Extraction of PHAs from *Rhodovulum Sulfidophilum DSM 1374* bacteria by non-chlorinated solvents

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Horizon 2020
European Union Funding
for Research & Innovation

BBI JU

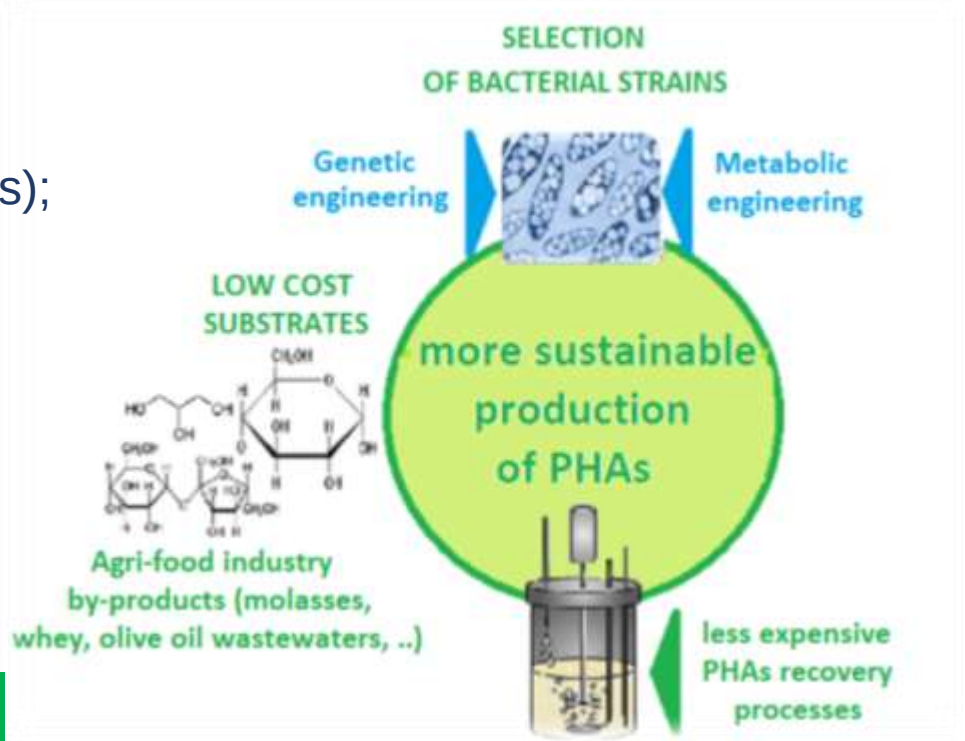
Bio-based Industries Consortium

Actions to increase the use of PHAs in commodities

The price of PHAs is still **very high** (**5-8 € / kg**) compared to conventional plastics (about 10 times higher) or to some other bioplastics (PLA) that limit their use mainly to high value medical applications. Given the global growth of bioplastics, at the end of 2021, the installed manufacturing capacity was of about 48 kt/annum and the PHA manufacturers aim for a manufacturing capacity of about 570 kt/annum by 2027 …. this will lead to a reduction in costs.

The other possible actions:

- ✓ Use of **low-cost carbon sources** (waste/surplus of agro-food industries);
- ✓ Development of **genetically modified microorganisms** more performing → greater PHA accumulation → higher productivity;
- ✓ Use of low-cost materials such as starch or low-cost and highly-available lignocellulosic fibers, coming from agricultural and industrial crops, into composites based on PHAs to **reduce the cost of the final products** used in different environments where the biodegradability is required (agriculture, marine restoration actions, and so on…)
- ✓ Development of **less expensive and more environmentally friendly** intracellular PHA extraction processes from microbial biomass (most used process based on chlorinated solvents (**chloroform**))



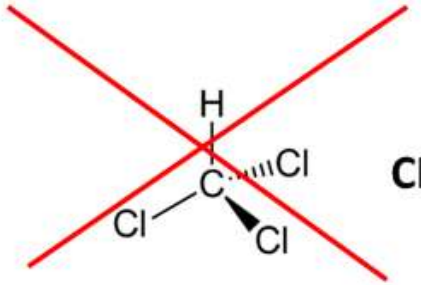
PHA extraction using non-chlorinated solvents

The high PHA price is greatly affected by the cost of the downstream processing necessary for their extraction and recovery from bacterial biomass that involves mainly the use of halogenated solvents, as chloroform (**CHL**). Non-chlorinated solvents such as **cyclohexanone** (**CYC**) and three ionic liquids (**ILs**) were tested:

1-ethyl-3-methylimidazolium dimethylphosphate, [EMIM][DMP]
1-ethyl-3-methylimidazolium diethylphosphate, [EMIM][DEP]
1-ethyl-3-methylimidazolium methylphosphite, [EMIM][MP]
to extract PHAs from the purple bacterium *Rhodovulum Sulfidophilum.*

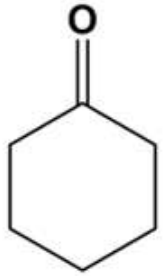
DIPARTIMENTO FARMACIA

PHA solvents and non-cell membrane solvents



CHL

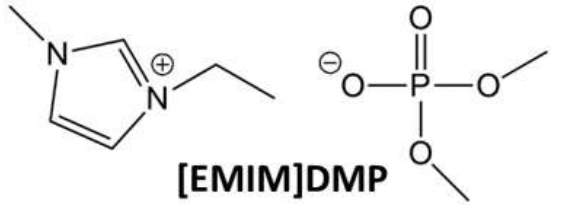
Boiling point 61.1 °C;
Vapour pressure 25.9 kPa (25 °C)
TLV, TWA 10 ppm
Viscosity 0.56 cP (20°C)



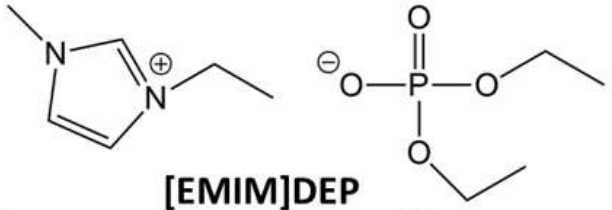
CYC

Boiling point 155.65 °C
Vapour pressure 0.67 kPa (20°C)
TLV, TWA 25 ppm
Viscosity 2.2 cP (20°C)

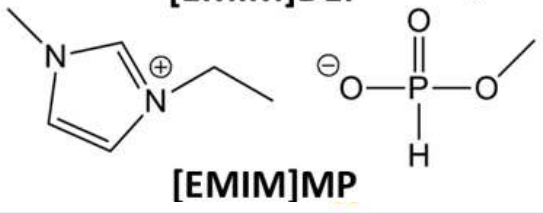
Non-PHA solvents and Cell Membrane Solvents



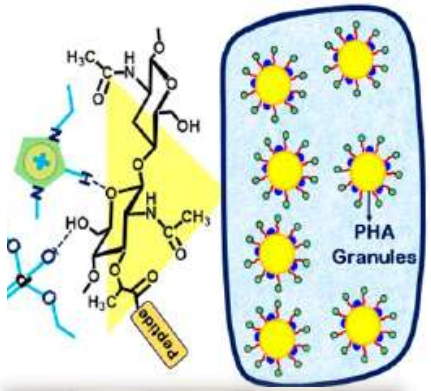
[EMIM]DMP



[EMIM]DEP



[EMIM]MP



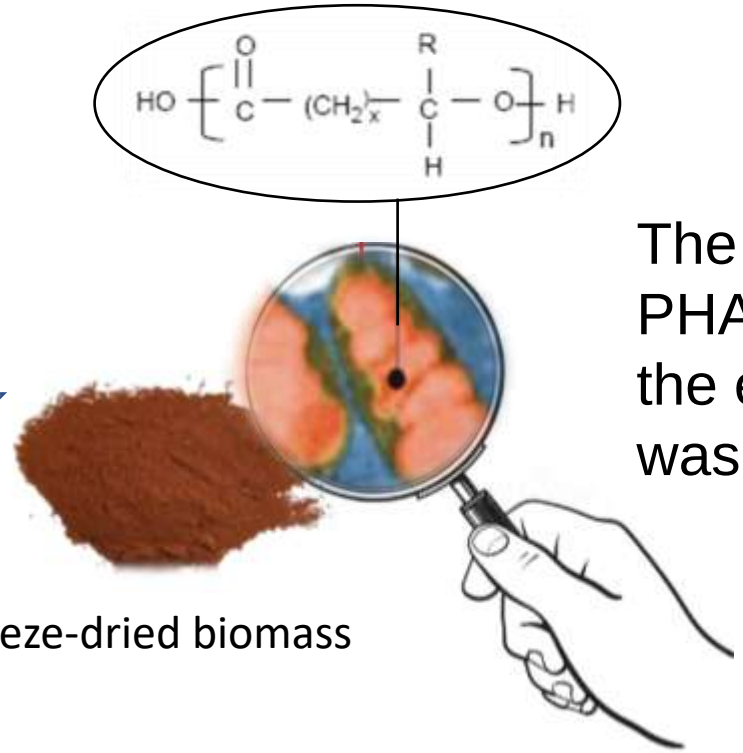
Production of PHA-containing *Rhodovulum sulfidophilum*

