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Italiadomani PIANO NAZIONALE DI RIPRESA E RESILIENZA
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Combination of membrane filtration and fermentation to produce poly-hydroxyalkanoates (PHA): the BIOCOSI' project

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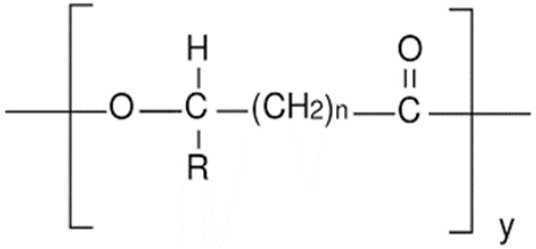
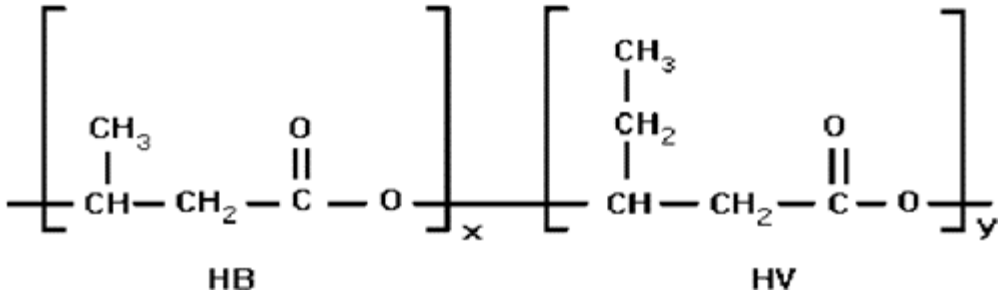
PHA - PHB - PHBV

PHA are biodegradable polyesters obtained by bacterial fermentation and considered the best candidates to produce bioplastics in place of conventional non degradable plastics. They are thermoplastic and differ in their chemical-physical properties depending on their chemical composition, bacterial species used and on the composition of the culture medium.



The main member of the PHA family is Polyhydroxybutyrate (**PHB**) and Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) (**PHBV**).



PHA, extracted from the bacteria cell, can be used for production of rigid and flexible plastic suitable for the most assessed medical applications, packaging, moulded goods, paper coatings, non-woven fabrics, adhesives, films and performance additives.

PHA

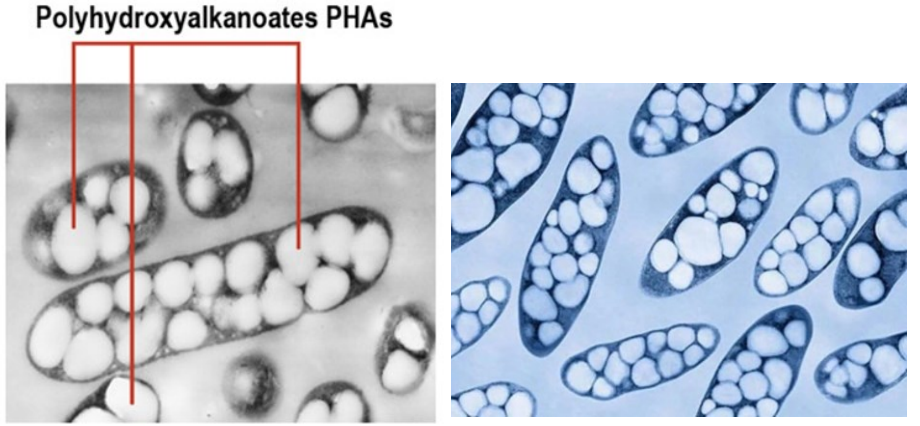
Some of the general characteristics of PHAs are:

- Biodegradable under aerobic and anaerobic conditions.
- Good barrier properties against oxygen, water and oil.
- Good ultra-violet resistance but poor resistance to acids and bases.
- Soluble in chloroform and other chlorinated hydrocarbons.
- Sinks in water, facilitating its anaerobic biodegradation in sediments.
- Non toxic.

PHA production

PHA are generally produced by various microorganisms during several different pathway as a best energetic molecule, which are both synthesized and stored in intracellular pseudo granules, known as *carbonosomes*.

Many bacterial, more than three hundred, in particular growth conditions such as carbon excess and limitation of essential nutrients (N, P, S), are able to accumulate PHA in the form of granules. These granules can constitute up to 90% of the dry weight of the bacterial mass.



TEM images of bacterial cells with PHA accumulation

Source: https://wisesociety.it/incontri/leco-plastica-che-si-scioglie-nellacqua-una-scoperta-tutta-italiana/
https://polymerinnovationblog.com/polyhydroxyalkanoates-natures-polyester/ [Online]

Microrganisms

Bacteria used for the production of PHAs are divided into two groups based on the culture conditions required for PHA synthesis.

