Development of eco-friendly procedures for PHA recovery from mixed microbial cultures

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Abstract

Today's society reached an emergency situation due to the consequences left by mankind. The scientific community is working to improve our future, one of the main goals is to achieve a circular economy and in this landscape this project was born. Our part in the project was to purify, using an eco-friendly procedure, a polyhydroxyalkanoate (PHA) from an enriched biomass grown using paper mill side streams as a substrate.

The strategy applied was to dissolve the non-polymeric cell mass with some inexpensive, low-impact reagents. The received biomass had a PHBV content of less than 30% and half of the polymer mass was water soluble, and thus was lost in all extraction processes implemented.

Nevertheless it was possible to obtain an extracted product with a PHA content of 70.8$\pm$1.5% recovering 33.7$\pm$1.8% of the polymer present in the initial biomass. This result was achieved with some state of the art techniques that did not allow an easy, ecological and low-cost scale-up, so the extraction process was adapted. With these changes, the extraction was quite unsuccessful, achieving the most promising result of 27.5$\pm$2.4% in PHA content and recovering 40.3$\pm$3.1% of the initial polymer.

All this tests allowed to conclude that, for this biomass, a dual extraction with a first quick step of acidification, followed by an basic digestion is needed to dissolve the impurities of different nature.

A lot of thought and work was put in the optimization of the producing and processing steps, prior to the arrival of the biomass at NOVA ID, but no improvements were made in the PHA content nor the quality of it. So, if the situation remains unchanged, no improvements are expected.

Keywords: Polyhydroxyalkanoates; Mixed Culture; Paper Industry Residues; Extraction; Alkaline Digestion

1. Introduction

One of the big problems contributing for the situation we are living nowadays is the accumulation of plastic both in soils and seas, wich is a key aspect to achieve carbon neutrality and a circular economy [1].

Our society is heavily dependent on plastics and the easiest way to fight this issue and continue with our lifestyle on this matter is to substitute the conventional plastics (mainly the single-use) with biodegradable plastics, such as polyhydroxyalcanoates (PHA's). These polymers are produced from living and renewable sources and, depending on their chemical structure, may be biodegradable if any living being can use it as a carbon source, metabolising it into water, carbon dioxide, methane, nitrate or ammonia [2]. Polyhydroxyalcanoates (PHA's) are biodegradable polymers and the aim of the project, in which this work is included, is to produce and purify a PHA.

PHA's are polyesters that occur naturally within some micro-organisms in the form of granular inclusions of up to 0.7 μm surrounded by a protein/lipid layer, serving as an energy reserve [3, 4]. It is a very versatile polymer being able to show rigid or ductile properties when the monomers are shorter or larger, respectively; because of such a marked contrast between the characteristics of short- and long-chain monomers, heteropolymers can be virtually tailor-made with the desired traits [5, 6].

The benchmark for production of PHA's is to use pure cultures and this method was extensively studied and optimised and it is now possible to produce a biomass with a polymer content up to 90% [7, 8, 9, 10]. In the last years a novel production strategy arised and became very popular achieving great results [11, 6], using Mixed Microbial Cultures

(MMC). Production of PHA with MMC assumes that in the fermenter, rather than there being just one controlled strain, there is a *cocktail* of microorganisms that adapt to the substrate being introduced. This specific peculiarity makes this strategy very suitable for productions with substrates derived from waste, whose constitution can vary substantially without compromising the yield of the process [12].

This method can be divided into 3 distinct phases [13] and is based on principles of natural selection, the conditions to which micro-organisms are subjected favour those that have the capacity to accumulate PHA [14]:

- The substrate is fermented to enrich it in volatile fatty acids (VFA's).
- The bacterial strains that will constitute the *cocktail* are selected in Sequencing Batch Reactors (SBR's)
- The biomass enriched in the step described above is fed with the substrate fermented in the initial step, under optimal conditions for the accumulation of PHA.

To extract the polymer accumulated in the biomass, the technique state-of-the-art involves the use of solvents that have a high affinity with the PHA of interest and can selectively dissolve it. The solvents used are usually chlorinated, very toxic and polluting, thus, difficult to implement at an industrial scale.