PHA containing biomass used: freeze-dried red photosynthetic bacteria of the *Rhodovulum Sulfidophilum* strain supplied by the Research Institute on Terrestrial Ecosystems (IRET) of the CNR of Florence.

Rhodovulum sulfidophilum DSM-1374 containing PHA was produced in batch phototrophic growth conditions, using lactate as carbon source, recovered by centrifugation and, subsequently, lyophilized.

Lab-PBR (4 L)

Exausted water broth



Freeze-dried biomass

The average content of PHA in the dry-biomass at the end of the experiments was **14.3 wt.%**

Batch growth conditions and **16/8 light/dark cycle**

Research Institute on Terrestrial Ecosystems

IRET

PHA extraction procedures with CHL and CYC

Extraction

CHL extraction

ratio solvent/biomass 30 mL/g

Temperature of 10-12°C for 24 h

CYC extraction

ratio solvent/biomass 17 mL/g

Temperature 100 and 125 °C for 5, 10, 20 and 30 min

Filtration of mixture under vacuum (Teflon filter 0.45 μm)

PHA precipitation by methanol addition

Ratio methanol/solution: 4 mL/mL

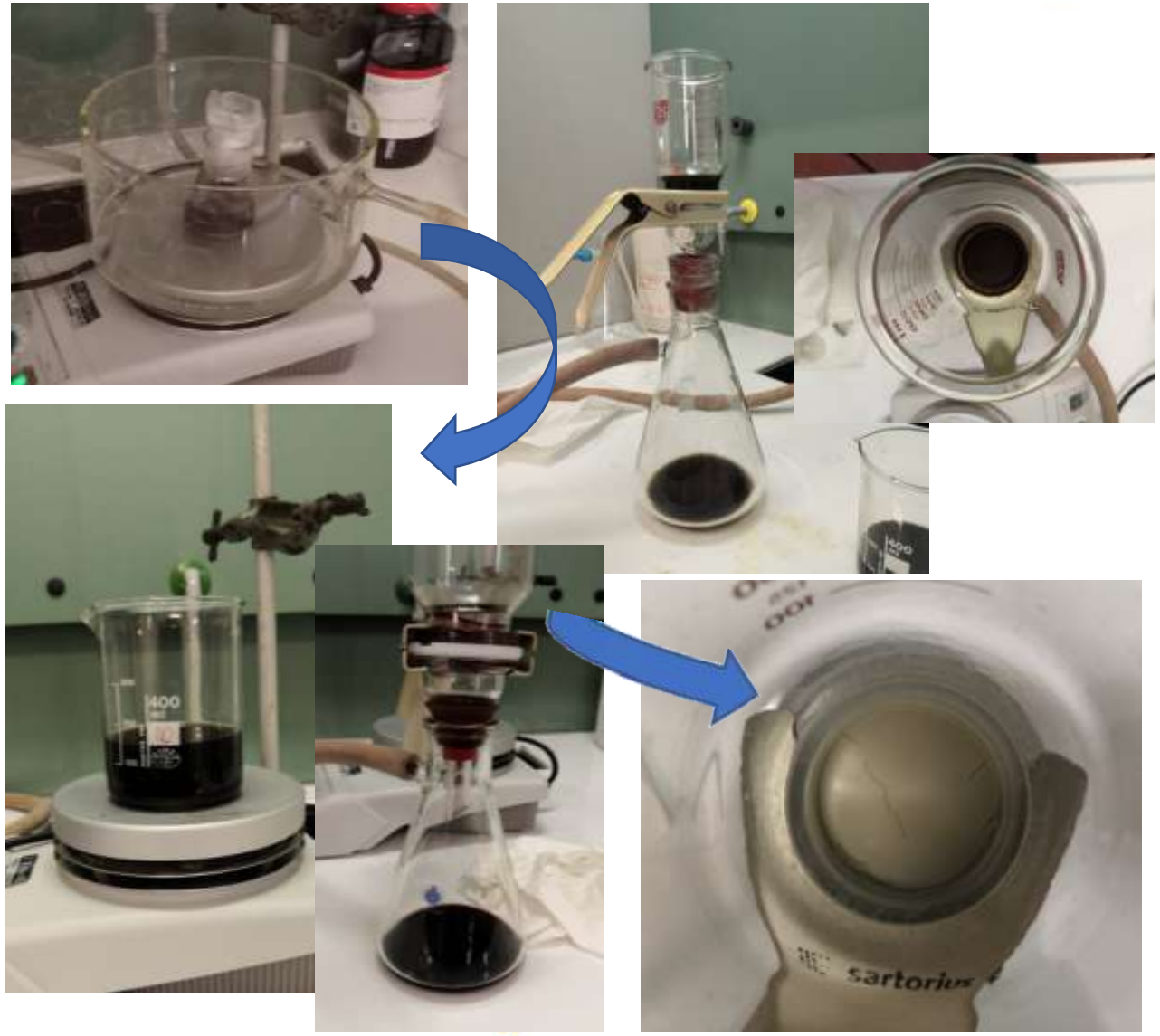
Room temperature

Under mixing for 5 min

Filtration of precipitated PHA under vacuum (Teflon filter 0.45 μm)

Cake washing with methanol

Ratio methanol/biomass: 10 mL/g



CYC and CHL extraction

Wet PHA cake from CYC extraction after precipitation by MetOH, filtration and MetOH washing

……. dried at room temperature (CHL) and 80°C (CYC) under vacuum at pressure of 10 mbar to remove the solvent and methanol

dried PHAs were characterized by:

- proton Nuclear Magnetic Resonance (^{1}H-NMR)
- Fourier-Transform Infrared (FTIR) spectroscopy in ATR (Attenuated Total Reflectance) mode
- Thermogravimetric Analysis (TGA)
- Differential Scanning Calorimetry (DSC)
- Gel Permeation Chromatography (GPC)
- Filmability by solvent casting method

