



# Submission to Journal of Polymers and the Environment Evaluation of Thermal Properties and Crystallinity in PHB-Based Systems – A DoE Approach

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## Abstract

Complex formulations based on poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate-*co*-valerate) (PHBV) were studied to statistically assess the effect of formulation (i.e., hydroxyvalerate (HV) content, plasticiser chemistry and content, filler type and content) on their thermal properties and degree of crystallinity ( $X_c$ ). In binary systems, thermal properties were mainly influenced by filler type rather than its content, while for plasticised systems the changes were dependent on both increasing plasticiser content and PHB-plasticiser compatibility. Variations in HV content affected the ability of the polymer chain to fold, leading to significant changes in both thermal properties and  $X_c$ . In ternary systems, presence of multiple additives and consequent changes in intermolecular interactions lead to multifaceted behaviours that were not easily predicted by results from binary systems alone. For example, melting temperature did not show dependence on filler presence in PHBV systems despite introducing variations in pure PHB systems. In general, thermal properties and  $X_c$  are affected by all parameters studied, with changes in system free volume (i.e. changes in HV content and plasticisation) playing the most significant role. These results expand the understanding of factors controlling crystallisation in complex polymer systems and can be used to control matrix properties in new generations of packaging materials.

**Keywords** Poly(hydroxybutyrate) · Crystallization · Melting · Nucleant · Plasticiser · DSC

## Introduction

Despite the recent progress in the field of green plastic packaging, currently used formulations are often petroleum-derived, single-use, non-recyclable and non-biodegradable due to technology and infrastructure limitations. These factors, together with lack of coherent waste disposal schemes, results in plastic packaging being the main contributor to plastic pollution [1] and generates a need for addressing this problem by replacing current plastic packaging formulations with their eco-friendly alternatives [2]. This movement is supported by consumer awareness as well as stricter policies and regulations [3–5], both national and international, with clear goals of eliminating single-use plastic packaging

and their replacement with recyclable or biodegradable solutions.

Biodegradable materials have attracted a lot of attention as potential candidates to replace synthetic thermoplastics and are widely regarded as solutions to alleviate demands on diminishing landfill space [6] and overall plastic pollution. Among these, poly(hydroxyalkanoates) (PHAs), natural polyesters produced by microorganisms as carbon and energy reserves [7,8], are particularly interesting as they are fully biodegradable and biocompatible [8,9], have mechanical properties similar to commonly used thermoplastics, good barrier properties, and are compatible with current manufacturing processes due to their melt-processability [10]. Poly(hydroxybutyrate) (PHB) is the best studied PHA as it contains no catalyst residues, has high stereoregularity [9] and crystallizes slowly [11]. These properties make PHB suitable to be used as a model nucleating system, enabling extension of the results from this polymer to other polymers with similar properties.

Despite these advantages, pure PHB systems are problematic in terms of processing due to the small processing

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window (i.e. difference between the degradation temperature and melting temperature) [12] and brittleness, which leads to several problems in terms of cost and broad industrial application [13,14]. These problems can be addressed by modifying the polymer matrix by changing HV content [6,15], controlling crystallisation conditions (e.g. crystallisation temperature,  $T_c$ ) [16], as well as introducing additives to the system [17]. The most popular additives are plasticisers (that reduce the brittleness of the matrix) and nucleating fillers (that influence the spherulitic density by acting as heterogeneous nucleation centres). As we have shown in our review [18], while many studies have investigated the effect of additives on the crystallisation behaviour of PHB at given conditions, it is currently challenging to provide an unambiguous trend in the system behaviour upon introducing different additives to the system due to variations in processing methods and additive properties. Moreover, the vast majority of research focuses on the influence of a single additive at fixed processing conditions. For more complex formulations, the traditional way of performing experiments made it impossible to deconvolute the effect of each additive due to complex interactions between each parameter. Therefore, there is no systematic way of assessing the influence of the effect of each parameter (e.g. changes in formulation or processing conditions) on the resulting system properties in complex formulations that mimic real-life products.

Design of experiments (DoE) is a method that uses analysis of variance (ANOVA) to plan and analyse experiments as well as interpret them to obtain information about the effects of each individual variable on the results, identify interactions between the variables, and model the outcome using a mathematical function. DoE enables making conclusions about simultaneous changes of multiple parameters, preventing often inconclusive results obtained from experiments where parameters are changed one at a time [19]. Specifically, full-factorial design (FFD) provides information about all of the potential interactions within a system, while other systematic designs such as response surface design (RSD) are better suited to account for non-linear relationships between control parameters and their outcomes. Results obtained from DoE allow the exclusion of statistically insignificant terms to minimise the number of parameters in the system, in turn minimising processing costs and enabling control of the final product.

We have shown in our previous work [20] that DoE can be successfully applied to assess the effect of each individual factor on spherulitic growth in PHB-based systems. Here, we are applying DoE to study the effect of formulation (HV content, plasticiser content and type, filler content and type) on thermal properties and crystallisation in PHB-based systems performed by Dynamic Scanning Calorimetry (DSC) to probe the importance of different length scales while investigating crystallisation and providing a natural extension to

previous work. This way, we will assess the statistical importance of each factor, assess interactions between the individual factors, conclude which parameters do not contribute to the outcome, and highlight the differences between crystallisation at various length scales, in turn enabling creation of design criteria for next-generation packaging materials.

## Materials and Methods

### Materials

Pure PHB and its copolymer, PHBV (HV contents of 7, 12, and 21 mol%, with molecular masses listed in Table 1), were obtained in a powder form from Zeneca Bio Products and purified by Soxhlet extraction in ethanol to remove residual cell debris, followed by drying at 40 °C and stored at low relative humidity environment (desiccator with silica gel) at room temperature prior to usage. The HV content of each sample was confirmed via nuclear magnetic resonance measurements. Triacetin (TA, Sigma-Aldrich, W200700, 99%, liquid) and acetyl tributyl citrate (ATBC, Sigma-Aldrich, W308005,  $\geq 98\%$ , liquid) were used as plasticisers, while Cloisite Na<sup>+</sup> and Ca<sup>++</sup> (BYK, in powder form, denoted as CNa<sup>+</sup> and CCa<sup>++</sup>, respectively, with particle size of ca. 2  $\mu\text{m}$  and 1  $\mu\text{m}$ , respectively), calcium carbonate (Sigma-Aldrich, C4830,  $\geq 99\%$ , powder, ca. 3  $\mu\text{m}$ ), and boron nitride (BN, Sigma-Aldrich, 255,475, 98%, powder, ca. 0.6  $\mu\text{m}$ ) were used as fillers. Plasticisers used in this study were proven to be compatible with PHB [21–23]. Plasticiser and fillers were used as received.

### DoE Parameters

FFD was utilised to create a matrix of formulations that need to be investigated to assess the parameters influencing thermal properties and degree of crystallinity ( $X_c$ ) in the PHB-based systems. This design was chosen as FFD has been proven to create better models compared to the other methods [24]. Further, alternative design choices such as central composite design were not appropriate for use in this study due to their incompatibility with categorical

**Table 1** Molecular masses of PHBV formulations used in this study expressed as the ‘polystyrene equivalent’ molecular masses. The numbers represent only soluble material

Sample type	$M_w$ /g·mol <sup>-1</sup>	$M_n$ /g·mol <sup>-1</sup>
PHB	621000 ± 1000	225000 ± 2000
7% HV	451000 ± 4000	179500 ± 1500
12% HV	453500 ± 500	178000
21% HV	429500 ± 3500	172000 ± 2000

**Table 2** Factors and factor values used for full- and partial factorial design (FD)

Factor	Code	Factor values					FFD concerned
		1	2	3	4	5	
HV content/ mol%	A	0	7	12	21	–	FF1
$X_{\text{filler}}$ / wt. %	B	0	1	5	–	–	FF1 & FF2
Filler type	C	No filler	CaCO <sub>3</sub>	CCa <sup>++</sup>	CNa <sup>+</sup>	BN	FF1 & FF2
$X_{\text{plasticiser}}$ / wt. %	D	0	10	20	–	–	FF2
Plasticiser type	E	No plasticiser	TA	ATBC	–	–	FF2

variables (i.e. plasticiser and filler types) and requirement for nonphysical formulations (i.e. negative values of additive content). The main drawback of FFD – using only first order equations (linear regression, the general form including 2- and 3-way interactions shown in Eq. 1)—was overcome by supplementing with RSD which implements quadratic terms in the response equation to account for possible non-linear correlations in the system (the general form shown in Eq. 2).

$$f(x_1, \dots, x_k) = \beta_0 + \beta_1 x_1 + \dots + \beta_k x_k + \beta_{12} x_1 x_2 + \dots + \beta_{k-1,k} x_{k-1} x_k + \dots + \beta_{123} x_1 x_2 x_3 + \dots + \beta_{k-2,k-1,k} x_{k-2} x_{k-1} x_k \tag{1}$$

$$f(x_1, \dots, x_k) = \beta_0 + \beta_1 x_1 + \dots + \beta_k x_k + \beta_{11}^2 x_1^2 + \dots + \beta_{kk}^2 x_k^2 + \beta_{12} x_1 x_2 + \dots + \beta_{k-1,k} x_{k-1} x_k \tag{2}$$

In Eqs. 1 and 2,  $x_k$  stands for the value of an independent factor used in the analysis,  $\beta_k$  refers to the corresponding coefficient, while  $k$  is the number of factors studied.

Minitab 20 was used for matrix creation and data analysis. The experiments were divided into six separate sets regarding formulation:

Set 1. Pure PHB samples.

Set 2. Pure PHBV samples.

Set 3. Plasticised PHB samples.

Set 4. Filled PHB samples.

Set 5. Filled and plasticised PHB samples.

Set 6. Filled PHBV samples.

For the analysis, two separate groups were created: 1) to assess the influence of HV content, filler type and content in unplasticised samples (sets 1, 2, 4, and 6) and 2) to assess the influence of plasticiser type and content as well as filler type and content for pure PHB matrix (without effect of HV content, sets 1, 3, 4, and 5) on thermal properties describing crystallisation in the given system ( $T_{m2}$ ,  $T_{cc}$ ,  $T_{ch}$ , vide infra, Table 3) and  $X_c$ , with separate analysis performed for each parameter. They were denoted as full-factorial 1 (FF1, with 36 formulations studied,  $k = 3$ ) and full-factorial 2 (FF2, with 45 formulations studied,  $k = 4$ ), respectively. The complete lists of samples analysed in FF1 and FF2 design are presented as tables s1 and s2 in the Supporting Information, while the complete set of thermal properties analysed in the study is presented as Table 3. Experiments were performed in random order (i.e. not by sets) to avoid any potential biases.