A novel approach was suggested and with it, this problems were mitigated. Why focus on extracting the polymer that usually constitutes 60-90% of the biomass? It is possible to focus on the digestion of the non-polymerical cell mass (NPCM), a minor fraction of the biomass and recover the polymer as it was produced. There are different ways to apply this concept, the one that allows an easier scale-up is the chemical digestion and can be executed using reagents of different natures:

Oxidants: When oxidized, most of the non-PHA material is solubilized. It is one of the most widely used methodologies in this type of extraction [15], with NaClO being the most commonly used oxidising agent. However, it is a method that, to achieve high purity ($>$97%), requires large amounts of reagent (in a ratio greater than 5:1) and drastically degrades the polymer [16, 17, 18, 19]. To reduce the degradation it is common to apply a pretreatment with surfactants that destabilises the envelope around the polymeric granule without disturbing the crystalline matrix.

Acid digestion: The application of acids induces the hydrolysis of lipids present in the various cellular lipid barriers, from the wall to the envelope of PHA granules. This effect increases membrane permeability allowing the exit of NPCM. The acids commonly used for this purpose are sulphuric (H_2SO_4) and hydrochloric (HCl), attaining quite positive results in terms of recovery and purity, but inducing extensive polymer degradation. It is seen as a poor option for industrial use due to the corrosive and toxic traits of these chemicals when used in concentrated form, as well as the need for high temperatures [18, 20].

Alkaline digestion: The effect of alkali and acidic reagents are physically similar, inducing saponification of lipid impurities. For this type of digestion, the most common and used reagents are NaOH and NH_4OH. In general, sodium hydroxide performs better, with higher PHA recovery, purity, molecular mass but higher polydispersity index (presence of greater variety in polymer length, indicative of differential degradation) [21, 22]. Still, for both reagents, the polymer degradation is considerable as the applied alkali hydrolyses PHA, a situation that can be minimised if the crystalline structure of the intracellular granules is preserved.

Comparing directly NaOH with NaClO (the reagent most used in the digestion of MCNP), the hypochlorite is generally more effective and the polymer obtained is also in better conditions [23, 24].

In various phases of the process it is needed a drying step, every one of them applied with ovens and the solid is spread onto trays without a considerable air flow, so, the drying is induced by heat conduction. This operation brings an important issue because although PHA's are thermoplastic, they have a low thermal resistance, wich varies between polymers but advises to proceed at a temperature below 60ºC [25].

2. Process

In the process that generates the biomass that was delivered to NOVA for PHA extraction, the production is made using as substrate an efluent from an Italian paper industry. One of the main objectives in processing the material that will make up the paper is to remove the lignin [26, 27], a hydrophobic polymer, similar to PHA, with a complex molecular structure that makes it resistant and it is also a component that is maintained throughout the productive process so it is expected that it remains intact up to the downstream phase.

The efluent shoud be enriched in VFA's through acidification but that step is dispensed in this process and it is fed directly to the accumulation reactor. After fermentation, the PHA-rich biomass is separated from the fermentation broth by centrifugation, assisted with a coagulant (polyamide). The resulting solid is acidified with acetic acid, to inactivate the cells and preserve the polymer content,

and then dried on trays in an oven at 60ºC for several days (>3). In some productions, this acidification step was not included as an attempt to simplify the process. At NOVA, the biomass is received in the form of dry pieces and sometimes they were obviously burnt.

In terms of accumulation, the received biomass had a polymer content ranging between 10 and 30% of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). For the extraction optimisation tests in the laboratory, the same biomass was used, IS201105, considered to be a standard biomass representing the production process, with PHA and moisture contents of approximately 24% and 5.4% respectively. Of the initial polymer, half was lost in the preliminary extraction tests, including in the control (incubation with only water). To be water soluble, the polymer needs to be already degraded in the form of monomers/oligomers, since the polymer granule, as it is produced intracellularly, is not soluble [28]. The molecular weight of the polymer (extracted with chloroform) was approximately 80 kDa as opposed to the 250 kDa measured for other polymer produced with MMC at NOVA. This analysis confirmed the hypothesis that the polymer was degraded and the root of this is probably in the drying step, prior to shipping the biomass to NOVA.

