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Enhanced 4-hydroxybutyrate incorporation into the PHA terpolymer of *Haloferax mediterranei* by heterologous expression of 4-hydroxybutyrate-CoA transferases/synthetases

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ABSTRACT

The polyhydroxyalkanoate terpolymer, P[(3HB)-*co*-(3HV)-*co*-(4HB)], is a promising plastic alternative for specialized applications, notably in medical and pharmaceutical sectors. *Haloferax mediterranei* (Hfx), an extreme halophile archaeon, is a P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer production host, however the native molar proportion of 4HB incorporated into the terpolymer is low. To improve incorporation, four 4-hydroxybutyrate-CoA transferases/synthetases from *Clostridium kluyveri* (OrfZ), *Clostridium aminobutyricum* (AbfT), *Nitrosopumilis maritimus* (*Nm*CAT), and *Cupriavidus necator* N-1 (*Cn*CAT), were heterologously expressed in *H. mediterranei*, and evaluated for their ability to supply 4HB-CoA for PHA terpolymer production. Growth, PHA synthesis, and polymer composition were evaluated for the four heterologous strains in shake-flask, with Hfx_*Nm*CAT demonstrating superior growth, terpolymer titre and 4HB molar ratio. Co-feeding with γ-butyrolactone was optimised, and Hfx_*Nm*CAT was further evaluated under fed-batch fermentation where a maximum PHA titre of 0.7 g/L, containing 52 mol% 4HB, was achieved. This is an order of magnitude improvement in 4HB terpolymer incorporation by *H. mediterranei*.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biopolymers produced by some bacteria and archaea for carbon and energy storage during unbalanced growth conditions. A number of PHAs possess similar physical, thermal, and mechanical properties to petroleum derived plastics, while further offering desirable characteristics such as biodegradability, biocompatibility and sustainable production routes. As such, the replacement of conventional plastics with PHA equivalents is of great interest [2]

The homopolymer poly-3-hydroxybutyrate P[(3HB)] is the most commonly synthesised PHA polymer by bacteria and archaea, however it is brittle, highly crystalline and possesses a high melting temperature which limits its processability by conventional methods [1,3]. However, a number of studies have previously demonstrated that the incorporation of different hydroxyacid monomers to create co- and terpolymers can alter the physiochemical properties of the polymer, thus broadening the application potential of these materials. Incorporation of 3-hydroxyvalerate (3HV) and/or 4-hydroxybutyrate (4HB) into the PHA polymer has been shown to increase tensile strength and elasticity and decrease crystallinity and melting temperature. Further, increased 4HB incorporation improves the biodegradation rate of the polymer in both sea water and within mammalian tissue, a desirable quality for both environmental bioremediation and biomedical applications [4–8]. As such, the development of microbial platforms for the production of P[(3HB)-*co*-(3HV)-*co*-(4HB)] PHA terpolymers with variable monomeric proportions, and thus variable physical and mechanical properties to P[(3HB)], is of current biotechnological interest as it would broaden the scope of potential commercial applications for these materials.

To date, few bacteria and archaea have been reported to synthesise P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer, namely *Hydrogenophaga pseudoflava*, *Cupriavidus necator*, *Cupriavidus malaysiensis*, *Aneurini bacillus* sp. H1, and *Haloferax mediterranei* [1]. *H. mediterranei*, an extreme halophile archaeon, is of particular interest as a Next Generation Industrial Biotechnology (NGIB) production host as it can be cultivated in

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open, non-sterile conditions and on complex biomass-derived substrates. *H. mediterranei* is unique among PHA producers as it can accumulate the P[(3HB)-*co*-(3HV)] copolymer on glucose as a sole carbon source, and possesses four different routes for the synthesis of the 3HV-CoA precursor, propionyl-CoA (See supplementary material) [9,10]. Further, PHA separation and recovery is simplified by exploiting an intra-extracellular osmotic pressure differential, simplifying processing and potentially reducing overall production cost [11,12].

P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer production has been demonstrated by *H. mediterranei* using exogenous supplementation of γ-butyrolactone to supply the 4HB-CoA monomer [3]. However even so, the molar content of 4HB in the PHA polymer remains low, with the highest proportion achieved being 5.1 mol% [13,14]. The rate limiting step to 4HB PHA polymer incorporation is the enzymatic conversion of 4HB to 4HB-CoA, which is achieved via the activity of a CoA transferase or synthetase. To date no 4HB-CoA transferase/synthetase has been identified in *H. mediterranei*, thus engineering improved 4HB incorporation into the terpolymer requires heterologous gene expression. Previously, the 4HB-CoA transferases, OrfZ and Cat2, from *Clostridium kluyveri* and *Porphyromononas gingivalis*, respectively, have permitted non-native 4HB incorporation into the PHA polymers of *Halomonas bluephagensis*, *Escherichia coli*, *Bacillus megaterium*, and *Synechococcus* sp. PCC7002. However, again, the molar ratio of this monomer has remained relatively low ($\leq$17 mol%), limiting the compositional tunability of the PHA polymer by these engineered hosts [1].