PHA extraction procedures with ILs

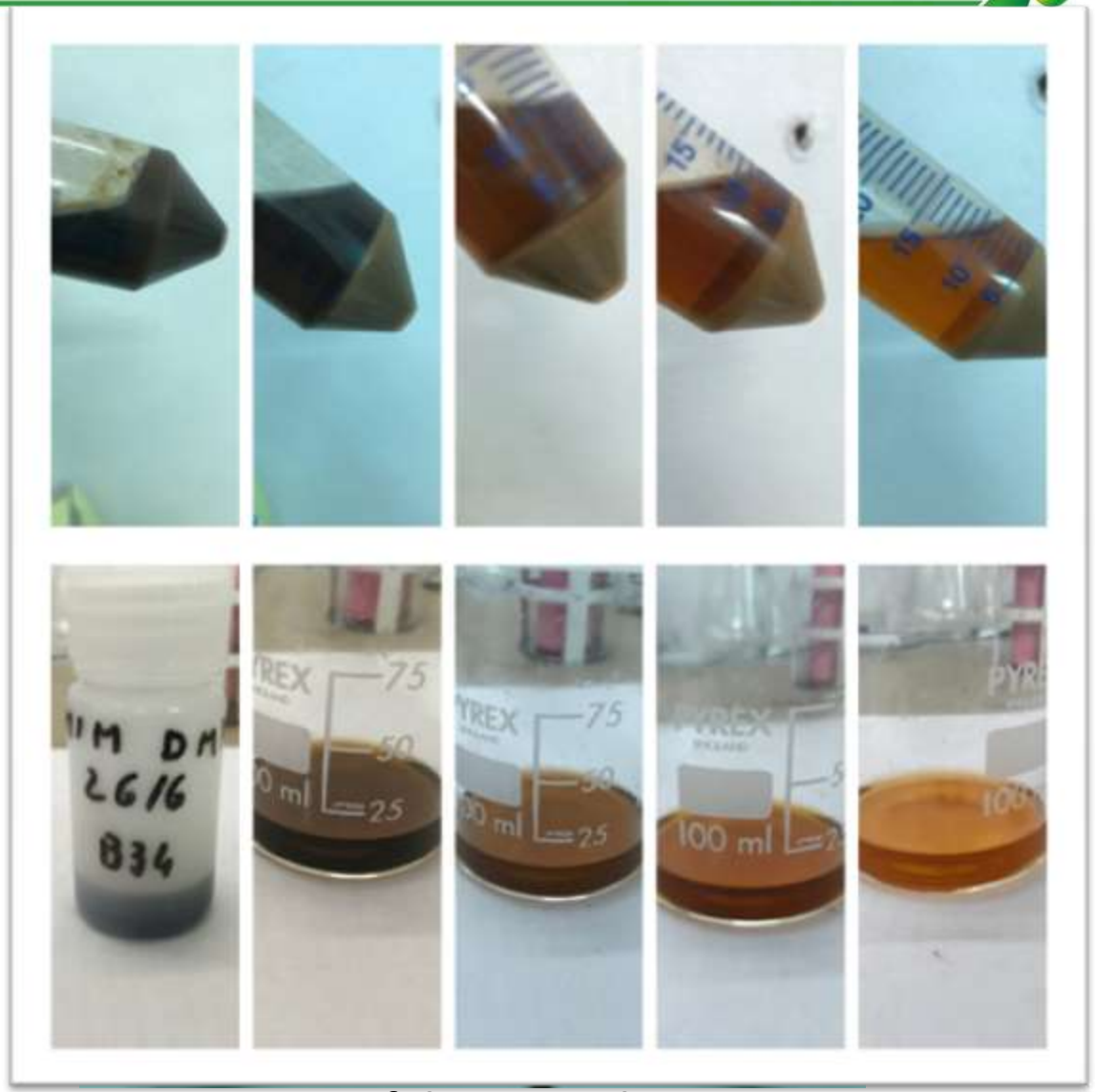
ILs extraction
Ratio solvent/biomass: 10 and 30 g/g
Temperature: 60 °C
Time: from 4 to 24 h

Centrifugation at 4000 rpm for 10 min

Resuspension washing with methanol (4 times)
Ratio methanol/biomass: 20 mL/g

Centrifugation at 4000 rpm for 5 min

High viscosities: at 60°C the viscosities are 58, 50 and 23 cP for [EMIM][DEP], [EMIM][DMP] and [EMIM][MP], respectively.