3. Methodology and materials

When the biomass arrives at NOVA it is ground manually with pestle and mortar or using a hammer mill Dietz DR80b/29. Then it was extracted in the lab, the procedures being developed throughout the experimental processes but some aspects remained unchanged to ensure comparability of results: the ground biomass is divided into fractions of 400 mg in 50 mL falcon tubes, where the reagents chosen for the test are added, obtaining a concentration of typically 20 g biomass/L. The mixture is incubated on a plate with constant agitation and temperature (agitation of 200 rpm; temperature varied between 30 and 50ºC depending on the trial). Masses are measured on a Sartorius CPA 225 D digital balance with a sensitivity of 0.01 mg. The moisture content of the samples was determined on a Mettler Toledo HB 43 drying balance. The centrifuge used for centrifuging/washing is the Sartorius Sigma 3-16K at 9300 g. The Thermo Scientific Orion Dual Star sensor and MQuant®Supelco®pH test strips were used to control the pH

The extraction at pilot scale was conducted in a 100L stainless steel reactor, the centrifugations were made in a continuous centrifuge CEPA Z 41 at 20,000 rpm ($\pm$10%).

For the drying trials, the reaction vessel used are 48 mL Nalgene tubes and the centrifuge is the Sartorius Sigma 4-16KS.

The reagents used were solid NaOH Labchem, H_2SO_4 Honeywell with 95,0-97,0% purity, ethanol Honeywell with $\geq$ 99,8% purity, acetic acid (AA) Fisher Chemical, H_2O_2 Fisher Chemical 60% m/v and NaClO Acros Organics 10-15%.

The polymer in the samples was quantified through gas chromatography (GC), all off them being lyophilised in a ScanVac CoolSafe 110-4, prior to the analysis. Calibration curves were obtained using commercial PHBV from Sigma-Aldrich (PHV content 12% mol) and hydroxydecanoate (HD) was used as internal standard. For this analysis, the methanolysis principle described by Cruz et al. [29] was followed with some slight modifications.

The apparatus used for the quantification is the *Chromatograph Trace 1300, Thermo Scientific* with a Restek column (60m, 0.53mm internal diameter, 1mum thick film, Crossbond, Stabilwax). The carrier gas used is helium with a flow rate of 1mL/min and a constant pressure of 14.50 psi. Each sample is injected at 2.0 μL that remain in the column for 32 min.

The PHA mass measured in each sample is taken as the sum of HB and HV monomer equivalents quantified in the analysis as shown in the following equation (1):

$$PHA = PHB + PHV(g) \quad (1)$$

The PHA content (%PHA) is calculated by dividing the polymer mass measured by the total mass placed in the digestion tube (2):

$$\%PHA(w/w) = \frac{PHA}{M_{total}} \cdot 100 \quad (2)$$

The recovery is determined by a quotient. The numerator multiplies the polymer content of the extraction product by the mass (M) extracted; the denominator is the product between the %PHA of the initial biomass and the initial biomass extracted in the test as shown in equation 3:

$$\%PHA(w/w) = \frac{\%PHA_{extractedprod} \cdot M_{extractedprod}}{\%PHA_{initialbiom} \cdot M_{initialbiom}} \cdot 100 \quad (3)$$

Also, to compare the different extractive strategies between themselves as well as with the initial biomass, the values for the PHA mass and for the NPCM are normalized. Equation 4 shows the calculation for the polymer, for the NPCM it is calculated in the same way, using the difference between the volatile solids and PHA contents in the samples.

$$PHA_{norm}(w/w) = \frac{\%PHA \cdot M_{extractedprod}}{M_{initialbiom}} \quad (4)$$

To dry the extraction product some preliminary tests were made on the drying conditions at 60, 70 and 105ºC. The initial moisture content of the samples was determined in the Mettler Toledo HB 43 drying balance. For the determination of the drying curves, the mass of the samples was measured using the Kern ALJ310-4A balance with a sensitivity of 0.1 mg. For the trials at 60 and 70ºC, the oven used was the Memmert BE 200 with natural air circulation; for the latter trial it was used the Memmert UN30 with natural air circulation. It was also used a microwave as pre-treatmen employing a Whirlpool MD 111 and a maximum *output* of 750 W.

4. Results and discussion

When I joined the NOVA ID team, the project had already been running for several months and some decisions had already been made. To ensure that during the incubations, the reagents were in contact with the biomass, a constant agitation of 200 rpm was established, transversal to all the tests performed.

An alkaline digestion with 0.84 M NaOH at 30 ºC had been chosen, the optimum conditions were obtained when the biomass was incubated for 0.8 h, i.e. 48 min. With this extraction, the product has a PHA content and a recovery of approximately 17 and 36%, respectively. The remaining impurities are predominantly organic (60-65%), of these, part is biomaterial from the PHA-producing microorganisms, other comes from the effluent of the paper industry, and finally there is have a small fraction of polyamide.