All datasets met the assumptions required for ANOVA (the residuals are independent, normally distributed, and

**Table 3** Symbols and meaning of parameters extracted from DSC analysis

Symbol	Meaning
$X_c$	Degree of crystallinity (%)
$T_{m1}$	Temperature at the maximum of the first melting peak (°C)
$T_{m2}$	Temperature at the maximum of the second melting peak (°C)
$\Delta H_m$	Enthalpy of melting (J/g)
$T_c$	Temperature of melt crystallisation (during cooling for HCR run) (°C)
$\Delta H_c$	Enthalpy of melt crystallisation (during cooling for HCR run) (J/g)
$T_{ch}$	Temperature of cold crystallisation in HCR run (°C)
$\Delta H_{ch}$	Enthalpy of cold crystallisation in HCR run (J/g)
$T_g$	Glass transition temperature (°C)
$T_{cc}$	Cold crystallisation temperature in quenching run (°C)
$\Delta H_{cc}$	Enthalpy of cold crystallisation in quenching run (J/g)
TRC	Temperature range over which crystallisation upon cooling occurs in HCR run (°C)
TRCC	Temperature range over which cold crystallisation in quenching run occurs (°C)
TRM	Temperature range over which melting occurs (°C)
TRCH	Temperature range over which cold crystallisation in HCR run occurs (°C)

with approximately equal variance; all input factors are independent of one another). The complete set of parameters and factor values used for each FFD is summarised in Table 2.

Contents of the fillers and plasticisers were based on the limits commonly used in literature [18] and industry [25] and were chosen as 1 wt.% and 5 wt.% for fillers and 10 wt.% and 20 wt.% for plasticisers, with respect to the polymer content.

### Sample Preparation

Plasticised polymer powder was prepared by manual mixing of polymer and plasticiser in the desired mass ratio at room temperature. As-prepared powders were left overnight to allow diffusion of the plasticiser into the polymer to improve its distribution. Filler was incorporated by manual mixing with either plasticised or unplasticised polymer in the desired mass ratio at room temperature [23,26,27]. The samples were named as PHBV/additive1/additive2 fashion, with the content of the additive calculated with respect to pure polymer (wt.%). The samples were melted during first DSC run as described below and were run in duplicates (powder samples prepared twice rather than running two experiments on the same batch to ensure no systematic error). For the presented set of data, the repeatability for all samples was good (error  $\pm 2\%$ ), with no significant clustering of the filler within PHB matrix observed [18, 20].

### Differential Scanning Calorimetry

Ca. 7 mg of mixed powder was placed in the DSC pans and used for analysis. Measurements were performed using a differential scanning calorimeter (DSC Q1000, TA Instruments), calibrated using indium standard, using both heat-cool-reheat (HCR) and quenching runs for each formulation under 20 mL/min nitrogen purge, with detailed procedure as specified below.

#### HCR Run

1. Cooling sample down from ambient to  $-40\text{ }^{\circ}\text{C}$ , followed by heating up to  $200\text{ }^{\circ}\text{C}$  at  $10\text{ }^{\circ}\text{C}/\text{min}$ , equilibration, and keeping sample isothermal for 2 min at  $200\text{ }^{\circ}\text{C}$ .
2. Cooling sample down from  $200\text{ }^{\circ}\text{C}$  to  $-40\text{ }^{\circ}\text{C}$  at  $10\text{ }^{\circ}\text{C}/\text{min}$ , with equilibration at  $-40\text{ }^{\circ}\text{C}$ .
3. Heating sample up from  $-40\text{ }^{\circ}\text{C}$  to  $200\text{ }^{\circ}\text{C}$  at  $10\text{ }^{\circ}\text{C}/\text{min}$ .

#### Quenching Run

1. Cooling sample down from ambient to  $-40\text{ }^{\circ}\text{C}$ , followed by heating up to  $200\text{ }^{\circ}\text{C}$  at  $10\text{ }^{\circ}\text{C}/\text{min}$ .
2. Removal of the sample and quenching it in liquid nitrogen.

3. Equilibrating DSC at  $-40\text{ }^{\circ}\text{C}$  and placing quenched sample back in the instrument, followed by heating it up from  $-40\text{ }^{\circ}\text{C}$  to  $15\text{ }^{\circ}\text{C}$ , cooling back down to  $-40\text{ }^{\circ}\text{C}$  and heating to  $200\text{ }^{\circ}\text{C}$  at  $10\text{ }^{\circ}\text{C}/\text{min}$ . Heat-cool-reheat loop within this run was applied to remove any artefacts related to enthalpic relaxation effects induced by the fast quench or from condensation from the quenching step that made it impossible to clearly establish  $T_g$ .

The first heating during either DSC run was used to erase thermal history of the material and create a melt-blended sample of polymer and additives that was further characterised during the second heating. Data were analysed using TA Universal Analysis software to assess thermal properties of the given formulation as specified in Table 3. The temperatures specified therein were taken as the maximum peak values of the respective endothermal and exothermal transitions, while the enthalpies were calculated using straight baseline.  $X_c$  was calculated using the equation [28]:

$$X_c = \frac{100 \cdot \Delta H_m}{w_{\text{PHB}} \cdot \Delta H_m^0} \quad (3)$$

where  $\Delta H_m$  and  $\Delta H_m^0$  ( $= 146\text{ J/g}$ ) [29] are the experimental melting enthalpy of partially-crystalline PHB and the estimated melting enthalpy of 100% crystalline PHB, respectively, and  $w_{\text{PHB}}$  is the weight fraction of polymer in the blend (i.e. the  $X_c$  corrected to account for the presence of filler and/or plasticiser).

## Results and Discussion

In “Effect of Changes in Formulation” Section, the behaviour of pure PHB is discussed, followed by the effect of formulation – changes in polymer chain by introducing HV groups as well as addition of the plasticiser or filler—on thermal properties ( $T_{m2}$ ,  $T_c$ ,  $T_{ch}$ ) and  $X_c$  in PHB-based systems. Then, in “Statistical Analysis of Crystallisation in PHB-Based Systems” Section, a statistical evaluation of multiple factors using DoE analysis implemented to deconvolute the importance of each factor is presented.

### Effect of Changes in Formulation

#### Thermal Behaviour of Pure PHB Samples

HCR runs are a popular way of performing measurements as they erase the thermal history of a sample (during the first heating cycle) [30] and establish the effect of changes in formulation on resultant thermal properties (during the second heating and cooling cycle). Here, the results of the cooling cycle and second heating cycle are discussed to show how the additives affect the thermal transitions of PHB.

PHB samples have shown bimodal melting peak distribution, with shoulder at 171 °C and peak maximum 177 °C, in good agreement with literature reports (Fig. 1a) [31]. Such high values of  $T_m$  can cause problems with PHB degradation during processing. While degradation of PHB usually occurs at temperatures above 200 °C [32], prolonged isothermal heating can result in significant production of volatiles at 200 °C and random polymer chain scission in the temperature range 170–200 °C [33].

Melting of PHB, defined as the temperature where the fusion process is 99% complete, takes place at temperatures ca. 180 °C [31].

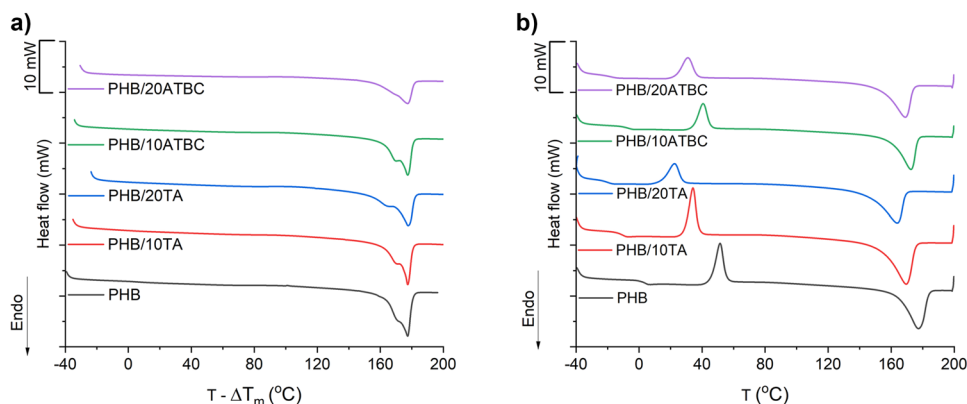
The melting behaviour deviating from unimodal distribution was widely reported for semi-crystalline polymers and are associated with five possible phenomena [34,35]: melting of unstable crystals – recrystallisation of more stable crystals at higher temperatures – remelting of more stable crystals events [36], isodimorphism or polymorphism of created crystals [37], differences in lamellar thickness distribution and morphology of the crystals [38], presence of species of various molecular weight ( $M_w$ ), either due to inherent polymer properties or as a result of random scission of PHB chain [39], and changes in crystallinity due to physical ageing and/or relaxation of the rigid amorphous fraction [40].

Upon cooling, PHB crystallises with  $T_c$  recorded at 79 °C for a cooling rate of 10 °C/min (Figure s1 in the Supporting Information). This value, similarly to other PHB properties, depends on molecular weight of PHB, its purity, and sample preparation method and has been reported to be in the 79–98 °C temperature range [41–46], suggesting good agreement with our data. However,  $\Delta H_c$  has lower values than  $\Delta H_m$  (equal to 74.2 and 91.7 J/g, respectively, Table 4). The temperature range over which crystallisation upon cooling occurs ( $TRC$ ) also varies compared to temperature range over which melting occurs ( $TRM$ ), with average values of 59 and 35 °C, respectively. These findings suggest that melting-recrystallisation-remelting events are the most likely explanation for bimodal melting peak occurrence in PHB systems, in line

with previous literature reports [36,47]. Further, the size of the first melting peak was reported as dependent on the crystallisation peak on cooling – the more crystallisation upon cooling, the greater the first crystalline melting peak [48].  $X_c$  calculated following Eq. 3 was equal to 62.8%, which appears to be in line for previous  $X_c$  measurements for this polymer (ranging from 56 to 64%) [45,49,50]. However, as-calculated  $X_c$  does not represent the degree of sample crystallinity prior to recrystallisation – instead, it refers to the overall contribution of reorganisation events [51] and will be used throughout this manuscript to assess how changes in system chemistry influence this value.

For the quenching run,  $T_{cc}$  was noted at 51 °C, with good agreement with temperature range of 37.4 – 50 °C reported elsewhere [52–54]. However, the melting peak showed unimodal distribution, with melting temperature  $T_m$  at 177 °C and the temperature range over which cold crystallisation occurred ( $TRCC$ ) equal to 26 °C (Fig. 1b, Table 4). The disparity between  $\Delta H_{cc}$  and  $\Delta H_m$  remained, with their values noted at 45.8 and 84.8 J/g, respectively, suggesting that significant recrystallisation occurs at elevated temperatures and increases the final degree of crystallinity in the sample (equal to 58.1%) and  $TRM$  (equal to 37 °C). Therefore, we propose that the quenching run leads to the creation of a higher number of smaller crystals during the cold crystallisation process compared to lower number of larger crystals that are created during the HCR run [36]. It is likely because of the differences in the crystallisation kinetics and the effective crystallisation time—shorter for the former as the crystallization peak upon heating from the quenched amorphous state occurs over a narrower temperature range than the crystallization peak upon cooling from the melt. The glass transition ( $T_g$ ) of pure PHB for quenching run is noted at 4 °C (Table 4, Fig. 1b) and it is within the  $T_g$  range reported for PHB elsewhere (– 12 to 16 °C). [55–63].