The first group of bacteria requires the limitation of an essential nutrient such as N, P, Mg, K, O or S for the efficient synthesis of PHA from an excess carbon source: *Alcaligenes eitrophus, Protomonas extorquens, Ps oleovorans* and many other bacteria.

The second group of bacteria does not require nutrient limitation for PHA synthesis and can accumulate polymer during growth: *Alcaligenes latus* and recombinant *E. coli.*

Microrganisms

The bacterial strains most frequently used and indicate as the promising candidate for industrial scale PHA production are:

- *Bacillus,*
- *Rhodococcus,*
- *Rodhospirillium,*
- *Pseudomonas,*
- *Alcaligenes/Ralstonia,*
- *Azotobacter,*
- *Rhizobium,*
- *Acinetobacter,*
- *Corynebacterium.*

PHA production

Currently, the limiting factor to produce bioplastics from PHA is the high cost of the polymer production process. Three important factors mainly contribute to the cost of PHA production: **substrate usage**, **fermentation process**, and **PHA recovery**.

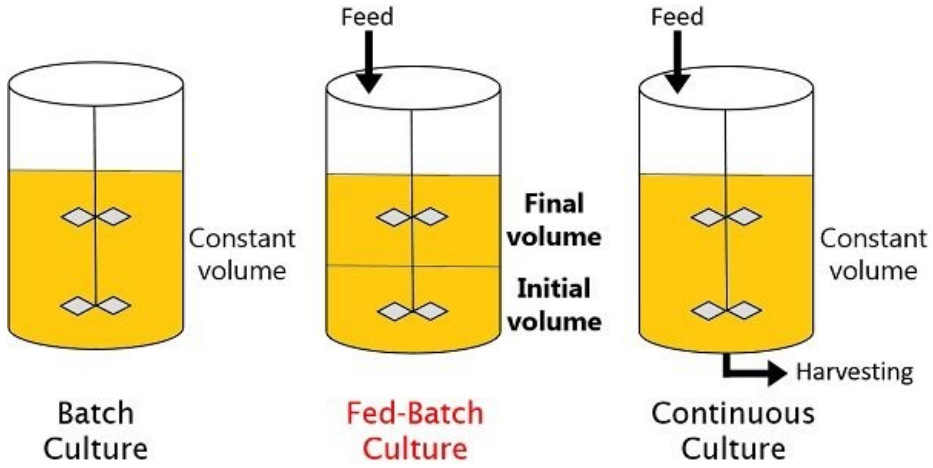
PHAs can be produced by both *pure* and *mixed microbial cultures* (MMC). The **pure cultures** are associated with the best process yields and PHA productivities, because they are chosen for their high storage capacity and high cell density, but they need a high purity substrates, which can account for 45% of the total production cost. On the other hand, **mixed cultures** have the advantage that they do not need sterile conditions and they are better able than pure cultures to adapt to complex substrates. The disadvantage of MMC is the low polymer yield, due to the low concentration of biomass, whose growth is inhibited by the organic acids produced during fermentation.

To compensate the high production cost resulting from low productivity involves the use of agrifood industry waste as a low-cost culture substrate. Agri-industrial wastes such as cheese whey, vinasse, olive mill wastewater, chitin waste from seafood industry have been already tested as carbon substrate for PHA synthesized by *Hfx. mediterranei*.

Bioreactors and Fermentation strategies

Fed-batch or continuous cultivation process can be used for the production of PHA with high productivity.
A bioreactor is a type of fermentation vessel that is used for the production of biomass, metabolites, and antibiotics.
It is a closed container with adequate arrangement for aeration, agitation, temperature and pH control.

In a **batch process**, all nutrients are provided at the beginning of the cultivation, without adding any more in the subsequent bioprocess; it is a closed system.



Continuous fermentation process is an open system, because a constant flow of culture medium through in and out of the system.

Fed-batch is a partially open system, because nutrients are constantly supplied during cultivation.

https://biologyreader.com/fed-batch-culture.html

Haloferax mediterranei

The halophiles microorganisms, having the ability to survive in hypersaline environments, are attracting the interest of the scientific and industrial research as a cost-effective tool to produce PHA. The foremost advantage is that the high salinity requirements reduces the chances of microbial contamination to a great extent, allowing to perform bioprocesses without expensive sterilization pretreatment of the substrates.