As for inorganic impurities, which still make up 40% of the total, it was theorized that they are primarily made up of calcium carbonate, which can be easily removed by acidification. This theory is supported by DSC and FTIR analyses, which strongly indicate the presence of this carbonate, and also by evidence of gas release upon contact with a strong acid (H_2SO_4).

Trial 1: The first trials that I was a part of had the objective to target specifically the lignin and the calcium carbonate, using some chosen techniques. The conditions applied are described in table 1 and the results depicted in figure 1.

The first condition tested, aimed to a lower temperature (30 vs 80ºC) for the the pre-incubation (considered close to an industrial ambient temperature) but the recovery of 26% and a polymer content of 41% were far behind the results achieved with a pre-incubation at 80ºC.

Observing the results referring to assays 2 and 3, it is possible to conclude that with wash water volumes of only 20 mL instead of the 40 mL, the performance of the extraction can be improved, with an increase of approximately 4% in the polymeric content and an increase in the polymeric recovery of over 10%. Condition 2, the best in this trial had a %PHA of 70.8$\pm$1.5% and a recovery of 33.7$\pm$1.8%.

In the fourth tests, pre-incubation combined with an oxidative extraction with sodium hypochlorite was analysed. The results were similar to those achieved with a alkaline extraction and, as it is a more toxic and polluting reagent than NaOH it was discarded; this was not an option for further trials.

Finally, in test 5, the attempt to reduce the amount of reagents required was clearly unsuccessful, resulting in both very low polymer contents and recoveries.

Although this trial was built on solid scientific foundations, it was not aligned with the purpose of scaling up the extraction process, since it included a long pre-incubation with heating (2 h at 80ºC) and a relatively high concentration of acetic acid (50%), a very expensive reagent. For these reasons, it was suggested to replace the acetic acid with a strong acid, such as sulphuric acid, but the mixture of strong acids and hydrogen peroxide is extremely corrosive and potentially explosive and is commonly called piranha solution. Therefore the use of such mixtures was discarded.

Since this type of approach could not be followed, although it showed promising results, it was decided that the next step would be to test the possibility of including acid pre-incubation without hydrogen peroxide in the extraction process.

Trial 2: The first step was to choose the concentration of acid to apply in the pre-treatment; preliminary trials allowed to conclude that the best option was to use 0,5 M of sulphuric acid (A). Then it was necessary to define the time of the acidic incubation and then to optimize the extraction as a whole, enabling the scale-up.

The results in figure 2 and table 2 (conditions 1,2 & 3) showed that a quick incubation of 5 minutes was as efective as a longer one but, for minimisation of errors and deviations, an incubation of 1 h was implemented for further optimization.

Condition 4 was applied to test the acid pre-treatment with 1 hour, an intermediate wash and the alkaline treatment. It was the condition that gave the best results of this trial and this optimization with a polymer content of 27.5$\pm$2.4% and a recovery of 40.3$\pm$3.1% of the initial polymer. In relation to the initial biomass, the extraction product was polymerically enriched by 11.5%.

The efect of the extraction was analyzed by studying the nature of the materials as shown in figure 3.

Table 1: Conditions applied in the trial 1

1	Pre-incubation with 4mL of 50% AA and H_2O_2 15% at 30ºC for 2h followed by a wash with 40 mL of water. The pellet is collected and incubated with 20 mL of 0.84 M NaOH at 30ºC for 0.8 h. Finally, the product is washed with 40 mL of water.
2	Pre-incubation with 4 mL of 50% AA and H_2O_2 15% at 80ºC for 2h followed by a wash with 20 mL water. The pellet is collected and incubated with 20 mL of 0.84 M NaOH at 30ºC for 0.8 h. Finally, the product is washed with 20 mL of water.
3	Pre-incubation with 4 mL of 50% AA and H_2O_2 15% at 80ºC for 2h followed by a wash with 40 mL water. The pellet is collected and incubated with 20 mL of 0.84 M NaOH at 30ºC for 0.8 h. Finally, the product is washed with 40 mL of water.
4	Pre-incubation with 4 mL of 50% AA and H_2O_2 15% at 80ºC for 2h followed by a wash with 40 mL water. The pellet is collected and incubated with 40 mL of NaClO 6.5% at 30ºC for 0.8 h. Finally, the product is washed with 40 mL of water.
5	Pre-incubation with 4mL of AA 10% and H_2O_2 3% at 30ºC for 2h followed by a wash with 40 mL of water. The pellet is collected and incubated with 20 mL of 0.84 M NaOH at 30ºC for 0.8 h. Finally, the product is washed with 40 mL of water.