**Fig. 1** **a** Changes in melting peak width upon introducing plasticiser to the system for reheating in HCR run. For samples with the addition of the plasticiser, the temperature was translated towards higher temperatures by an amount of  $\Delta T_m$  (difference between the maximum of the melting peak of pure PHB and plasticised sample) for ease of comparison of the  $TRM$ ; **b** quenching cycle for pure PHB and plasticised PHB samples





**Table 4** Thermal properties of plasticised PHB samples

Sample	HCR run									
	$T_c$ (°C)	TRC (°C)	$\Delta H_c$ (J/g)	$T_{ch}$ (°C)	TRCH (°C)	$\Delta H_{ch}$ (J/g)	$T_{m2}$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)
PHB	79	59	74	–	–	–	177	35	92	63
PHB/10TA	73	49	66	–	–	–	171	32	74	56
PHB/20TA	67	51	55	–	–	–	166	35	65	56
PHB/10ATBC	78	57	68	–	–	–	173	35	81	62
PHB/20ATBC	72	57	56	–	–	–	168	40	71	61
Sample	Quenching run									
	$T_g$ (°C)	$T_{cc}$ (°C)	TRCC (°C)	$\Delta H_{cc}$ (J/g)	$T_m$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)		
PHB	4	51	26	46	177	37	85	58		
PHB/10TA	– 10	34	22	38	170	40	78	60		
PHB/20TA	– 20	22	35	28	164	40	69	60		
PHB/10ATBC	– 7	41	28	41	173	32	79	59		
PHB/20ATBC	– 18	31	30	32	169	36	70	60		

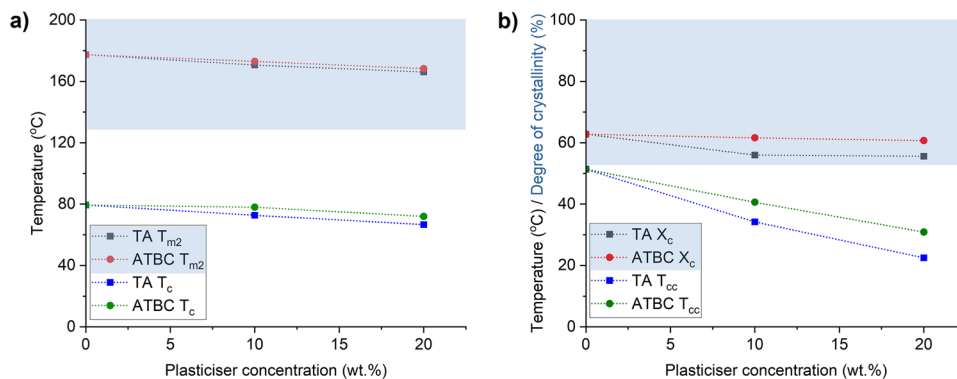
### Effect of Plasticiser in PHB Systems

Compatible plasticisers present in polymer matrix increase the free volume in the system and improve overall processability due to a decrease in formulation viscosity [64]. In plasticised PHB systems, a decrease in  $T_{m2}$  with increasing plasticiser content was noted, in turn resulting in broadening of the polymer processing window. These changes are dependent on plasticiser type, with more significant changes reported for TA addition (Fig. 2a). ATBC was previously reported to occupy mainly the amorphous region of the PHBV [48]- hence, it is unlikely than plasticiser inclusion in the crystallites is responsible for the observed changes. Instead, the crystallite size, perfection, and amount all vary upon changes in polymer chain mobility, leading to decrease in  $T_{m2}$ . The variations in melting temperature are in line with the ones observed in literature for ATBC, with ca. 10 °C decrease in  $T_{m2}$  upon adding 20 wt.% of this plasticiser [65]. However, a larger decrease was noted here compared to the one reported for 20 wt.% TA addition [66].

Similar shift towards lower temperatures is noted for  $T_g$ , recorded at – 10.4 and – 19.7 °C for PHB/10TA and PHB/20TA, respectively, and – 6.9 and – 17.7 °C for PHB/10ATBC and PHB/20ATBC, respectively (Table 4). For ATBC, this decrease is less pronounced compared to literature reports where PHB with 20 wt.% plasticiser blend had a  $T_g$  of – 28.5 °C, although with similar values noted upon 10 wt.% plasticiser addition [65]. Similar to the shift in  $T_{m2}$ ,  $T_g$  reported for TA addition also varied compared to similar formulations reported elsewhere, with less significant difference observed in work by Quispe et al [66]. These variations might be caused by differences in processing – powder samples studied here vs film samples in the referenced study [66]—and changes in polymer composition after prolonged exposure of PHB to high temperatures in the cited work.

The observed shift in thermal properties upon plasticiser addition is a result of increased mobility and freedom of movement of individual polymer chains hence lower energy required for crankshaft motion or melting of polymer crystals, the size or perfection of which is affected by

**Fig. 2** Effect of the plasticiser content and chemistry on **a**  $T_{m2}$  (shaded area) and  $T_c$  and **b**  $T_{cc}$  and  $X_c$  (shaded area). The dotted lines represent trends in properties changes for reader guidance



plasticiser presence. Crystallisation (decrease in  $T_c$  with increasing plasticiser content, independent of its chemistry, Fig. 2a, Figure S1 in the Supporting Information) happens at the same value of undercooling for both pure and plasticised samples (equal to ca. 95 °C). However, the difference between  $T_{m2}$  and  $T_g$  increases upon plasticiser addition independent of its chemistry and is equal to 173, 181 and 186 °C for pure PHB, samples plasticised with 10 wt.% and 20 wt.% plasticiser, respectively.

Addition of the plasticiser did not lead to significant changes in double-peak shape of melting peak for HCR run as this shape was visible for all formulations, with the least visible features noted for PHB/20ATBC (Fig. 1a). All plasticisers decrease  $\Delta H_c$  – the higher the plasticiser content, the more significant the  $\Delta H_c$  decrease—however, differences between various chemistries are insignificant (Table 4). This changes for  $\Delta H_m$  as while both plasticisers result in decrease of this value, TA reduces it in a more pronounced way. In terms of  $TRM$ , the differences between pure and plasticised systems are only slight, with overall decrease in  $TRM$  for samples with 10 wt.% TA and increase for samples with 20 wt.% ATBC (Table 4, Fig. 1a). Further, while slight decrease in  $TRC$  is noted for formulations plasticised with ATBC, the decrease is more significant for samples with TA (Table 4). Similar effect of widening main peak towards lower temperatures and consequent increase in the crystal population with lower thicknesses and perfection upon addition of the plasticiser was previously observed in literature [30]. The results presented here therefore suggest both changes in the crystallite size distribution (likely appearing as a result of plasticiser influencing primary crystallisation during sample cooling) and melting-recrystallisation-remelting events affecting crystallite perfection and final distribution.

Overall, the trend of decreasing  $X_c$  with introducing plasticiser to the system is clear – independent of the plasticiser chemistry, the higher the content of the plasticiser, the lower  $X_c$  (Fig. 2b), with TA addition leading to more substantial changes. We suggest that more significant effect of TA on thermal properties of PHB compared to ATBC action is a result of its higher compatibility of TA with PHB. This conclusion is in line with theoretical investigations based on Hansen solubility parameters and results obtained for spherulitic crystallisation presented previously. [20]

Upon introducing plasticiser to the system,  $T_{cc}$  changes in a manner similar to  $T_c$  and decreases with increasing plasticiser content, with more significant changes noted for TA addition compared to ATBC addition. Alike pure PHB samples,  $\Delta H_{cc}$  values are noted at ca. 50% of the  $\Delta H_m$  for all plasticised formulations, with overall  $X_c$  values similar to those obtained from HCR runs (Table 4). Further,  $TRCC$  remains lower than both  $TRC$  and  $TRM$ , suggesting that cold crystallisation is responsible for only part of the final crystallinity created in the system and significant reorganization

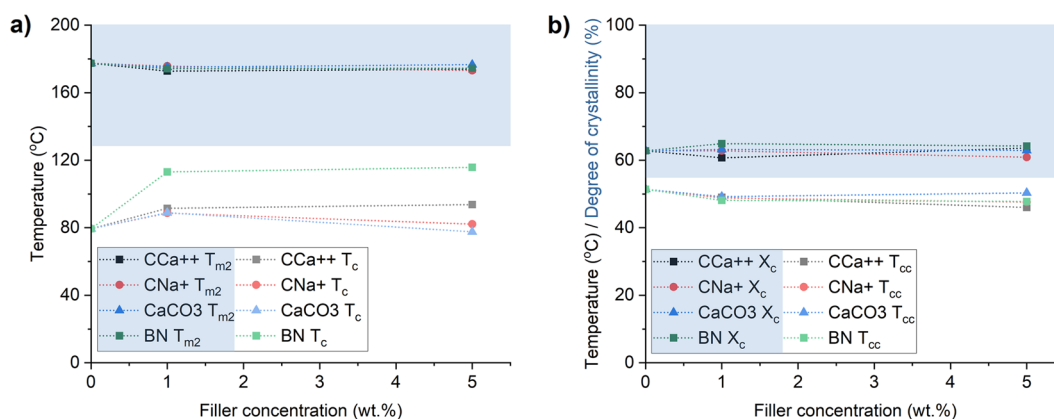
occurs at higher temperatures closer to  $T_{m2}$ . Interestingly, for quenching run  $X_c$  values for all plasticised systems are slightly higher than values noted for pure PHB (Table 4). We suggest that these differences in  $X_c$  might be a result of changes in the polymer chain mobility and faster creation of smaller and less perfect crystals where plasticiser presence affects crystallite structure.

### Effect of Filler in PHB Systems

Fillers present in polymer systems are widely used as nucleating agents that increase the number of heterogeneous nucleation centres through interactions at the polymer-filler interface. The nature of the interface determines the efficacy of the filler as a nucleant [67]. Here, we reported the behaviour of well-studied nucleating agent, BN, widely regarded as model nucleant for PHB systems. However, as its production requires application of extremely high temperatures, other nucleating agents are preferred. We therefore compare nucleating action of BN with green fillers – safe to use chemicals, production of which is less energy-intense – to make conclusion about the effect of filler type and content on thermal properties of the final formulations.

The nucleating action of the fillers can be observed as shift in  $T_c$  towards higher values compared to pure PHB formulations. BN shows the most significant shift ( $T_c = 79, 113$  and  $116$  °C for pure PHB, PHB/1BN and PHB/5BN formulations, respectively, Fig. 3a, Figure S2a in the Supporting Information), proving the most prominent nucleating action and the most significant increase in crystallisation rate among fillers studied. Introducing BN to the system at either content leads to significant increase in  $\Delta H_c$  compared to pure PHB formulations that is approximately equal to  $\Delta H_m$  (Table 5). These observations are in line with previous reports regarding BN action in PHB systems [46,68]. Presented results suggest that while BN addition in general does not lead to increase in  $X_c$ , most of the crystals are developed during cooling. These structures might then undergo reorganization into more stable structures during reheating, however, small difference between  $\Delta H_c$  and  $\Delta H_m$  shows that mainly melting of previously created crystals take place. This can be further supported by similar shape of the crystallisation upon cooling and melting peak for these samples – monomodal peak compared to bimodal peak observed for PHB, in line with literature reports [69].