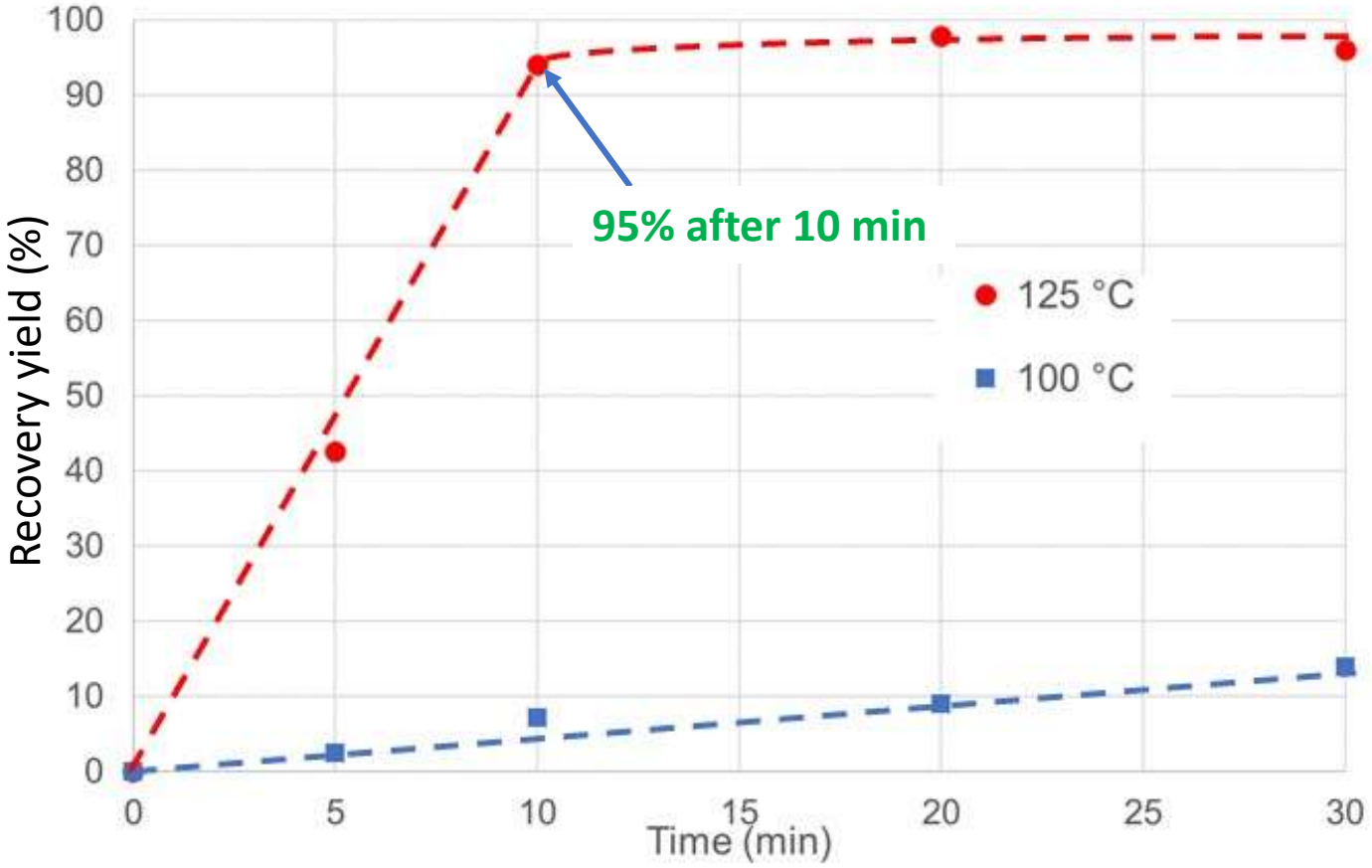
Images of the run with EMIM DMP

Consumption for 1 g of biomass: 10/30 g of IL and 80 mL of methanol

CHL and CYC extraction

PHA recovery yield by CHL (10°C for 24 h) = **98.5%**

PHA recovery yield by CYC

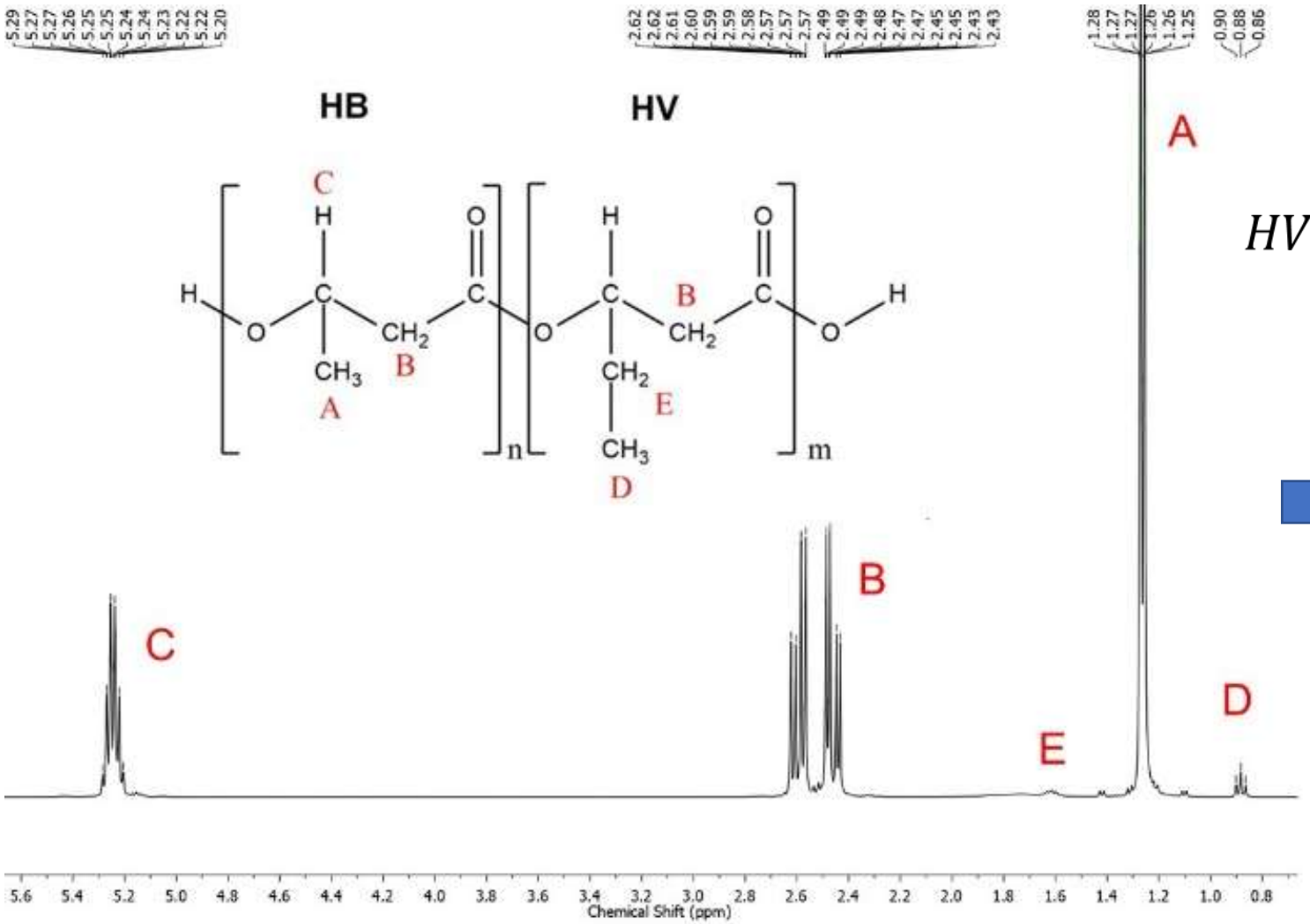


Recovery yields using CYC as function of temperature and time

PHA extraction with CYC – A comparison with literature data

Paper	Bacterium strain	PHA content (wt.%)	bacteria/CYC ratio w/v % (% g/mL)	Extraction temperature (°C)	Extraction time (min)	Recovery yield (%)
this work	Rhodovulum Sulfidophilum DSM-1374	14.2	6.0 6.0 6.0	125 125 125	5 10 20	43 **95** **98**
[Jiang et al. 2018]	Cupriavidus necator H16	82.3	2.0 2.0 2.0	80 100 120	1200 5 3	16 90 99
[Rosengart et al, 2015]	Burkholderia sacchari DSM 17165	57.7	1.5 6.0 6.0	120-130 120-130 120-130	15 15 30	94 84 85
[Van Walsem et al., 2010]	Escherichia coli	80.0	7.0	90	35	80

Proton Nuclear Magnetic Resonance (^{1}H-NMR)



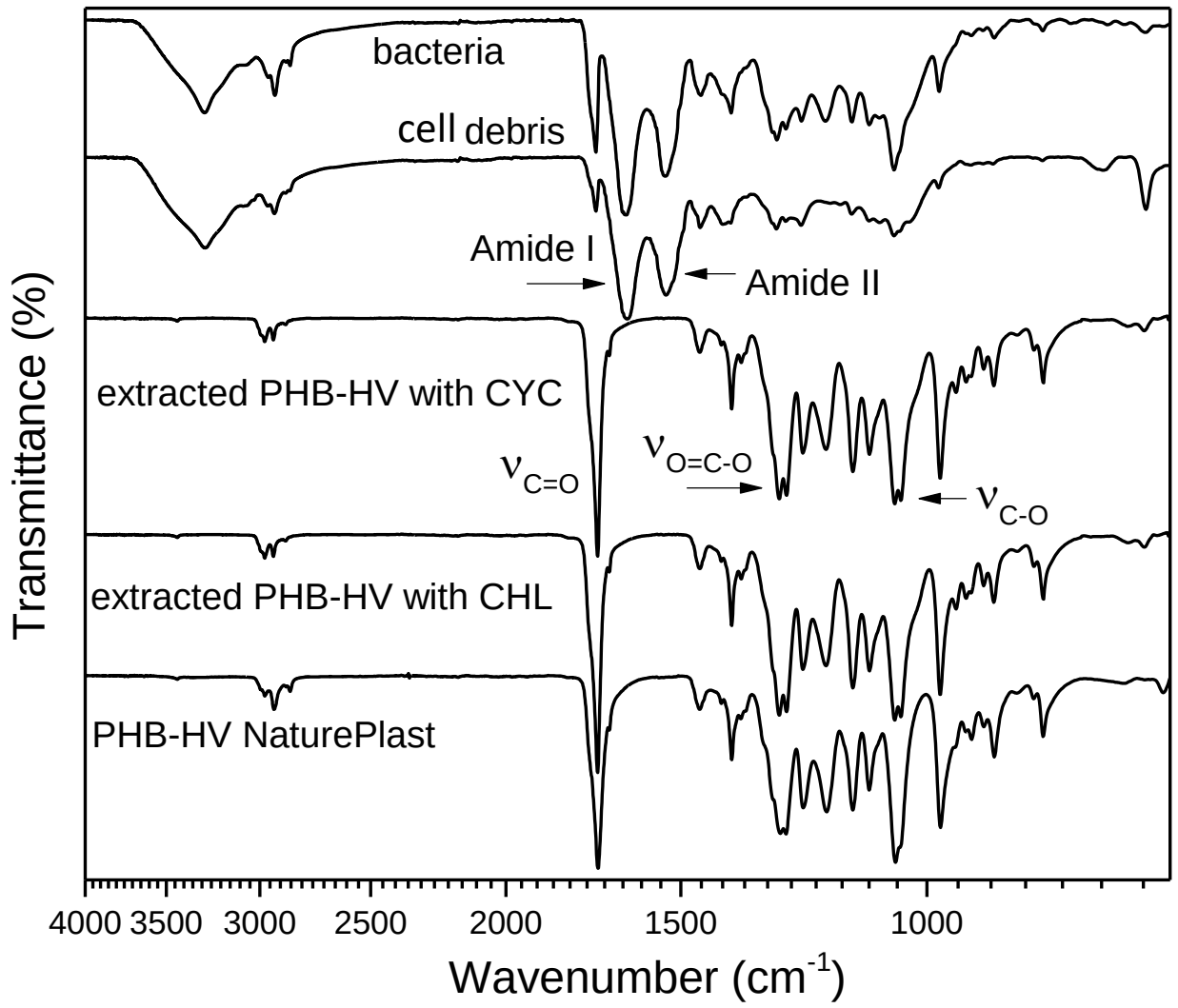
$$HV\ (\%) = \frac{Area\ CH_3(HV)}{Area\ CH_3(HV) + Area\ CH_3(HB)} \cdot 100$$

PHB-HV extracted by CYC has 2.7 mol % of HV

The commercial PHB-HV (by NaturePlast): 0.9 mol%

Correspondences between the 1HNMR signals of PHA extracted using CYC at 125°C for 10 min and the protons of the polymer structure.