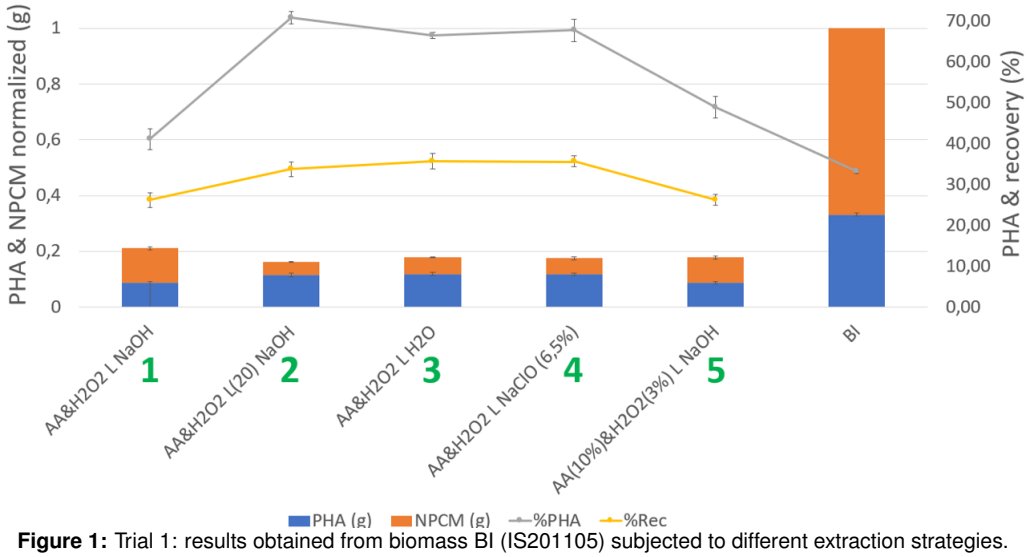

Figure 1: Trial 1: results obtained from biomass BI (IS201105) subjected to different extraction strategies.

Table 2: Conditions applied in the 2 trial

1	Incubation with 20 mL of 0.5 M A at 30 ℃ for 1 h, followed by a wash with 40 mL of water.
2	Incubation with 20 mL of 0.5 M A at 30 ℃ for 2 h, followed by a wash with 40 mL of water.
3	Incubation with 20 mL of 0.5 M A at 30 ℃ for 3 h, followed by a wash with 40 mL of water.
4	Pre-incubation with 20 mL of 0.5 M A at 30ºC for 1 h, followed by washing with 40 mL (water), then the pellet is collected and incubated with 20 mL of 0.84 M NaOH at 30ºC for 0.8 h. Finally, the product is washed again with 40 mL of water.
5	Pre-incubation with 20 mL of 0.5 M A at 30ºC for 1 h, followed by an acidity adjustment up to pH 10 with 10 mL of NaOH. Finally the product is washed with 40 mL of water.

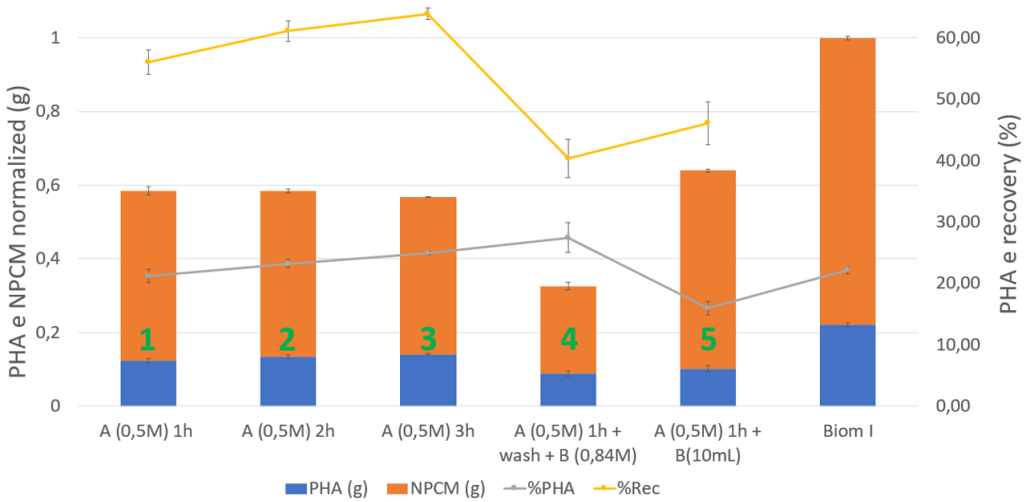

Figure 2: Trial 2: results obtained from biomass BI (IS201105) subjected to different extraction strategies.

