefore further optimisation of JA meal hydrolysis to increase the concentration of fructose (and glucose) in the final hydrolysate will be necessary in order to develop fed-batch fermentations in a bioreactor. Moreover, future studies will focus on bioprocess design to assess the commercial potential of this bioprocess.

In the process comprising production of PHAs from crude glycerol and rapeseed hydrolysates, both residues from the biodiesel industry, future studies will focus on optimization of complex PHA production using crude glycerol and hydrolysates from oilseed meals and bioprocess design to assess the commercial benefits of this bioprocess.

Flour-based food for infants (FBFI) did not contain enough nitrogen, therefore bioreactor experiments showed that further optimisation of media composition and operating conditions is necessary.

Concerning to the design and economic evaluation of PHB

production proceses, future studies will focus on a deeper economical evaluation of the PHAs producing processes. Nevertheless, not only cost competitiveness must be taken in account, but also competition of fermentation raw materials in the food markets, thus production of PHAs shouldn't represent a threat for the global food security, such as it could be in the case of wheat utilization.

Moreover, future studies will also focus on the evaluation of more PHA production processes that are available in the literature and the application of sensitivity analysis in order to assess the effect of production capacity and other parameters on the final production cost.