For other fillers studied here,  $T_c$  increase upon introducing filler to the system was less significant (Fig. 3a, Figure S2a in the Supporting Information).  $CCa^{++}$  shown the most significant nucleating action among  $CCa^{++}$ ,  $CNa^+$  and  $CaCO_3$  group, in line with results obtained for spherulitic growth measurements [20]. Further, PHB/5 $CaCO_3$  sample has shown anti-nucleating action (reducing  $T_c$ ), showing that at higher contents, restriction of polymer



**Fig. 3** Effect of the filler content and chemistry on **a**  $T_{m2}$  (shaded area) and  $T_c$  and **b**  $T_{cc}$  and  $X_c$  (shaded area). The dotted lines represent trends in properties changes for reader guidance

**Table 5** Thermal properties of filled PHB samples

Sample	HCR run									
	$T_c$ (°C)	TRC (°C)	$\Delta H_c$ (J/g)	$T_{ch}$ (°C)	TRCH (°C)	$\Delta H_{ch}$ (J/g)	$T_{m2}$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)
PHB	79	59	74	–	–	–	177	35	92	63
PHB/1BN	113	56	93	–	–	–	174	38	94	65
PHB/5BN	116	55	92	–	–	–	174	36	89	64
PHB/1CCa <sup>++</sup>	92	50	79	–	–	–	173	33	88	61
PHB/5CCa <sup>++</sup>	94	52	80	–	–	–	174	38	88	64
PHB/1CNa <sup>+</sup>	89	56	78	–	–	–	176	37	91	63
PHB/5CNa <sup>+</sup>	82	56	74	–	–	–	173	35	85	61
PHB/1CaCO <sub>3</sub>	89	67	82	–	–	–	175	35	91	63
PHB/5CaCO <sub>3</sub>	78	65	72	–	–	–	177	35	87	63
Sample	Quenching run									
	$T_g$ (°C)	$T_{cc}$ (°C)	TRCC (°C)	$\Delta H_{cc}$ (J/g)	$T_m$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)		
PHB	4	51	26	46	177	37	85	58		
PHB/1BN	3	48	35	44	176	39	91	63		
PHB/5BN	4	48	42	38	176	38	83	60		
PHB/1CCa <sup>++</sup>	3	49	33	46	176	35	87	60		
PHB/5CCa <sup>++</sup>	3	46	31	41	175	38	86	62		
PHB/1CNa <sup>+</sup>	3	49	27	45	175	33	85	59		
PHB/5CNa <sup>+</sup>	2	48	28	43	175	39	85	62		
PHB/1CaCO <sub>3</sub>	–1	49	28	34	176	35	87	60		
PHB/5CaCO <sub>3</sub>	5	50	28	37	177	36	71	51		

mobility due to filler presence might be dominant over presence of additional nucleation centres [70,71]. This phenomenon was previously correlated with reduction of lamellar thickness upon introducing filler to the system [72]. The variations of TRC for HCR run compared to pure PHB systems were dependent on filler type, implying possible changes in overall crystallite size distribution or their perfection compared to pure PHB. In general, CaCO<sub>3</sub> led to increase in TRC, CCa<sup>++</sup>—decrease, while

lack of significant changes was observed for CNa<sup>+</sup> and BN systems (Table 5). No such trend was observed for TRM, which remained similar to values noted for pure PHB samples.

While there are differences in the maximum of the first melting peak,  $T_{m1}$  (related to melting crystals formed during primary crystallisation upon sample cooling) [48], the overall trend of its changes upon filler addition was difficult to assess. However, formulations with the



filler, independent of its content or chemistry, showed a decrease of  $T_{m2}$  (associated with the melting of crystallites formed by reorganisation during heating, Fig. 3a, Figure S2b in the Supporting Information). Hence, despite fillers promoting nucleation in PHB systems, they are likely to introduce imperfections or inhibit the crystal reorganisation process due to restrictions in chain motion that lower  $T_{m2}$  [48]. This behaviour is unlike that previously reported for PHB/CNa<sup>+</sup> composites, where no changes in melting temperatures were noted for filler addition [73]. Further, the difference between  $\Delta H_c$  and  $\Delta H_m$  remained significant, but was overall lower than the value noted for pure PHB.

The addition of the filler did not lead to significant changes in  $X_c$  compared to pure polymer, even for the most effective nucleating agent studied, BN. These results are consistent with studies for other clay additives [48,73] and BN where the addition of the latter above 0.2 wt% did not affect  $X_c$  [74]. Fillers present in PHB matrix therefore affect crystallite size, distribution, as well as crystalline microstructure rather than  $X_c$ .

In terms of quenching runs, for all formulations but PHB/5CaCO<sub>3</sub> a slight increase in  $X_c$  values compared to pure PHB sample was noted. Introducing any filler also leads to shift of  $T_{cc}$  towards lower temperatures (Fig. 3b, Figure S2c in the Supporting Information). Further, the difference between  $TRCC$  and  $TRM$  for all filled samples is lower than that of pure PHB, with  $TRCC$  values overall increasing and  $TRM$  values remaining similar. Despite the nucleating action of the fillers,  $\Delta H_{cc}$  remains at ca. 50% of the  $\Delta H_m$  values, suggesting that crystallisation time remains a limiting step despite introduction of heterogeneous nucleation centres, with reorganization occurring during melting.

In general,  $T_g$  was only slightly altered upon filler addition and shifted towards lower temperatures except for PHB/5CaCO<sub>3</sub> sample, where no difference compared to pure polymer was noted (Table 5, Figure S2c in the Supporting Information). This behaviour therefore suggests slight improvement in processability of the formulation and minimally lower stiffness for filled samples [75] due to lubrication of polymer chain with lower  $M_w$  additives [76] and is consistent with previous studies for CNa<sup>+</sup> addition into PHB system [73]. This result is, however, not consistent for all fillers in PHB-based systems as addition of lignin was reported to increase  $T_g$  of PHB [75].

### Effect of Filler and Plasticiser in PHB Systems

In complex formulations, the effect of multiple additives (their chemistry and content) is difficult to establish due to overall changes in system compatibility, intermolecular

interactions, and polymer chain flexibility compared to binary systems with only one additive. In plasticised ternary systems studied here (FF2 design), there was lack of significant changes in  $T_{m2}$  for any formulation, with notable exception of PHB/20TA/BN system (both with 1 and 5 wt% BN content, Figure S3f in the Supporting Information), for which a decrease in  $T_{m2}$  was noted, similarly to the corresponding binary polymer/filler systems. This behaviour highlights the importance of possible reorganization in the system as the changes in  $T_c$  were present and dependent on the chemistry and contents of the filler and plasticiser (Figure S3 a,e in the Supporting Information).

BN exhibited the highest nucleation efficiency in ternary systems amongst the fillers studied (Figure S3 in the Supporting Information), in line with the results obtained from binary systems (Fig. 3). Together with CCa<sup>++</sup>, it was the only filler showing nucleating action despite system plasticisation. This result is consistent with observations noted in our study of spherulite nucleation and growth in PHB-based systems where CCa<sup>++</sup> was better nucleant than CNa<sup>+</sup> or CaCO<sub>3</sub> [20]. BN was also the only filler that consistently increased  $X_c$  in the plasticised PHB matrix (Figure S3 d,h in the Supporting Information). For  $X_c$  in other filled and plasticised systems no clear trends were observed. A previous study on PHB/plasticiser/filler systems showed that presence of these additives leads to overall changes in  $X_c$ , as well as variations in torsion angles in the PHB helical crystalline structure [48]. The authors concluded that plasticiser affects mainly the size of the crystalline domains within spherulites, while filler reduces overall  $X_c$  – however, they were not able to draw clear conclusions about the  $X_c$  changes with additive and HV content. We also observed no clear trends for changes in  $T_{cc}$  (Figure S3 c,g in the Supporting Information) – here, changes were highly dependent on filler chemistry and content, highlighting importance of overall interactions in the system.

### Effect of HV Content in PHB Systems

Introducing the HV side group to PHB chain affects its ability to fold due to internal plasticisation [6,51] and disruption within the crystal lattice because of the additional steric obstructions. This phenomenon results in slight decrease in  $T_g$  noted with increasing HV content – equal to 4, 2, 1 and –1 °C for PHB, PHB/7HV, PHB/12HB and PHB/21HV, respectively (Fig. 4c), which is consistent with previous studies [3,32]. Further, increase in HV content also results in decrease in  $T_m$  – both  $T_{m1}$  and  $T_{m2}$ , Fig. 4b – leading to broader processing window for PHBV compared to pure PHB and improved melt stability at lower processing temperatures [3,32].

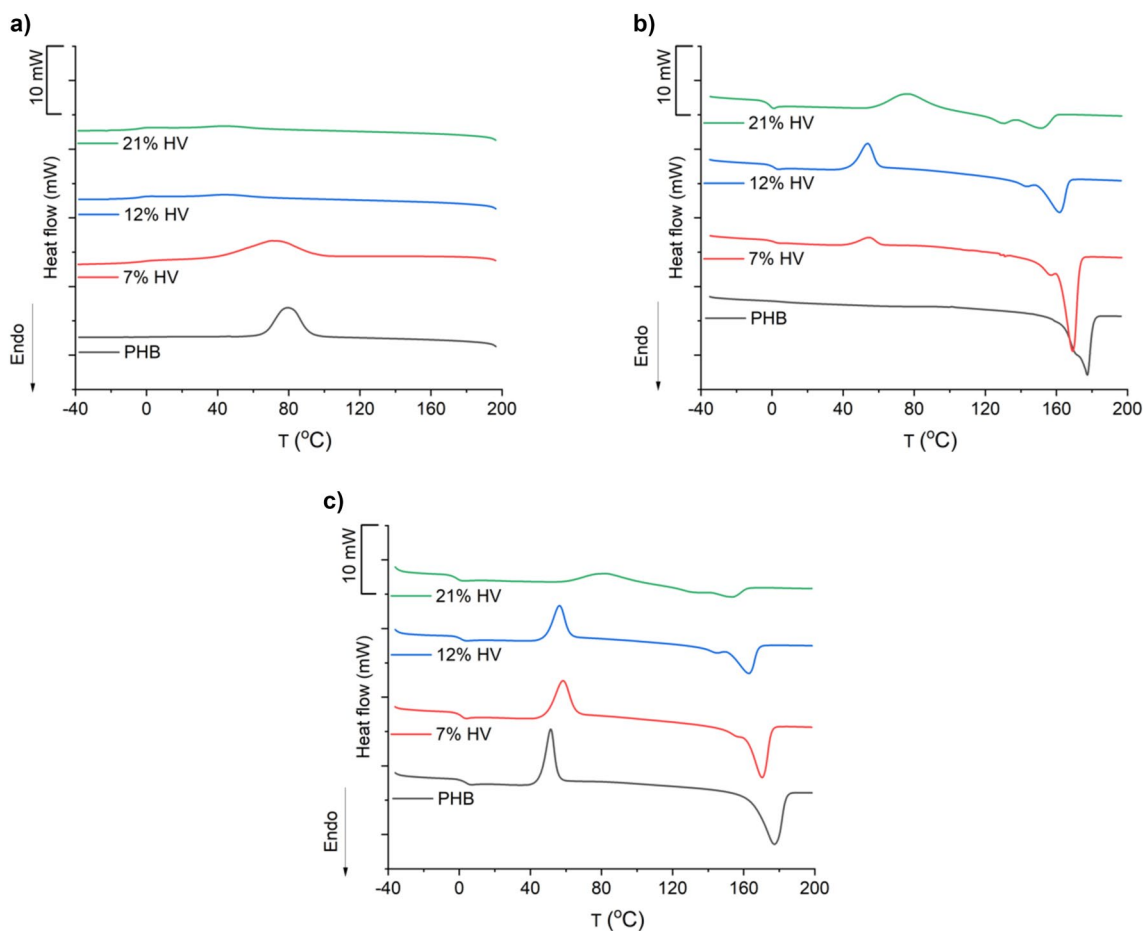
Lower  $T_m$  values also imply creation of less stable crystals due to larger side group inclusion in polymer chain.