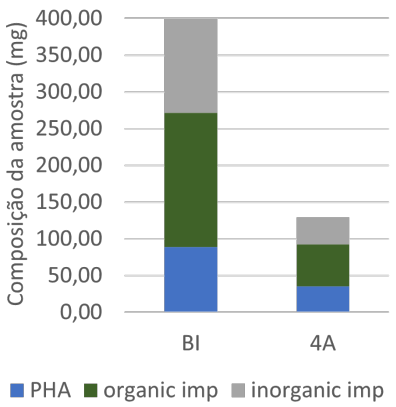

Figure 3: Quantitative and qualitative analysis of the nature of the samples and their impurities (imp) showing the effect of the degradation imputed by the extraction process from the initial biomass (BI) to the extraction product (4A).

A major part of the different impurities were digested successfully, but almost 60% of the polymer was also lost.

Finally, the fifth test was inspired by the literature that suggested a correction up to pH 10 before centrifugation [14, 18, 30]; this was carried out by adding 10 mL of a 2 M NaOH solution. The products obtained show lower recovery and polymer content than products 1 that received the same treatment without the adjustment.

Trial 3: To further optimise the downstream another trial was applied. The conditions implemented and the results of the assay are depicted in the table 3 and figure 4, respectively.

It should be noted that for this assay, the vessel for extraction was changed: instead of 50 mL falcon, 48 mL round-bottom Nalgene flasks were used. This test had to be divided in two days, so, two equal controls were made: control 1 referring to the first day and control 2 to the second day; in the figure the testes applied in different days are divided by a dotted line.

It is important to clarify how each condition was applied, highlighting some consequences of the strategies used and that differentiate this test from the previous ones.

As described in table 3, in conditions 1, 2 and 3 an acidic pre-incubation was applied with 20 mL of 0.5 M sulphuric acid. This volume makes for an optimum biomass concentration of 20 g/L, as the initial mass weighed for each Nalgene was 400 mg. Now for the second incubation, the total volume is higher as only a certain amount of a 2M NaOH solution is added diluting the biomass concentration. For the second incubations we then have a biomass concentration of: **1** - 13.5 g/L; **2** - 13.3 g/L; **3** - 10.7 g/L. This concentration decreases from condition to condition and deviates more and more from the optimal value.

A general analysis of these results shows that this test was unsuccessful as none of the conditions applied resulted in an improvement of the polymer content compared to the initial biomass.

In the first day trials, the best condition was condition number 2, where the pH adjustment was applied using calculated dosage. It would be expected that the most favourable condition would be the third one, where the second incubation was done in a much more concentrated basic environment (0.84 M). This divergence is due to the volumetric difference in the incubation where condition 3 is more deviated from the optimal value of 20 g biomass/L.

Regarding the second part of the test, the aim was to explore new alternatives, keeping the intermediate wash. Test 5 had a very interesting result: when changing the order of the contrasting treatments, basic incubation and acid post-incubation, the polymer degradation is very large resulting in a recovery of only 14.7±3.3%. Condition 6 was the most favourable of the assay, achieving a polymer content of 20.24% and recovery of 26.5% yet below what was achieved before.

A scale-up extraction was also tried when the extraction development was still at an early stage. Even knowing that a low quality product would be obtained, the trial was carried out so that the partners would have at least 1 kg of product to test their processing method and final application.

As there was not enough IS201105 biomass for this test, several batches with the highest PHA were selected and are listed in the table 4. It was used an extraction purely alkaline with NaOH (0.84 M) at 30ºC for 0.8 h where the biomass is at a concentration of 20 g/L followed by a wash.

Table 4: Biomass applied in the pilot scale extraction trial

Biomass	Mass (kg)	%PHA
IS201130	0.38	25.5±2.0
IS201217-A1	0.68	27±8.5
IS201217-A-1&2	0.3	28.4±0.3
IS201217-NA-2&3	0.51	29.6±1.0
IS210112-NA-1&2	0.3	29±0.1
IS210112-NA-2	1.39	24.9±1.4
Pilot biomass	3.56	26.7

In a subsequent GC run this biomass was analysed giving a %PHA of 25.1% close to the calculated in the table.