FTIR analysis

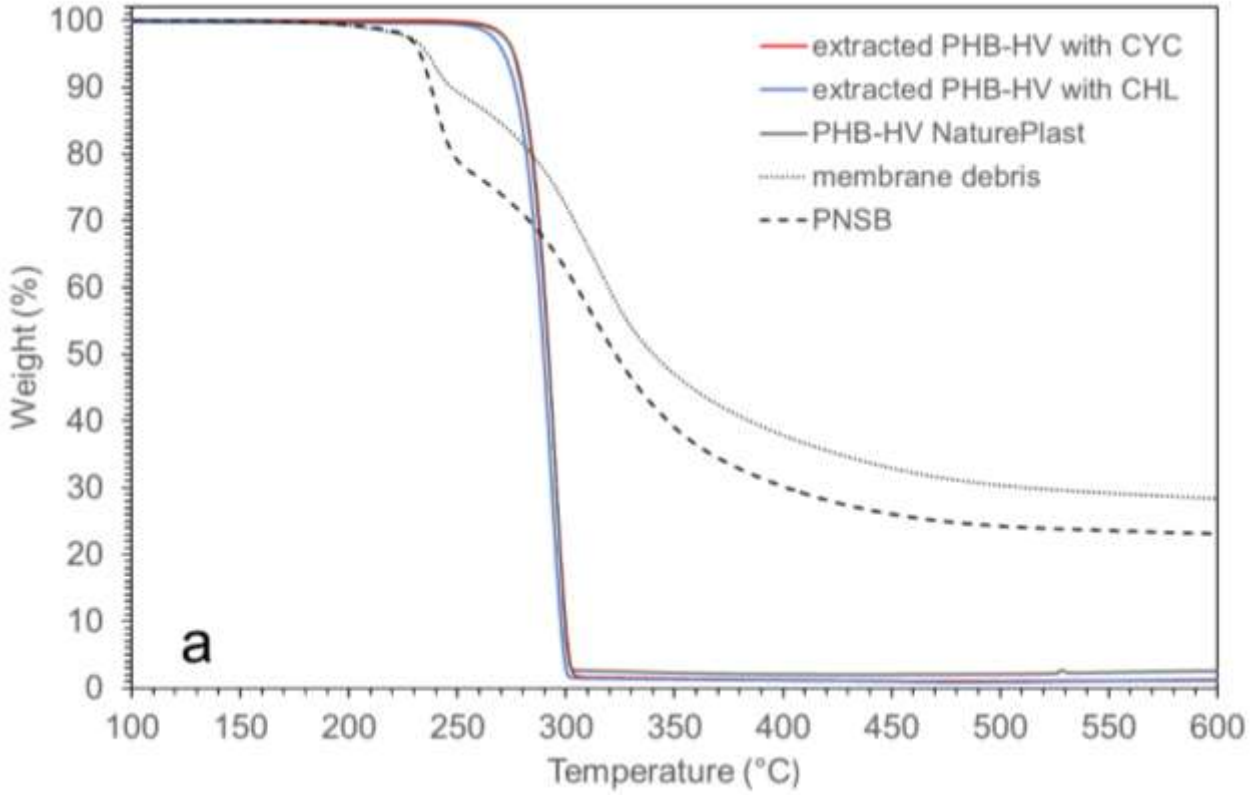


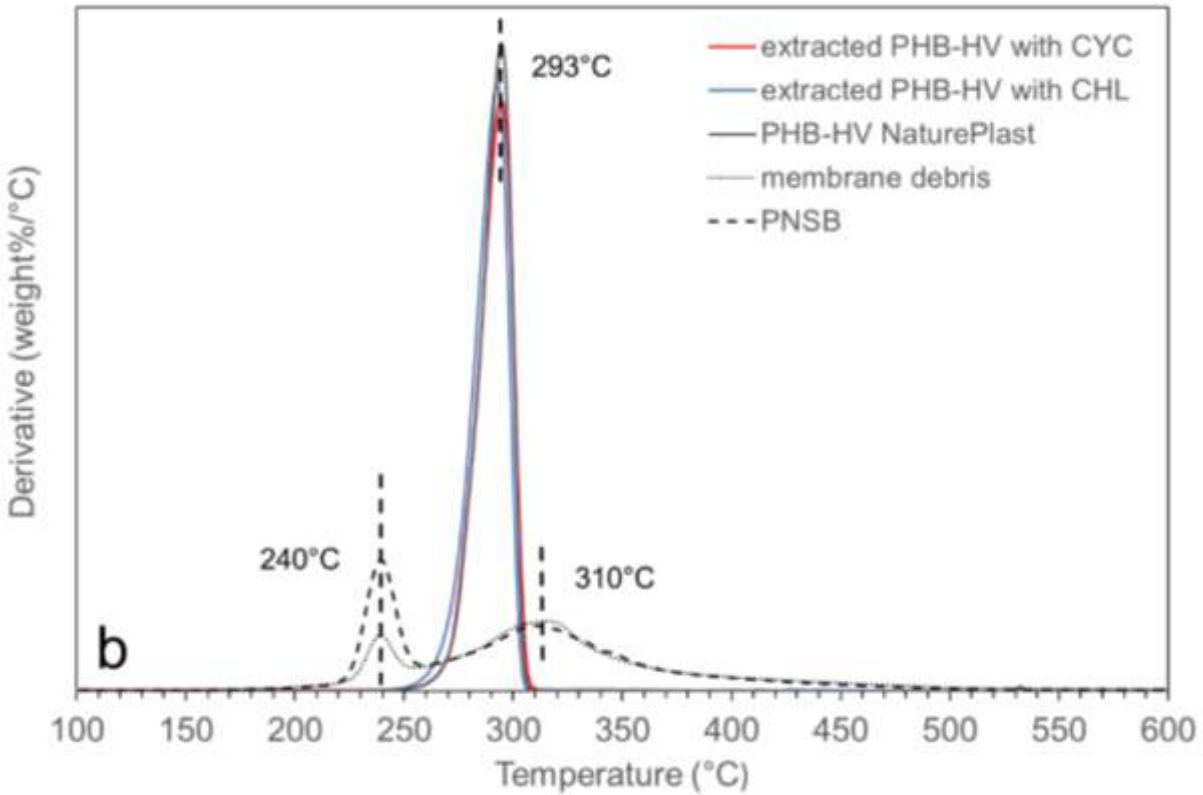
FTIR analysis of PHB-HV extracted with CHL, CYC (125°C, 20 min), commercial PHB-HV, starting purple bacteria and membrane debris after extraction with CYC.

the two prominent bands at 1645 and 1545 cm−1, corresponding to C=O stretching vibrations of the peptide bond and the C-N stretching vibrations and N-H bending modes, respectively, are completely absent in the PHB-HV patterns.