PHBV copolymers exhibit isodimorphism – mutually co-crystallizable repeating units [77] both in the PHB and PHV crystalline lattices [15,78]. For random PHBV copolymers containing <25 mol% HV units in PHBV chain, the PHB lattice was proven to withstand the disruption caused by presence of the larger side group [79], with the strength of the  $\text{CH}_3\cdots\text{O}=\text{C}$  hydrogen bond remaining similar to that of pure polymer for HV content <21 mol% [80]. HV units therefore reside as defects within the PHB crystal lattice with deviations from perfect crystal increasing with increasing HV content [35]. These changes can be observed as small expansion of  $d$  spacing in the (1 1 0) plane of the PHB lattice upon increasing HV content [35,48].

The plasticisation of the polymer chains and presence of crystal imperfections leads to  $X_c$  changes with increasing HV content. A significant decrease in  $X_c$  for both HCR (Fig. 5b) and quenching run (Table 6) that can be represented as a linear trend for HV content vs  $X_c$  function was noted. These results are similar to values obtained from X-ray diffraction reported elsewhere [35], however, with overall decrease in  $X_c$  being less significant with increasing HV content for

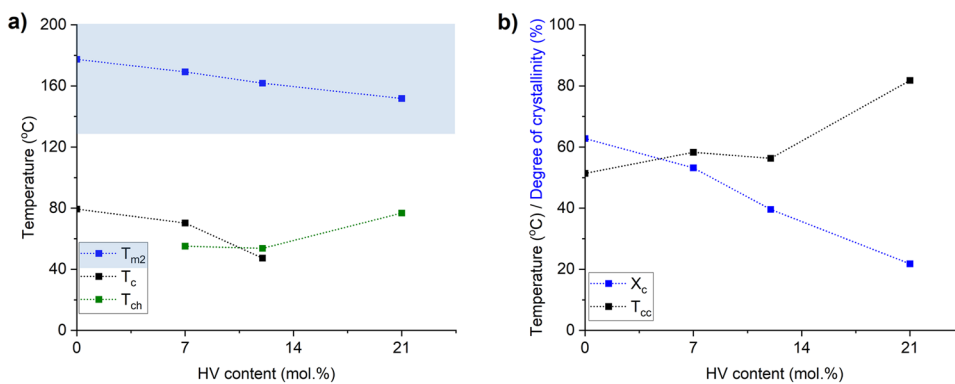
results presented here. Further, in previous literature reports, not only  $X_c$ , but also crystallisation half-time reduces and lamellae thickness decreases for increased HV content compared to pure PHB samples [35], which complements results obtained here for changes in  $X_c$ .

The steric effect is also apparent when considering crystallisation for these samples. It occurs both during sample cooling and reheating for samples of PHB/7HV and PHB/12HV.  $T_c$  (Fig. 4a, Fig. 5a) and  $\Delta H_c$  (Table 6) decrease with increasing HV content, with no significant differences in  $TRC$  for PHB, PHB/7HV and PHB/12HV [51]. This is likely due to increase in free volume upon increasing HV content in the PHBV backbone. The opposite trend is noted for  $T_{cc}$  (Fig. 4c, Fig. 5b) and  $\Delta H_{cc}$  (Table 6). Therefore, despite both additives (plasticisers and fillers) and HV side group affecting PHB ability to fold, they show opposite effect on  $T_{cc}$  in the investigated formulations. For PHB/21HV sample, however, the crystallisation occurs only during second heating cycle and  $\Delta H_{cc}$  decreases compared to PHB/12HV sample (Fig. 4a, b). PHB/21HV is also the only HV content that results in



**Fig. 4** DSC runs for **a** cooling and **b** second heating during HCR run as well as **c** quenching run for samples with increasing HV content

**Fig. 5** Effect of the HV content on **a**  $T_{m2}$  (shaded region),  $T_c$  and  $T_{ch}$  and **b**  $T_{cc}$  and  $X_c$ . The dotted lines represent trends in properties changes for reader guidance



**Table 6** Thermal properties of PHBV samples

Sample	HCR run										
	$T_c$ (°C)	TRC (°C)	$\Delta H_c$ (J/g)	$T_{ch}$ (°C)	TRCH (°C)	$\Delta H_{ch}$ (J/g)	$T_{m2}$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)	
PHB	79	59	74	–	–	–	177	35	92	63	
7% HV	70	58	49	28	28	7	169	43	78	53	
12% HV	47	59	8	37	37	34	162	41	58	40	
21% HV	–	–	–	44	44	31	152	53	32	22	
Sample	Quenching run										
	$T_g$ (°C)	$T_{cc}$ (°C)	TRCC (°C)	$\Delta H_{cc}$ (J/g)	$T_{m2}$ (°C)	TRM (°C)	$\Delta H_m$ (J/g)	$X_c$ (%)			
PHB	4	51	26	46	177	37	85	58			
7% HV	2	58	32	49	170	40	70	48			
12% HV	1	56	29	39	163	48	53	36			
21% HV	–1	82	47	19	154	47	20	14			

lack of notable differences between  $\Delta H_{cc}$  and  $\Delta H_m$ . It is suggested that the high HV content leads to a lack of significant reorganization upon heating for this sample, with complete melting of crystals created during the initial cold crystallisation only. The difference between  $TRCC$  and  $TRM$ , as well as the shape of melting peak for this sample (Fig. 4c) might therefore indicate the creation of crystallites population of that can be characterised by polymodal distribution.

This trend of no significant differences between  $\Delta H_{cc}$  and  $\Delta H_m$  for PHB/21HV remains consistent for quenching run, with  $TRCC$  and  $TRM$  also showing similar values (Table 6). For other samples, however, reorganization is likely to take place as evidenced by differences between  $\Delta H_{cc}$  and  $\Delta H_m$  as well as  $TRCC$  and  $TRM$ . It is suggested that cold crystallisation led to creation of wider distribution of less stable crystallites compared to the HCR run, which were then remelted into more stable structures upon heating.

### Effect of Filler in PHBV Systems

Introducing fillers of any studied chemistry and content to PHBV samples (FF1 design) led to a decrease in  $T_{cc}$

compared to unfilled samples (Figure S4c in the Supporting Information), in line with results obtained for filled PHB systems. This behaviour is likely related to an increased number of heterogeneous nucleation centres in the polymer matrix. BN again showed the most prominent nucleating action (signified by the difference in  $T_c$ ,  $T_{cc}$  and  $T_{ch}$  between pure and filled PHBV sample), followed by both Cloisites, with  $\text{CaCO}_3$  being the least efficient nucleant in these systems. The addition of any filler, however, led to overall decrease in  $X_c$  in the system (Figure S4d in the Supporting Information), suggesting possible hindering effect of fillers on overall polymer mobility and hence decrease in crystallite size and perfection [81]. The presence of filler also did not result in significant changes in  $T_{m2}$  (Figure S4b in the Supporting Information). However,  $\text{CCa}^{++}$  and BN addition increased  $T_c$  values, with BN remaining the most effective nucleating agent as it was the only filler that either led to full crystallisation upon cooling for all studied HV contents (at 5 wt.%, Figure S4a in the Supporting Information) or decreased  $T_{ch}$  (at 1 wt.%, Figure S4e in the Supporting Information) compared to pure polymer. Further, BN was the only filler that caused crystallisation for PHB/21HV sample upon cooling, consistent with previous literature reports [46].

## Statistical Analysis of Crystallisation in PHB-Based Systems

The dependence of crystallisation behaviour on filler type suggests competition between increase in polymer flexibility (either due to increased HV content, FF1 design, or plasticiser presence, FF2 design) and nucleating action of the fillers. With increase in chemical complexity of the formulations, the nature of intermolecular interactions between species present in the system changes [57]. In order to systematically identify the effect of each parameter on thermal properties ( $T_m$ ,  $T_c$ ,  $T_{cc}$ ) and  $X_c$ , as well as to identify parameters which significantly interact with one another, factorial experimental design combined with ANOVA were implemented.

FFD and RSD were applied to investigate the effect of each parameter on thermal properties ( $T_m$ ,  $T_c$ ,  $T_{cc}$ ) and  $X_c$  both in unplasticised (i.e. filled systems with various HV content, Table 2, FF1) and plasticised (i.e. filled PHB systems of various plasticiser content, two types of plasticiser studied, Table 2, FF2) formulations. FFD was used as the primary screening tool to investigate the complex interactions (included in the model as  $x_1 \cdot x_2$  term, Eqs. 1 and 2) between the considered factors. Despite the model having four (FF1) or five (FF2) parameters, only interactions up to third order were considered for this initial analysis as higher-level interactions are often too complex to produce unambiguous results. Third-order interactions are unlikely to be important in real-life settings [82], however, they can provide useful insight into the interplay between components in the systems studied here and were therefore not omitted. These results were further expanded by RSD to account for any non-linearities in the investigated system.

## Crystallisation Upon Cooling ( $T_c$ ) in Ternary Systems

For filled PHBV systems (FF1 design), linear regression of  $T_c$  against the system parameters including 3-way interactions resulted in perfect fit ( $R^2 = 1$ ), therefore it was not possible to establish the importance of each parameter on the response. Hence, linear regression including 2-way interactions was used instead to access this information, resulting in fit of high accuracy ( $R^2 = 0.987$ ,  $R^2$  adjusted = 0.9643). This signifies a very good description of the system without overfitting errors despite using lower number of parameters (i.e. 2-way interactions instead of 3-way interactions).