Among halophiles, the *Haloferax mediterranei* has several advantages such as adaptivity, high growth rate, genetic stability, and an efficient synthesis of the polymer. It was also demonstrated that *Hfx. mediterranei* can efficiently use carbon sources from different industrial and household wastes for synthesizing PHA. Furthermore, the **PHA recovery** is easily carried out by complete cell lysis, using tap-water.

Microorganism and Growth Conditions and Fermentation process to produce poly-hydroxyalkanoates (PHBV)

foods
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Article

Production of the Polyhydroxyalkanoate PHBV from Ricotta Cheese Exhausted Whey by *Haloferax mediterranei* Fermentation

Susanna Raho [1], Vito Emanuele Carofiglio [1], Marco Montemurro [2], Valerio Miceli [3], Domenico Centrone [1], Paolo Stufano [1], Monica Schioppa [3], Erica Pontonio [2] and Carlo Giuseppe Rizzello [2,*]

Cultivation conditions: *Haloferax mediterranei* DSM1411 was cultivated in synthetic highly saline media Halobacterium Medium 372 (**HM372**); T = 37 ◦C; t: 24 h; stirring conditions: at 150 rpm; Inoculum consisted of 20% (v/v) of the fresh culture on total working volume.

Most organisms require 3HV precursor for PHBV synthesis whereas *Hfx. mediterranei* can efficiently synthesize PHBV without any external precursor, thus greatly reducing the production cost.

Enzymatic Pre-Treatment: *Hfx. mediterranei DSM1411* incapability to metabolize lactose, so an enzymatic hydrolysis of the disaccharide was carried out in **R-NF (nano-filtration retentate)** using two different liquid commercial preparations of **β-galactosidase.**

All experiments were carried out in 1 L flasks (containing 250 mL of substrate) at orbital shaker (150 rpm) for 72–144 h or in a 3 L bioreactor for 120 h.

PHBV was extracted from dry biomass and to achieve hypo-osmotic shock and complete cell lysis was resuspended in deionized water. The pellet, corresponding to raw PHBV, was resuspended in $CHCl_3$:H_2O and, after centrifugation, recovered and weighed.

The polymer yield (%) was calculated as [PHBV (mg/L)/CDM (mg/L)] × 100.

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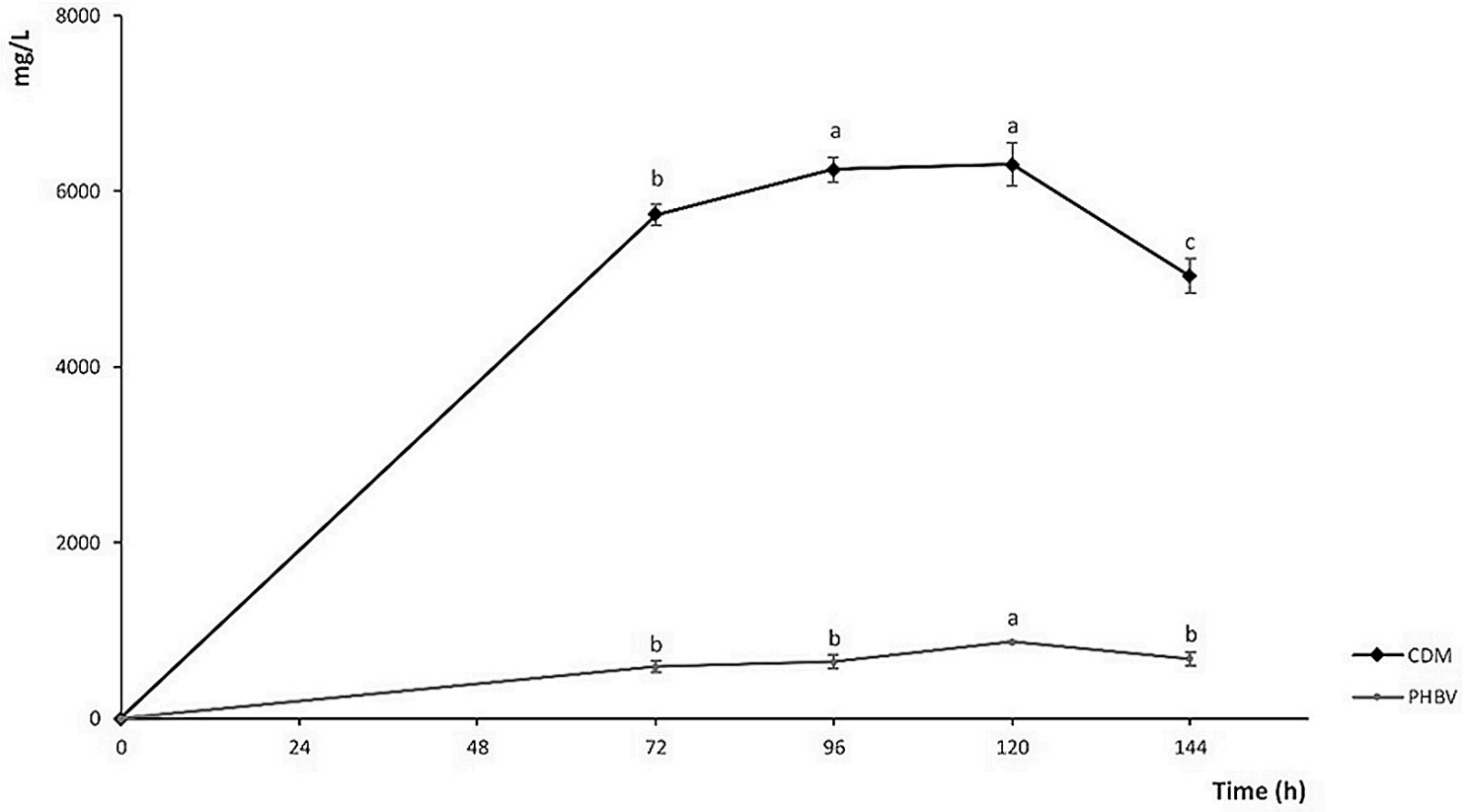
PHBV Productivity in-flasks trials