High purity of the extracted PHB-HVs

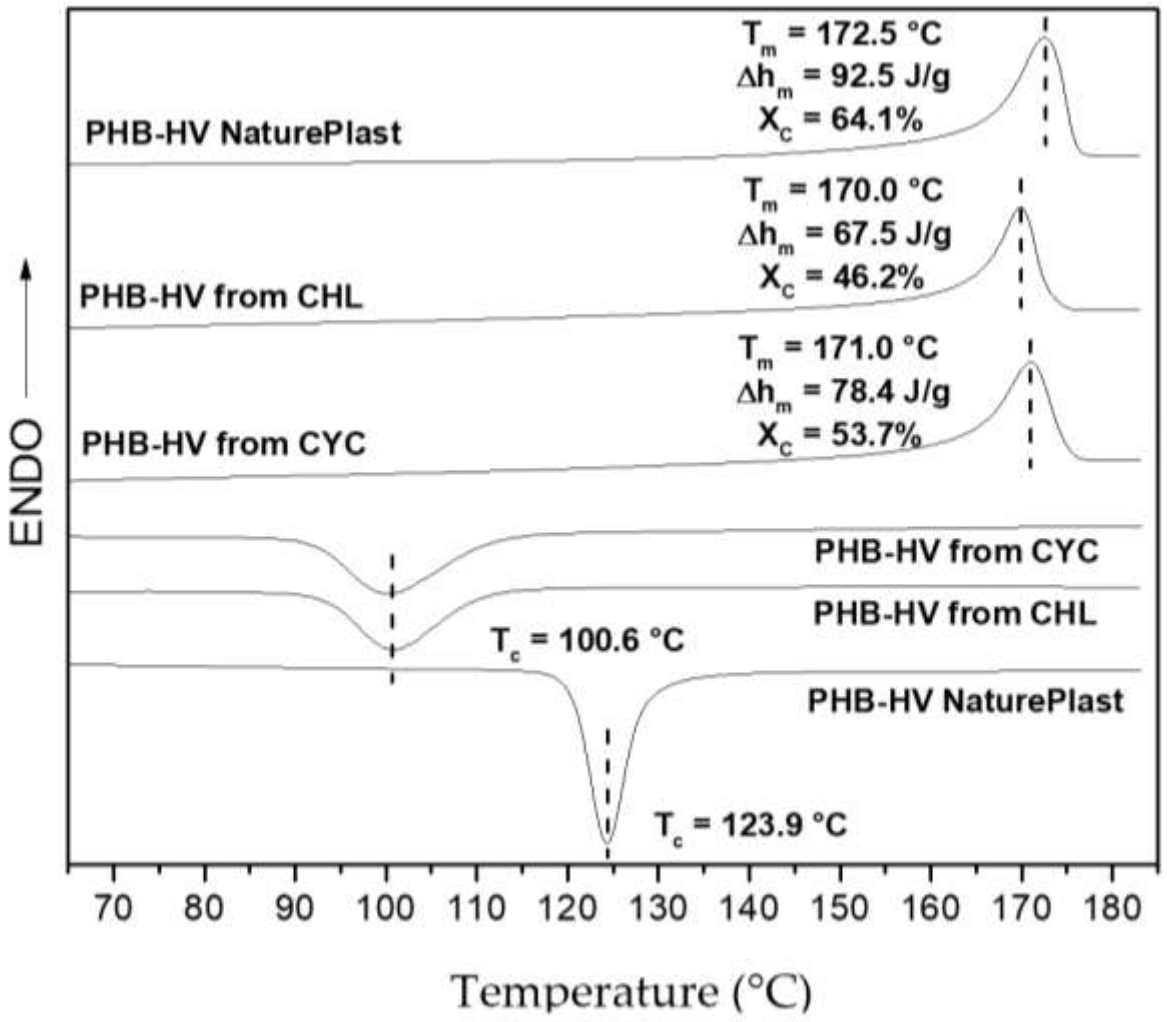
Thermogravimetric Analysis (TGA)





a) TG and **b)** DTG curves of PHB-HV extracted by CHL, CYC (125°C, 20 min), commercial PHB-HV, purple bacteria and cell debris after extraction by CYC.

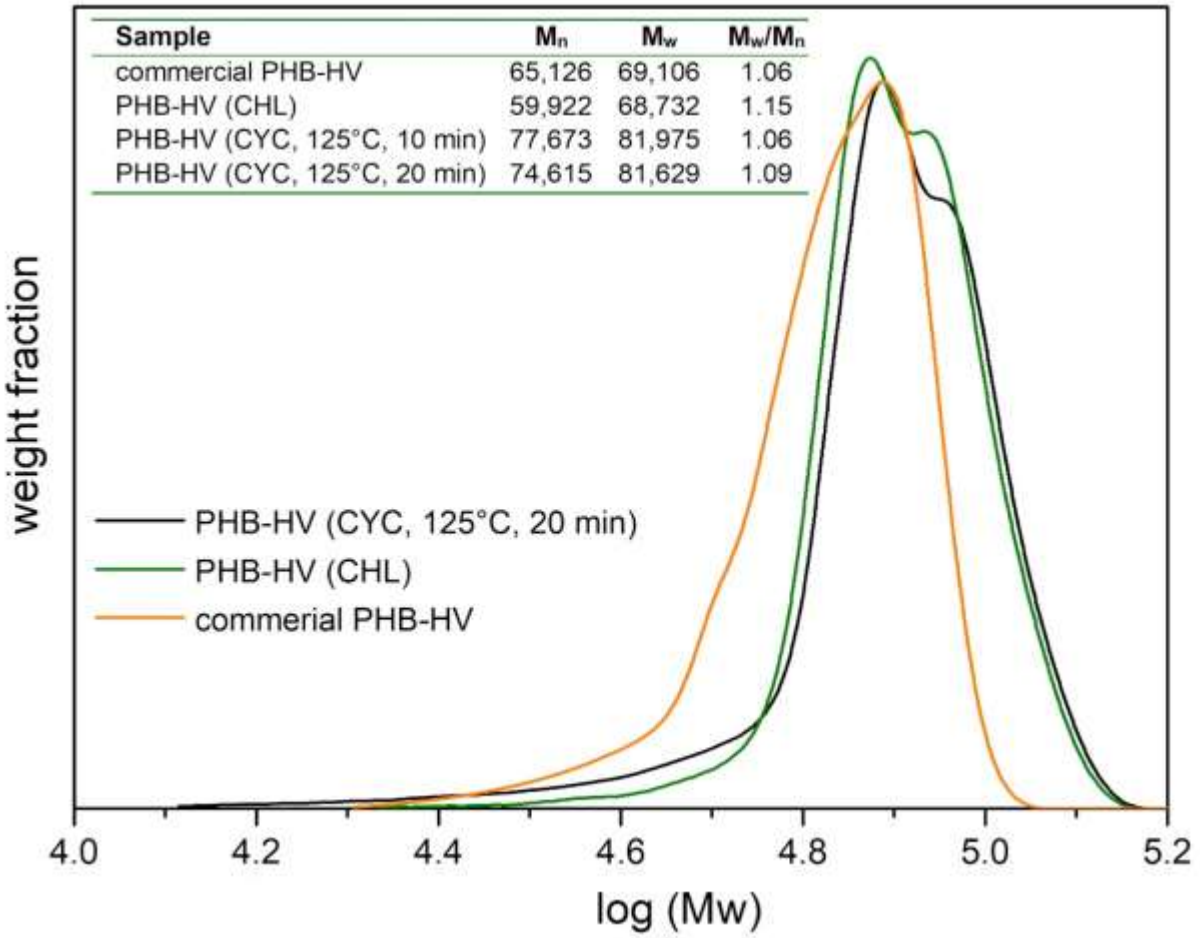
Differential Scanning Calorimetry (DSC)



The crystallization process is kinetically slower than that of commercial sample as shown by the lower T_c (100.6° C) respect to 124° C of the commercial sample. These data are perfectly in accordance with the higher HV content (2.7 mol%) of the extracted PHB-HVs compared to that the commercial sample (0.9 mol%).

2nd heating and cooling DSC curves and derived calorimetric data of PHB-HV extracted with CYC (125° C, 20 min), extracted with CHL (10° C, 24 h) and commercial PHB-HV

Gel Permeation Chromatography (GPC)



CYC-based extraction procedure did not adversely affect the average polymer chain length compared to CHL-based procedure at lower temperature (10°C).

The increase of the extraction time, from 10 to 30 min, had no significant effect on Mn and Mw.

Number average molecular weight: Mn $$Mn = \frac{\Sigma N_i M_i}{\Sigma N_i}$$

Weight average molecular weight: Mw $$Mw = \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i}$$

GPC results for PHB-HV extracted with CHL and CYC and commercial PHB-HV

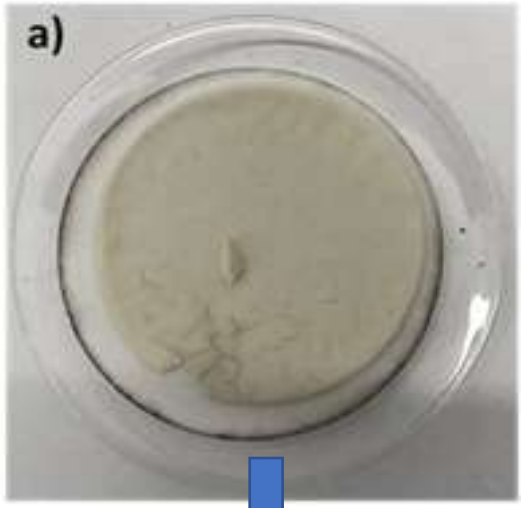
Filmability by solvent casting

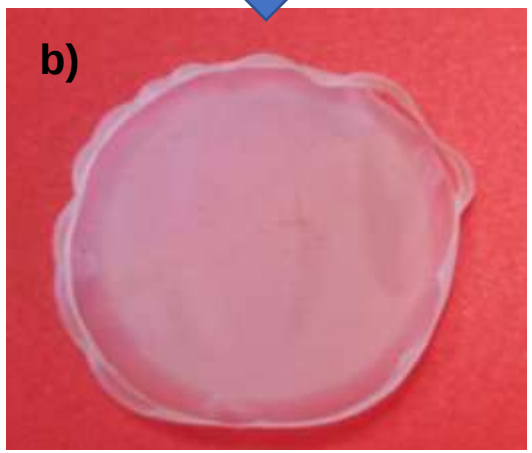
a) PHB-HV cake after CYC-based extraction;

b) corresponding film obtained by chloroform casting;

c) pellets of commercial PHB-HV (NaturePlast);