To detect the effect of individual factors and their interactions on  $T_c$ , a Pareto plot was used that shows the magnitude of the standardised effects – from the largest to the smallest – as well as a reference line, the value of which is dependent on the defined  $\alpha$  value [83] (here 0.05). Any factor that

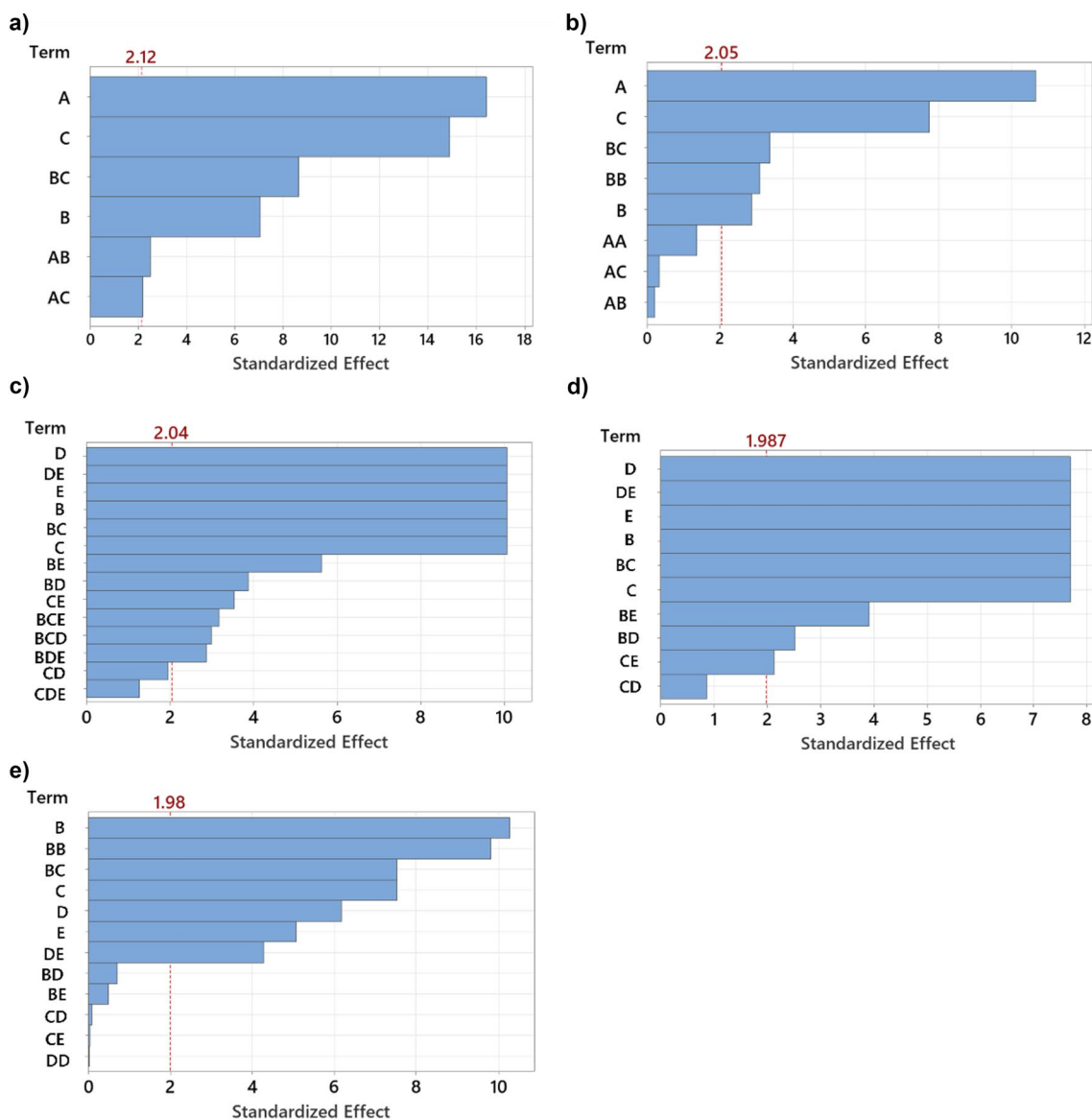
surpasses the reference line is likely to have a profound effect on the response function. From the Pareto plot for FFD analysis in the unplasticized system (Fig. 6a) it is clear that all the parameters influence the outcome. HV content holds the most significance, followed by filler type, filler type-filler content interactions, and filler content, highlighting the choice of filler chemistry as more important parameter than increasing content of less efficient nucleating agent. This conclusion is in line with that obtained from binary systems, where BN has shown the most prominent change in thermal properties of the sample, independent of its content.

RSD analysis was performed on the same set of data as FFD analysis to account for possible non-linearities in system behaviour, resulting in a fit of good accuracy ( $R^2 = 0.8975$ ,  $R^2$  adjusted = 0.8329). The variation in the  $R^2$  values compared to FFD analysis suggest that second-order polynomial is less accurate description of system behaviour than linear regression or is a result of accumulation of error due to double-fitting. Regardless, the conclusions from RSD agree with those for FFD analysis as the most important parameters are listed in the same order (Fig. 6b). The only exception is noted for filler content – here, the second-order term is more important than first-order one, implying non-linearity in changes of  $T_c$  with increasing heterogeneous nucleation density introduced by the presence of the filler. The effect of HV content remains linear. Interestingly, neither HV content-filler type nor HV content-filler content are of statistical importance, which is changed compared to FFD analysis.

Interaction plots can be used to visually represent if the effect of one variable is dependent on another variable by comparing the differences in sensitivity from low to high levels (i.e. slope of obtained lines). In simple terms, they can be described as: no interactions for parallel lines, synergistic interactions for diverging lines, antagonistic interactions for converging lines [84].

However, the interaction plots alone cannot be used to assess the statistical importance of the discussed interactions and need to be paired with ANOVA. In the systems studied here, the interactions change with increasing HV content and in general can be divided into two groups: first, for pure PHB and PHB/7HV samples and second, for PHB/12HV and PHB/21HV (Figure S5a in the Supporting Information). At 7% HV, distinct changes in interactions were seen, rendering drawing overall conclusion for HV content range investigated here impossible. In general, the character of changes is the same upon introducing filler to the system, with the slope changing with increasing filler content in the system, and different character of changes noted for pure polymer.

Application of DoE in plasticised, filled PHB systems (FF2 design) and inclusion of 3-way interaction parameters result in close-to-perfect fit ( $R^2 = 0.9976$ ,  $R^2$  adjusted = 0.9901), with a small decrease in fit accuracy



**Fig. 6** Pareto plot of the standardised effects with response as  $T_c$ ,  $\alpha=0.05$ , obtained from: **a** FFD analysis (two-way interactions) and **b** RSD analysis for FF1 design of pure and filled PHBV formulations, as well as **c** FFD analysis (three-way interactions), **d** FFD analysis

(two-way interactions) and **e** RSD analysis for FF2 design of plasticised and filled PHB formulations. Symbols in the figure correspond to parameters as presented in Table 2

upon including only 2-way interactions ( $R^2=0.9870$ ,  $R^2$  adjusted = 0.9802). From 3-way FFD analysis, it is visible that almost all parameters except CD and CDE interactions (Table 2) are of statistical importance in discussed system (Fig. 6c). This is in line with results from 2-way FFD analysis and implies that BCE, BCD and BDE interactions (Table 2) influence the result only slightly (Fig. 6d), as suggested by differences in  $R^2$  for both designs. While looking at the interaction plots, although the only obvious interactions are between pure PHB and systems with additives (Figure S5b in the Supporting Information), it is clear that even small changes are important here, likely because

the magnitude of importance for each parameter in the system. In general, the nature of the interaction is complex and changes within chosen conditions, with all parameters affecting  $T_c$  in the same way (Fig. 6c, d).

The importance of each parameter is easier to establish following results from RSD analysis (Fig. 6e) – however, it comes at a cost of decreased accuracy of the fit ( $R^2=0.8815$ ,  $R^2$  adjusted = 0.8458). Here, filler content is the most prominent factor, which is opposed to the results from FF1 design, where filler type was more important than its content and unexpected considering results from binary systems. The importance of this parameter is



further confirmed by three parameters including filler content – first- and second-order term as well as filler content-filler type interactions. Filler type, plasticiser content and plasticiser type, as well as plasticiser content-plasticiser type interactions are other parameters that hold statistical importance here, therefore all parameters should be included in the model in the form of first-order terms, independent of the model used (FFD or RSD). The contour plot from RSD analysis for both designs (FF1 and FF2) are presented as Figure S6a and S6b in the Supporting Information.

From statistical analysis one can conclude that chemistry of the system has a significant effect on  $T_c$  as all parameters contribute to the response function. The results from FFD and RSD are in good agreement with expected trends from binary systems for FF1 design. In FF2 design, however, either all main parameters equally affect  $T_c$  (for FFD), or filler content appears as the most important factor (for RSD), which cannot be concluded from results from binary systems alone.

### Cold Crystallisation ( $T_{cc}$ ) in Ternary Systems

While modelling  $T_{cc}$  for FF1 design, equation including 3-way interactions results in  $R^2 = 1$ , hence making it impossible to make conclusions about the effect of each parameter, similarly to  $T_c$ . Elimination of 3-way interactions and including only 2-way interactions in linear regression leads to very good fit ( $R^2 = 0.984$ ,  $R^2$  adjusted = 0.9606). HV content is the most significant parameter, similarly to results obtained for  $T_c$ . Further, interactions of HV with any other parameter (i.e. filler content or filler type) are statistically unimportant, while filler content-filler type interactions should be included in the model (Fig. 7a). The importance of filler type over filler content is therefore highlighted and is in line with results obtained for binary systems.

The results from RSD analysis ( $R^2 = 0.9268$ ,  $R^2$  adjusted = 0.8972) show that HV content remains the most important parameter but also non-linearly affects the output, with second-order term that should be included in the final function (Fig. 7b). Filler content proves to be less important than filler type. However, filler content should be included in the model both in the first- and second-order term. In this model, none of the inter-parameter interactions are statistically significant, suggesting that RSD analysis provides the extension of the FFD analysis – the importance of each parameter remains of the same order, with the addition of second-order parameters.

In terms of interactions, the trend of changes remains similar for each pair of parameters, independent on filler type, filler content, or HV content (Figure S7a in the SI), indicating lack of interactions between parameters. The significant deviation from this behaviour is observed only

for filler content-filler type graph, where the gradient of the function changes for each filler type, showing why only this pair of parameters is statistically important for FFD function.