From the 3 kg of pilot biomass, 1348.60 g of extraction product with a PHA content of 8.17±0.4% was obtained. As expected, the extraction was not very effective in digesting the inorganic impurities, since there was no acid pre-treatment to solubilise the calcium carbonate. Even so, the characteristic of this extraction that stands out is the very small

Table 3: Conditions applied in the 3 trial

1	Pre-incubation with 20 mL of 0.5 M A at 30ºC for 1 h, the pH is adjusted by practical neutralization (9.6 mL of 2M NaOH). This is followed by incubation at 30ºC for 0.8 h and the product obtained is washed with 40 mL of water.
2	Pre-incubation with 20 mL of 0.5 M A at 30ºC for 1 h, the pH is adjusted by calculated neutralization (10 mL of 2M NaOH). This is followed by incubation at 30ºC for 0.8 h and the product obtained is washed with 40 mL of water.
3	Pre-incubation with 20 mL of 0.5 M A at 30ºC for 1 h, NaOH was added to obtain a final concentration of 0.84 M OH^- (17.24 mL). The mixture is incubated at 30ºC for 0.8 h and the product obtained is washed with 40 mL of water.
4	It is pre-incubated with 20 mL of water at 30ºC for 1 hour and then incubated with 40 mL of water at 30ºC for 0.8 hour. Finally, the product obtained is washed with 40 mL of water.
5	Incubation with 20 mL of 0.84 M NaOH at 50ºC for 0.8 h, followed by a wash with 40 mL of water, the pellet is collected and submitted to a post-incubation with 20 mL of 0.5 M A for 1 h. Finally, the product is washed with 40 mL of water.
6	Pre-incubation with 20 mL of 0.5 M A at 50ºC for 1 h, followed by washing with 40 mL (water), then the pellet is collectet and incubated with 20 mL of 0.84 M NaOH and 2.5% H_2O_2 at 30ºC for 0.8 h. Finally, the product is washed again with 40 mL of water.
7	Pre-incubation with 20 mL of water at 30ºC for 1 hour and then incubated with 40 mL of water at 30ºC for 0.8 hour. Finally, the product obtained is washed with 40 mL of water.

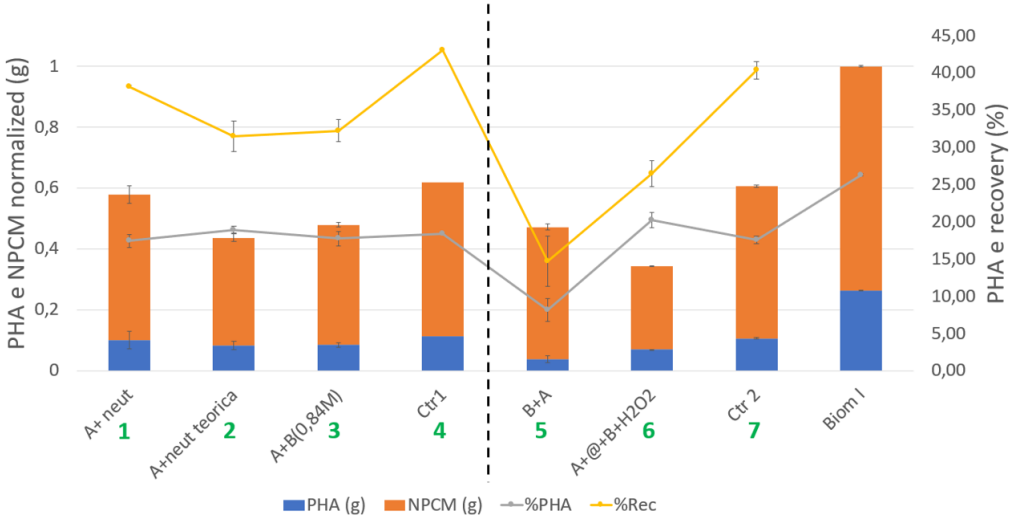

Figure 4: Trial 3: results obtained from biomass BI (IS201105) subjected to differente extraction strategies.

amount of polymer that was reclaimed, with a recovery of only 22.1%, a value much lower than the one obtained in the laboratory with the corresponding extraction, in which the recovery exceeds 35%. This difference could be related to an big deviation in the GC analysis since, in this run, the initial biomass was quantified with a polymer content of only 18.0±1.8% as opposed to the 25.1% read before.