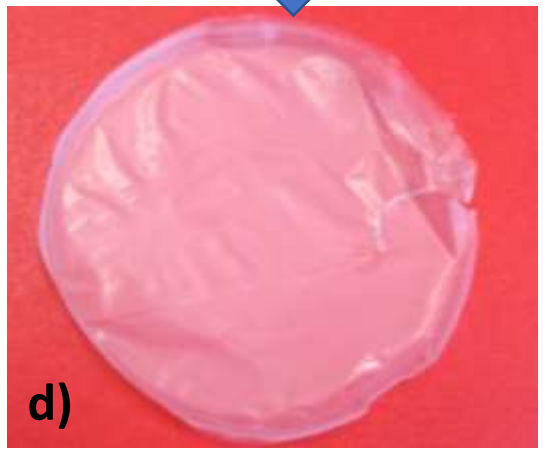
d) film obtained by using commercial PHB-HV by chloroform casting.





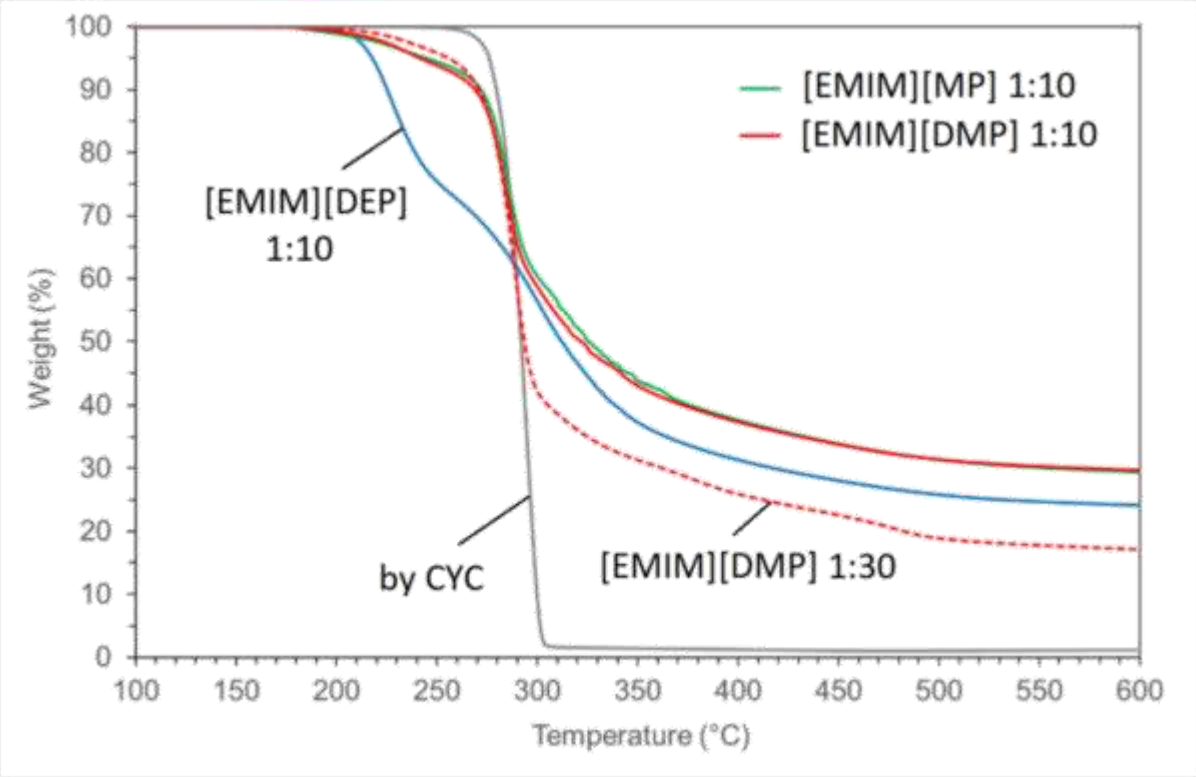
c)

PHB-HV (NaturePlast)