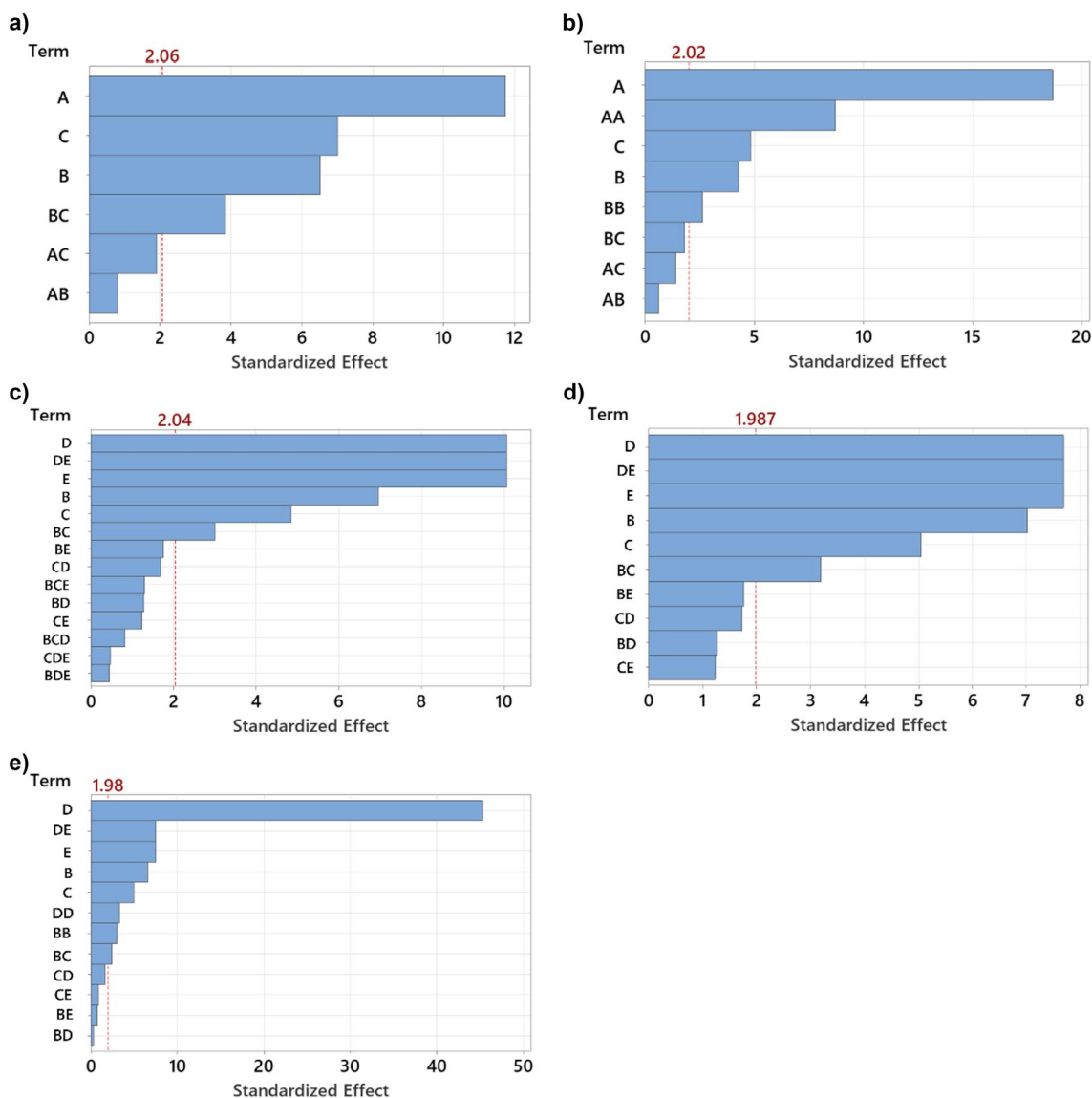
While modelling  $T_{cc}$  in FF2 design, inclusion of 3-way interactions parameters result in almost perfect fit ( $R^2 = 0.9955$ ,  $R^2$  adjusted = 0.9811), with a small decrease in fit accuracy upon including only 2-way interactions ( $R^2 = 0.9872$ ,  $R^2$  adjusted = 0.9805). Here, the number of parameters influencing the outcome decreases compared to  $T_c$  function – however, first-order terms for all parameters (i.e. plasticiser content and type, filler content and type) appear as statistically significant, together with plasticiser type-plasticiser content and filler content-filler type inter-factorial interactions. These conclusions are independent of the model (either including both 3- and 2-way interactions or just 2-way interactions, Fig. 7c, d).

For FF2 design, RSD analysis is an extension of FFD analysis and provides a good fit ( $R^2 = 0.9811$ ,  $R^2$  adjusted = 0.9754). From this analysis one can conclude that plasticiser content is the most prominent factor, which is expected from the results obtained for binary systems. Further, not only first-order term, but also second order term of both plasticiser and filler content were shown as statistically significant in the system. The interactions of importance are in line with FFD analysis (i.e. plasticiser content-plasticiser type and filler content-filler type), with no other additions to the model. These interactions are the only ones that show deviations from parallel behaviour observed for other pairs of parameters (Figure S7b in the Supporting Information), implying that weak or lack of interactions are observed for the pairs that are not included in the model. The contour plot from RSD analysis for both designs (FF1 and FF2) are presented as Figure S8a and S8b in the Supporting Information.

Statistical analysis suggests that both  $T_c$  and  $T_{cc}$  are strongly dependent on formulation. Variations in matrix free volume (either by introducing plasticiser or increasing HV content) cause the most significant variations in temperature values, while choice of additive chemistry plays a more important role than increasing its content. Independent of the model used, all parameters are statistically significant and affect output function.

### Crystal Melting ( $T_{m2}$ ) in Ternary Systems

Similarly to two other parameters discussed above, including 3-way interactions in the linear system for FF1 design of  $T_{m2}$  results in perfect fit ( $R^2 = 1$ ). However, these interactions affect the final effect only slightly as after their elimination the decrease in fit quality is insignificant ( $R^2 = 0.9963$ ,  $R^2$  adjusted = 0.9909). For this parameter, also the fit to RSD is the best among parameters studied



**Fig. 7** Pareto plot of the standardised effects with response as  $T_{cc}$ ,  $\alpha=0.05$ , obtained from: **a** FFD analysis (two-way interactions) and **b** RSD analysis for FF1 design for pure and filled PHBV formulations, as well as **c** FFD analysis (three-way interactions), **d** FFD analysis

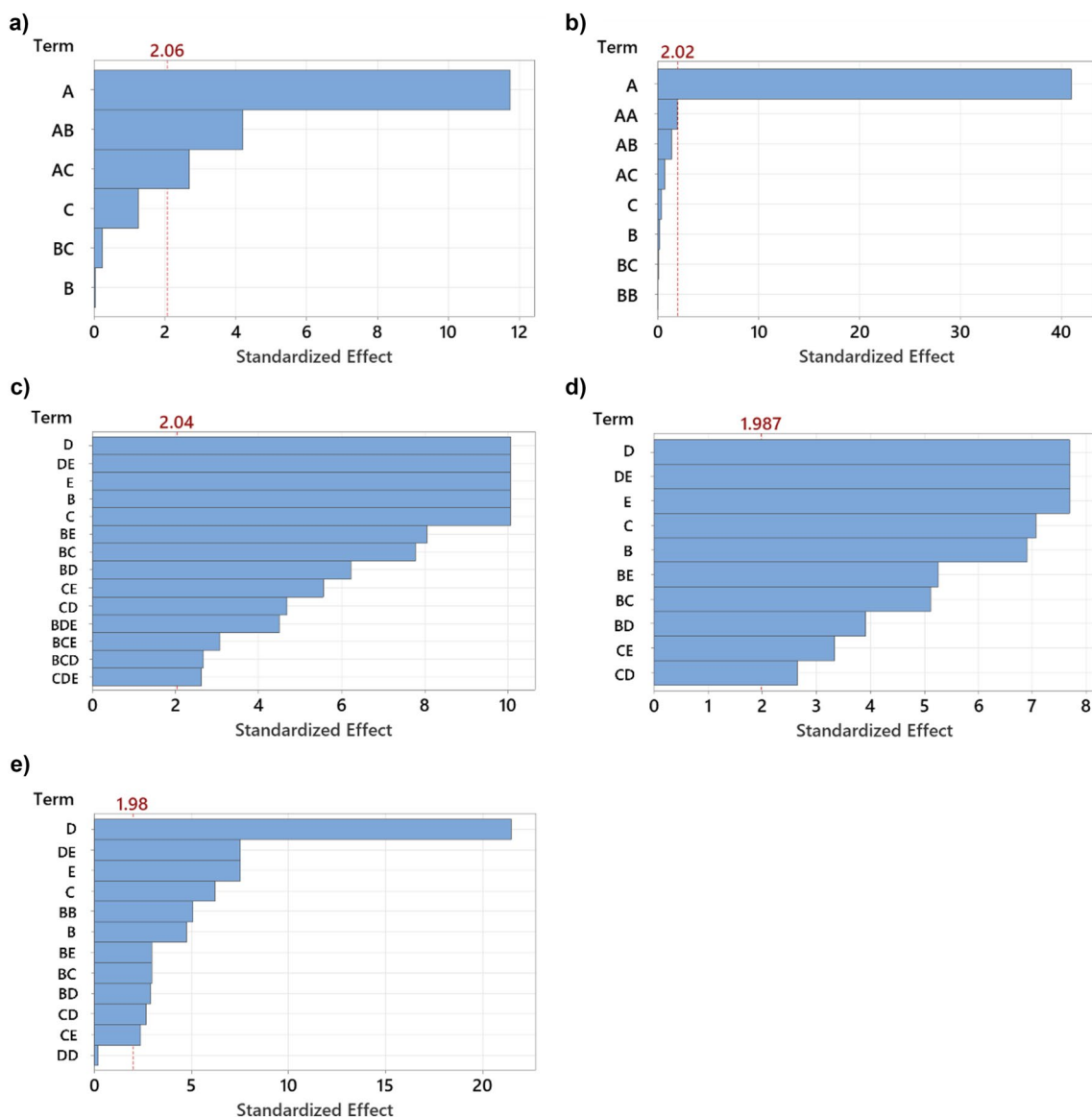
(two-way interactions) and **e** RSD analysis for FF2 design of plasticised and filled PHB formulations. Symbols in the figure correspond to parameters as presented in Table 2

( $R^2=0.9774$ ,  $R^2$  adjusted = 0.9683), suggesting that it provides a good description of the investigated system.

From the screening results of FFD analysis, HV content is the only parameter that substantially influences  $T_{m2}$  in the system – both as a first-order parameter and in the form of interactions with other two parameters (i.e. filler content and chemistry, Fig. 8a). In RSD model, however, HV content is the only parameter that affects the outcome, without any pairs of interactions included (Fig. 8b). In general, only small deviations from general trends are observed for given pair of parameters (i.e., functions are approximately parallel,

Figure S9a in the Supporting Information), indicating lack of strong inter-parameter interactions. These results signify the importance of polymer chain structure and flexibility as the main parameter that affects changes in crystal conformation and stability in the system because the inclusion of additives results in less significant shift in  $T_{m2}$ , in line with binary systems. It is therefore likely that reorganization happens in filled PHBV systems, reaching a final crystal structure and crystal perfection similar to that of pure PHBV systems.

For FF2 design, modelling of  $T_{m2}$  using linear regression with 2-way interaction results in worse quality fit



**Fig. 8** Pareto plot of the standardised effects with response as  $T_{m2}$ ,  $\alpha=0.05$ , obtained from: **a** FFD analysis (two-way interactions) and **b** RSD analysis for FF1 design for pure and filled PHBV formulations, as well as **c** FFD analysis (three-way interactions), **d** FFD analysis

(two-way interactions) and **e** RSD analysis for FF2 design of plasticised and filled PHB formulations. Symbols in the figure correspond to parameters as presented in Table 2

compared to that using 3-way interactions ( $R^2=0.9935$ ,  $R^2$  adjusted=0.9730 for 3-way interactions model and  $R^2=0.9578$ ,  $R^2$  adjusted=0.9357 for 2-way interactions model). The quality of fit is further decreasing while using RSD model ( $R^2=0.9272$ ,  $R^2$  adjusted=0.9053), likely due to estimation of the function by set of 2-order polynomials and accumulation of errors by approximating final function as a polynomial.

Independently of model used, all parameters are statistically important, with the only exception being quadratic term of plasticiser content in RSD model (Fig. 8c–e). While this parameter lacks quadratic dependence, plasticiser

content is highlighted as the most important parameter in the system and likely causing interaction pair plasticiser content-plasticiser type to arise as the second most important parameter in all models. Other first-order parameters are more important than remaining pairs of interactions and change in the same way for all models used:

Plasticiser content > Plasticiser type > Filler type > Filler content.