The PHBV yield was **10.33% (w/w)** at 72 h and no significant ($p > 0.05$) differences were observed at 96 h, while the highest value was observed at 120 h.

The highest CDM was obtained at 96 h of fermentation, with values stable until 120 h, while a significant ($p < 0.05$) decrease was observed at 144 h.

Haloferax mediterranei Cell Dry Mass (CDM) and PHBV production in R-NF supplemented with 1% (v/v) SL-6 and 10% (w/v) NaCl. Inoculum was made with 20% v/v active colture of *Hfx. mediterranei* DSM1411 grown (24 h at 37 °C) in HM372. Fermentation was carried out at 37 °C for 144 h. Herror bars represent the standard deviation of three replicates. $^{a-c}$Values in the same data series, with different superscript letters, differ significantly ($p<0.05$).

Susanna Raho1, Vito Emanuele Carofiglio1, Marco Montemurro2 , Valerio Miceli3, Domenico Centrone1, Paolo Stufano1, Monica Schioppa3, Erica Pontonio2 and Carlo Giuseppe Rizzello2,* Production of the Polyhydroxyalkanoate PHBV from Ricotta Cheese Exhausted Whey by *Haloferax mediterranei* Fermentation. Foods 2020, 9, 1459; doi:10.3390/foods9101459 www.mdpi.com/journal/foods.

PHBV synthesis in Bioreactor System

Overall, both CDM and polymer synthesis were significantly ($p < 0.05$) higher compared to the in-flasks trials; nevertheless, the yield resulted significantly ($p < 0.05$) lower. The highest PHBV synthesis was observed in the range of 400–500 rpm (1.18±0.06 – 1.27±0.09 g/L). Stirring at values higher than 500 rpm did not cause further increases in PHBV production.

CDM, PHBV production and yield obtained after 72 h of fermentation in bioreactor system.

Stirring	CDM (g/L)	PHBV (g/L)	Yield (% *w/w*)
300 rpm	10.75 ± 0.14 [d]	0.94 ± 0.05 [b]	8.74 ± 0.09 [a]
400 rpm	12.66 ± 0.17 [c]	1.18 ± 0.06 [a]	9.36 ± 0.55 [a]
500 rpm	18.32 ± 0.15 [a]	1.27 ± 0.09 [a]	7.03 ± 0.32 [b]
600 rpm	14.65 ± 0.10 [b]	0.95 ± 0.05 [b]	6.48 ± 0.12 [c]

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Conclusions

- Ricotta cheese exhausted whey (RCEW) is considered as one of the large wastewater from the agri-food compart and is proposed as a sustainable carbon source for the synthesis of the polymer.
- A membrane multi-step fractionation was used for lactose enrichment (and contemporary recover whey proteins).
- Among the different microorganisms recently investigated for PHA biosynthesis, *Hfx. mediterranei*, able to accumulate PHBV, is considered the most promising for the large-scale production.

Currently, PHA production is not an efficient process due to both high costs and low yield. The main limitations could be overcome by:

1. changing the cultivation approach, thus using mixed microbial cultures (MMCs) instead of pure cultures;
2. using waste as carbon sources instead of selected and pure substrates;
3. improving downstream steps (extraction/purification) using green and sustainable extraction tool aids instead of toxic and not-environmental friendly solvents and additives.

Thank you for your attention

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