Another factor that may have contributed to the higher losses of the material of interest in this assay lies in the characteristics of a pilot scale extraction. Firstly, it is a test in which it is more difficult to control all the conditions when compared to a laboratory test. Then, the product was transferred from container to container on multiple occasions, in addition to the centrifugation yield these are a big source for material loss in this process.

Last but not least, three drying trials of the extraction product were conducted. In each experiment the temperature was kept unchanged at 60, 70 and 105ºC. The extraction on which these tests were based was the best one achieved on Nalgene flasks (condition 2, trial 3), had an acidic pre-incubation with sulphuric acid, followed by a calculated dose neutralisation after which a second incubation was applied and, finally, a wash. Three different extraction conditions were applied, including one of them, a post-treatment:

- **Ctr**: Control condition where an acidic pre-incubation with 20 mL of 0.5 M sulphuric acid (for 1 h at 30ºC) was applied followed by a calculated neutralisation (with 10 mL of NaOH), the alkaline mixture was incubated (for 0.8 h at 30ºC) and finally the product was washed with 20 mL of H_2O.
- **EtOH**: Acidic pre-incubation with 20 mL of 0.5 M sulphuric acid (for 1 h at 30ºC) was applied followed by a calculated neutralisation (with 10 mL of NaOH), the alkaline mixture was incubated (for 0.8 h at 30ºC) and finally the product was washed with 20 mL of ethanol (70%).
- **MW**: Acidic pre-incubation with 20 mL of 0.5 M sulphuric acid (for 1 h at 30ºC) was applied followed by a calculated neutralisation (with 10 mL of NaOH), the alkaline mixture was incubated (for 0.8 h at 30ºC) and finally the product

was washed with 20 mL of H_2O. Finally, immediately before drying, the solid was heated in a microwave oven (duration variable according to the test).

This test had a exploratory character and its main objective was to investigate the degradation caused by the different drying conditions. However, the analyses that would allow us to conclude on this subject could not be carried out because the GC apparatus broke down at the beginning of August 2021 and could not be used for the present report.

It is also considered that the results of this assay cannot be extrapolated to a larger scale, since the drying was applied to relatively small disks of extraction product (diameter of 3 to 4,5 cm and height of 0,5 cm), while on a large scale they would always be on large plates and the mass/surface area ratio would be higher, which increases the mass transfer resistance.

These trials, even with some characteristics that make comparison between them difficult, made it possible to evaluate the strategies applied. adding a polishing step with ethanol brings a higher inefficiency to the extraction, resulting in a reduction of the final polymeric content. This setback associated with the greater complexity of drying with ethanol, which necessarily involves a strategy to recover this vapour, and the higher cost of this liquid compared to water, allows us to conclude that it is not a favourable strategy.

The samples that underwent microwave pretreatment (MW), presented a shattered disc, so, the mass/contact area ratio decreases, favouring drying. This characteristic makes the results obtained unreliable. Microwave pre-treatment also proved to be an impractical technique in terms of feasibility of scale-up because, for the treatment to be effective, a large amount of solid is projected bringing practical difficulties upon application.

The preferred temperature was 70ºC, the Ctr samples dried completely in 115 min. At 60ºC the drying procedure was much slower with the assay being stoped at the minute 200 for technichal reasons and the Ctr sample was not even close to be completely dried. Finally, the 105ºC process dried the control sample in 100 min, only 13% faster than the assay conducted at 70ºC. However, it is essential to ascertain the degradation caused by the heat treatment, as it is advisable to apply it at a temperature below 60ºC to reduce the impacts on polymer quality [25].

5. Conclusions

It was not possible to develop an environmentally friendly process to extract the polymer of interest from available biomass that would make scaling up the process feasible.

Optimisation attempts were clearly constrained by the characteristics of the biomass coupled with the extraction strategy since the NPCM digestion is designed to extract PHA from biomasses with high polymer content [31, 32].

The purified polymer quality has a relatively low molecular weight (below 80,000 g/mol), therefore the applications of this PHA are very limited.

Although the extraction was not successful, this type of ecological extractive strategy has shown success all over the world, when applied to biomasses with high polymer contents. Although this project has not yet achieved positive results, it does not mean that NPCM digestion, and specifically basic digestion, is not a method with potential, and I believe that it may even be prevalent in the PHA industry in a few years.

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