In RSD model, the 2-order term of filler content is also statistically important, implying non-linear character of  $T_{m2}$  changes with this parameter. The contour plot from RSD

analysis for both designs (FF1 and FF2) are presented as Figure S10a and S10b in the Supporting Information.

The role of inter-parameter interactions is highlighted while looking at the interaction plots in this system (Figure S9b in the Supporting Information). The nature of interactions remains complex, however, the changes within investigated borders are noted, which explains the importance of all interaction pairs in the final models. In order to make conclusions about character of changes, the division of region of interest into smaller one is required. For instance, while plasticised samples show antagonistic behaviour for filler contents between pure polymer and sample with 1 wt.% filler, they become synergistic between sample with 1 wt.% and 5 wt.% filler addition.

Overall, the significance of filler presence (both content and chemistry) varies in studied designs – it can be excluded from final function in FF1 design, which is not the case in FF2 design. This behaviour highlights the differences not only between PHBV and plasticised PHB systems, but also between pure PHB and PHBV system. In a PHB matrix, introducing filler resulted in a marginal  $T_{m2}$  shift towards lower temperatures in some instances, therefore indicating a limited effect on crystallite perfection and distribution. As the group of parameters affecting  $T_c$ ,  $T_{cc}$ , and  $T_{m2}$  vary, the importance of reorganization in the system is highlighted. During reorganisation, any potential crystal imperfections can be removed, hence filler is not seen to contribute to the  $T_{m2}$  in ternary systems in a profound way.

### Degree of Crystallinity ( $X_c$ ) in Ternary Systems

In filled PHBV systems (FF1 design), FFD analysis using 3-way interactions again results in perfect fit ( $R^2 = 1$ ). Hence, the model using 2-way interactions was implemented instead, resulting in an excellent fit without overparameterization ( $R^2 = 0.9972$ ,  $R^2$  adjusted = 0.9931). Similarly to results obtained from analysis of  $T_c$  and  $T_{cc}$ , all the parameters and interactions influence the final output, with individual parameters being more important than pairs of interactions. HV content is noted as the most important parameter, while filler type is least important one (Fig. 9a). Interestingly, the effect of filler content appears as more important parameter than its chemistry despite the strong nucleating action of BN in investigated systems, the result in line with that obtained from analysis for  $T_{cc}$ . Further, the nature of the interactions in the system remains complex (Figure S11a in the Supporting Information).

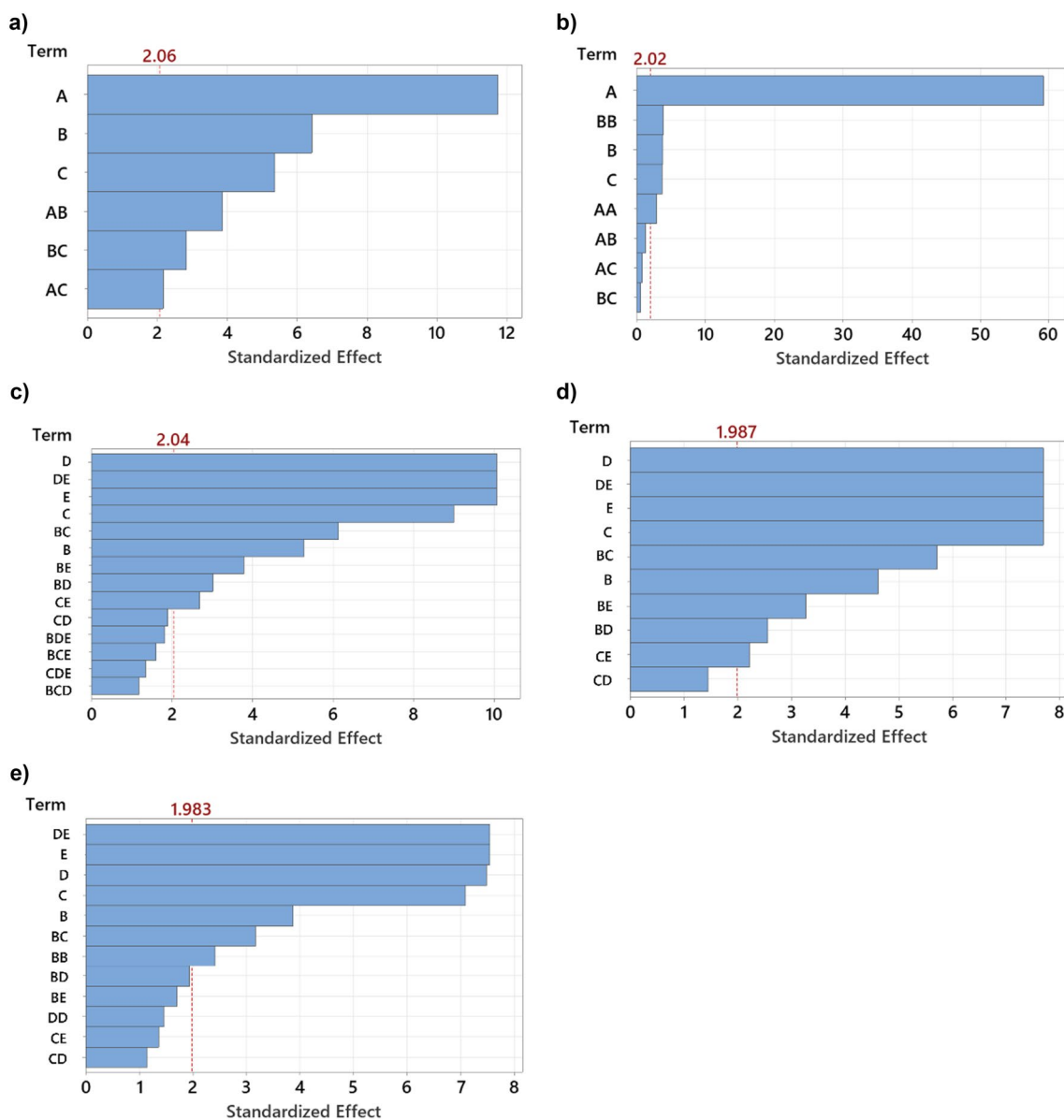
While considering RSD analysis in this system, very good fit without overparameterization is noted ( $R^2 = 0.9898$ ,  $R^2$  adjusted = 0.9844). Here, none of the pairs of interfactorial interactions are important – instead, quadratic terms of both numerical values are included, with HV content remaining undoubtedly the most significant parameter and the presence

of fillers in PHBV matrix affecting  $X_c$  in a significant way (Fig. 9b). The importance of filler content over filler type is highlighted again, showing the importance of statistical analysis that reveals parameters of importance difficult to extract from analysis of binary systems alone.

While considering  $X_c$  in filled, plasticised PHB systems (FF2 design), there is an observed decrease in the quality of the fit for all models considered compared to FF1 design: FFD with 3-way interactions ( $R^2 = 0.9708$ ,  $R^2$  adjusted = 0.8777), FFD with 2-way interactions ( $R^2 = 0.8922$ ,  $R^2$  adjusted = 0.8358) and RSD analysis ( $R^2 = 0.8049$ ,  $R^2$  adjusted = 0.7461). Significant difference between  $R^2$  and  $R^2$  adjusted suggest overparameterization in the system independent of the model used.

Regardless of decreasing fit, plasticiser presence remains the major parameter in the system, with plasticiser content appearing as more important than its type except of RSD analysis (Fig. 9c–e). The observation from RSD design is expected as introducing more compatible plasticiser to the system results in profound increase in system free volume of magnitude dependent on intermolecular interactions. Similarly, the choice of filler type holds more significance than variations in its content (Fig. 9c–e), in opposition to results from FF1 design. Both filler content-filler type and plasticiser content-plasticiser type interactions are of importance in all considered models. In addition, in RSD analysis, filler content should be included as quadratic term. In terms of interactions, ones not included in the system (plasticiser content-filler type) are represented by almost parallel lines in the investigated range, while character of other pairs is complex and cannot be generalised using one general term (Figure S11b in the Supporting Information). The contour plot from RSD analysis for both designs (FF1 and FF2) are presented as Figure S12a and S12b in the Supporting Information.

The investigations into  $X_c$  are a natural extension of investigations into  $T_{m2}$  and  $T_c$  (HCR run) as both primary crystallisation and recrystallisation contribute to the  $\Delta H_m$  and consequently  $X_c$ . The dependence of  $X_c$  on all studied parameters is therefore expected as for majority of the functions all factors affect the output in a statistically significant way. For all designs and parameters studied, HV content and presence of plasticiser are the most important ones and highlight the effect of increase in system free volume. These changes are more prominent than introducing heterogeneous nucleation centres (i.e. nucleating agents) to the system despite plasticiser being mainly located in the amorphous phase and its previously reported limited influence on system crystallinity [48]. The results obtained here are in line with these obtained from analysis of spherulitic crystallisation [20], providing their extension into smaller scales.



**Fig. 9** Pareto plot of the standardised effects with response as  $X_c$ ,  $\alpha=0.05$ , obtained from: **a** FFD analysis (two-way interactions) and **b** RSD analysis for FF1 design for pure and filled PHBV formulations, as well as **c** FFD analysis (three-way interactions), **d** FFD analysis

(two-way interactions) and **e**) RSD analysis for FF2 design of plasticised and filled PHB formulations. Symbols in the figure correspond to parameters as presented in Table 2

## Conclusions

In this study, factors influencing crystallisation in filled and plasticised PHBV systems were investigated using systematic experimental design and statistical analysis. We have found that in binary systems, the filler type plays a more significant role than its content, with BN being the most effective nucleating agent, followed by  $CCa^{++}$ . In

general, fillers present in the system increase  $T_c$ , decrease  $T_{cc}$  and  $T_{m2}$ , with no significant changes in  $X_c$ . Plasticiser addition results in different trends – decrease in  $T_c$ ,  $T_{cc}$ ,  $T_{m2}$  and  $X_c$ . TA was shown to be more compatible plasticiser with PHB, in line with theoretical calculations and our previous results. Finally, changes in HV content in the polymer backbone led to the most significant changes, with no crystallisation upon cooling noted for 21% HV, significant decrease in  $T_{m2}$ ,  $X_c$  and high  $T_{ch}$ .



In ternary systems (filled PHBV or plasticised and filled PHB), thermal properties show strong dependence on all investigated parameters and interactions, except for  $T_{m2}$ . In filled PHBV systems,  $T_{m2}$  was dependent only on HV content and its interactions with filler content and type, without dependence on these parameters alone.  $X_c$  in all systems showed dependence on all investigated parameters signifying effect of both crystallisation and recrystallisation events on final  $X_c$  in studied systems. Overall, changes in system free volume (either via plasticisation or via an increase in HV content) was the most important parameter that affected the outcome function, with the importance of other factors dependent on the parameter studied.

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**Data Availability** Data underpinning this publication are openly available from the University of Strathclyde Knowledge Base at <https://doi.org/https://doi.org/10.15129/77d20d47-183e-41ff-bd9a-907bf73657f3>.

## Declarations

**Conflict of interest** Authors declare no conflict of interest.

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