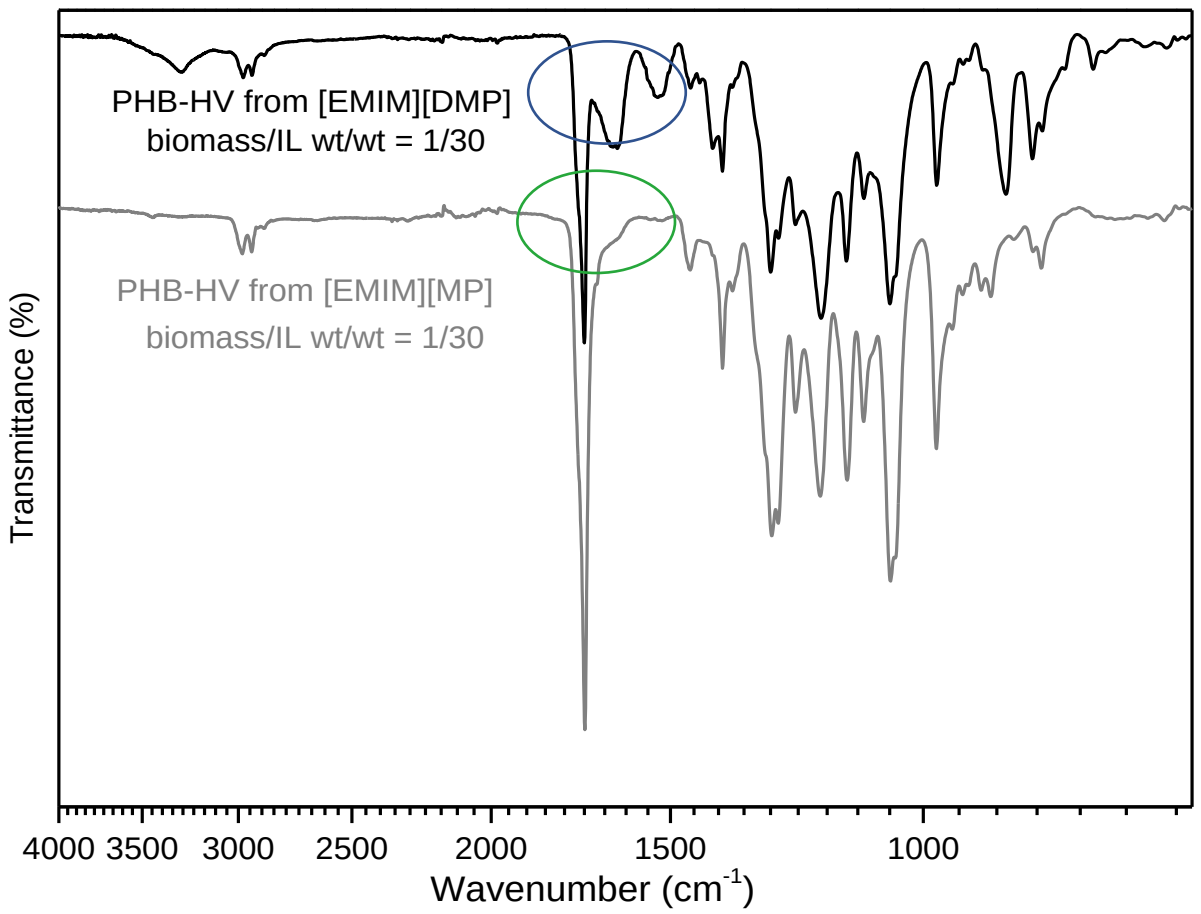
PHA extraction by ILs

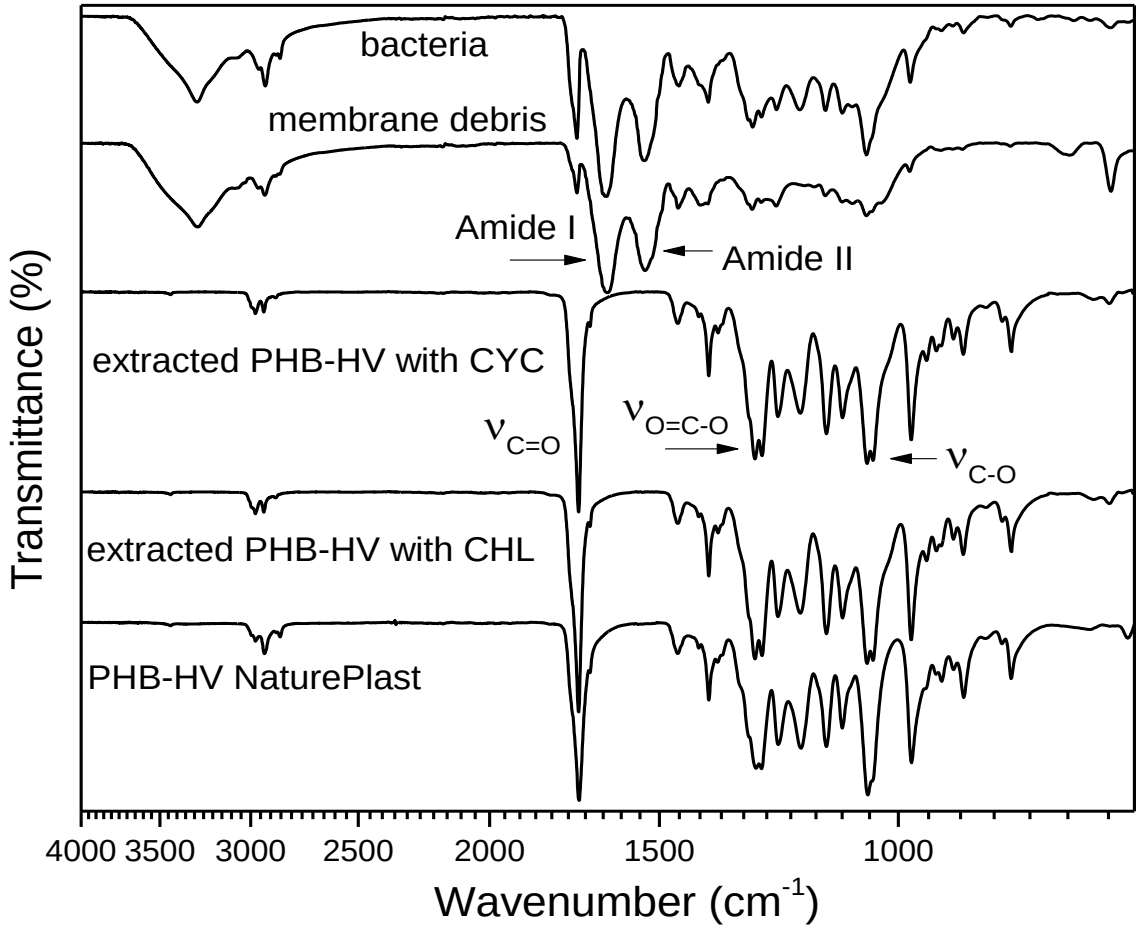
The wet centrifugated solid was washed with methanol → the amount of dried solid residues obtained for 1 g of treated biomass (1:10) resulted 0.45, 0.49 and 0.72 g by using [EMIM][MP], [EMIM][DMP] and [EMIM][DEP], respectively → **relevant presence of non-PHA cellular matter in the recovered PHB-HV**



TG curves of PHB-HV extracted with ILs for 24 h at 60°C compared with that of the samples extracted by CYC and CHL and commercial PHB-HV.

PHA extraction by ILs





Further tests are need to obtain higher NPCM removal efficiencies and, consequently, higher PHB-HV purity to make the procedure with ILs applicable on industrial scale.

In the case of EMIM MP 1/30 the extracted PHB-HV showed a purity of about 60 %.

Conclusions …..

- ✓ CYC and three ionic liquids ([EMIM][DEP], [EMIM][MP] and [EM-IM][DMP]) were investigated for extracting PHA from the *Rhodovulum sulfidophilum* DSM-1374 , as alternatives to commonly used halogenated solvents as CHL.

About the extraction by CYC:

- ✓ Recovery yields higher than 95% were obtained using CYC at 125 °C after 10 min with a cell/solvent ratio of 6 % (w/v).
- ✓ The extracted PHA was confirmed to be PHB-HV with 2.7 mol.% of HV, having purity, thermal properties, molar mass and dispersity like those of the PHB-HV extracted with CHL, used as reference solvent, and of a commercial PHB-HV.

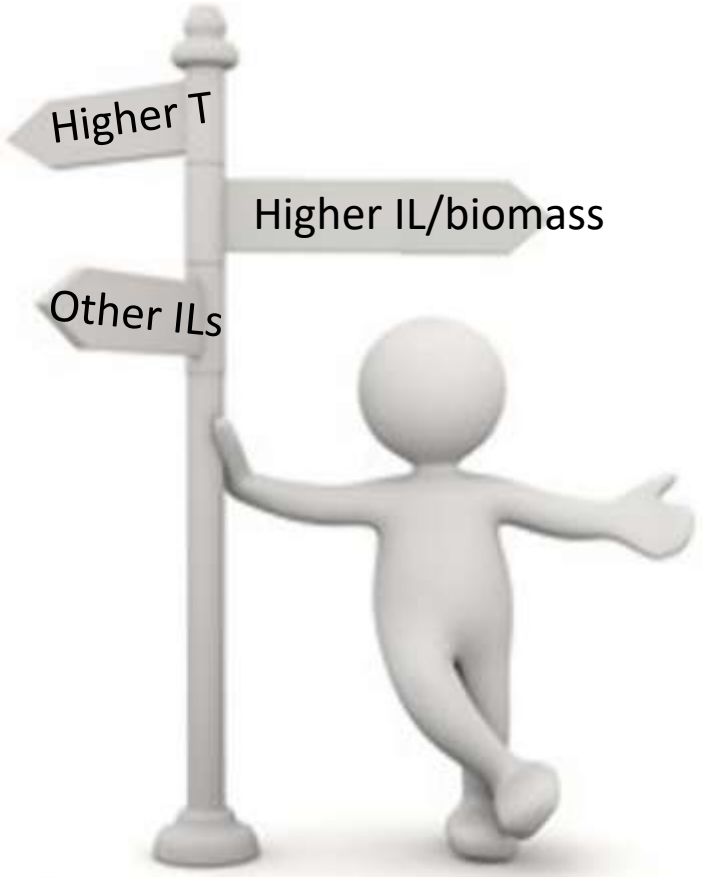
CYC at 125 °C is an **interesting alternative to CHL** for the PHA extraction from bacterial biomasses with very limited extraction times required (**10-20 min**) → simpler, less expensive solvent recovery (no azeotrope CYC-MetOH respect to the CHL-MetOH azeotrope) → The distillation itself can provide the solvent (CYC) at temperature close to its boiling point (155°C), without extra heating costs prior to extraction.

Conclusions …..

About the extraction by ILs:

✓ The three investigated ILs allowed to obtain **PHAs strongly contaminated by cellular material** and only using [EMIM][MP] with a cell/IL ratio of 1/30 (w/w) after 24 h at 60°C, the extracted PHB-HV showed a purity of about 60%.

Consequently, further investigations are need regarding the use of ILs as PHA extraction solvents to increase the selectivity of extraction and overcome the difficulties that occur during the step of filtration/centrifugation/washing because of their high viscosity.



Thank you for the attention

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Open Access Article

Extraction of Polyhydroxyalkanoates from Purple Non-Sulfur Bacteria by Non-Chlorinated Solvents

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