In this work, several heterologous 4HB-CoA transferases/synthetases were evaluated for their impact on 4HB incorporation into the P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer of *H. mediterranei*. These include 4HB-CoA transferases from *C. kluyveri*, *C. aminobutyricum*, and *C. necator* N-1, as well as a 4HB-CoA synthetase from *N. maritimus* – none of which has been previously evaluated for the purpose of 4HB incorporation into PHA in *H. mediterranei*. The most promising candidate was then carried forward into fed batch bioreactor cultivations to demonstrate the highest 4HB PHA molar ratio achieved by *H. mediterranei* to date (52 mol % 4HB). This work illustrates the potential use of engineered *H. mediterranei* as a platform host for the production of PHA terpolymer of variable monomeric composition.

2. Materials and methods

2.1. Plasmid construction

Plasmid pTA231-p.Syn-PAmCherry1Hxf was acquired from Addgene (#164661) and modified by, first, replacing the *trpA* selection marker with a mutated *Haloferax* gyrase B (*gyrB**) and truncated gyrase A (*gyrA*) to confer novobiocin antibiotic resistance [15]. The putative 4HB-CoA transferases *CnCAT* from *C. necator* N-1 (CNE_2c13270) and *AbfT* from *C. aminobutyricum* (JYB65_02205), as well as the 4-hydroxybutyrate--CoA ligase, *NmCAT,* from *N. maritimus* (Nmar_0206) were then manually codon-optimized to the genome of *H. mediterranei* and cloned into the multiple cloning site of the modified plasmid. DNA constructs and oligonucleotide primers were synthesized by Integrated DNA Technologies (See supplementary materials). DNA amplification for cloning was performed using Q5® Hot Start High-Fidelity 2X Master Mix (NEB), and DNA amplification for colony PCR was performed using Phire Green Hot Start II Master Mix (Thermo Fisher Scientific). Plasmid assembly was carried out using the NEB® NEBuilder HiFi DNA Assembly Master Mix. Correct plasmid assemblies were validated via Sanger sequencing (Source Bioscience, UK).

2.2. Transformation

Transformation into *H. mediterranei* followed the method described in [16]. Briefly, a culture was grown in Hv-YPC media (68 g/L yeast extract, 13.6 g/L peptone, 13.6 g/L casamino acids, 20 % sea water (SW)) at 45 °C and 200 rpm to an OD_{600} of 0.8 before undergoing centrifugation and resuspension into 600 μL of spheroplasting solution (58.4 g/L NaCl, 2 g/L KCl, 150 g/L sucrose, 10 mM Tris-HCl pH 8.5). 20 μL of 0.5 M EDTA pH 8.0 was then added to the suspension, followed by a 10-min incubation before the addition of 1–2 μg of plasmid DNA and 250 μL of 60 % PEG600. The suspension was incubated for a further 60 min at room temperature. Following this, 1.5 mL of spheroplast dilution solution (150 g/L sucrose, 760 mL/L 30 % SW) was added before centrifugation and resuspension in 1 mL of regeneration solution (150 g/L sucrose, 5 g g/L yeast extract, 1 g/L peptone, 1 g/L casamino acids, 600 mL/L SW). Samples were then incubated at 45 °C for an initial 2 h at stationary followed by rotary incubation for 3 h. Cells were then plated onto Hv-YPC + 0.2 μg/mL Novobiocin and incubated at 45 °C for 5 days.

2.3. H. mediterranei seed cultivation

H. mediterranei (DSM 1411, ATCC 33500) cultures were initiated by inoculating cryo-stored cells into 10 mL of MSM media (156 g/L NaCl, 13 g/L $MgCl_2{\cdot}6H_2O$, 20 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L $CaCl_2{\cdot}6H_2O$, 4 g/L KCl, 0.2 g/L $NaHCO_3$, 0.5 g/L NaBr, 2 g/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.005 g/L $FeCl_3$, 15 g/L 1,4-piperazinediethanesulfonic acid (PIPES), and 1 mL/L 10x SL6 trace element solution) supplemented with 10 g/L glucose and 0.2 μg/mL novobiocin. Seed cultures were then incubated at 37 °C and 200 rpm for 24 h before being transferred to 200 mL MSM media + 10 g/L glucose and 0.2 μg/mL novobiocin for a further 36 h at 45 °C and 200 rpm. Cultures were then centrifuged and cell pellets were resuspended in MSM for inoculation into fermentations.

2.4. PHA fermentations

2.4.1. Shake flask fermentations

All shake flask fermentations were performed in 100 mL flasks containing 20 mL of MSM media supplemented with 10 g/L glucose and 0.2 μg/mL novobiocin, and 10 g/L of γ-butyrolactone (GBL). Cultures were inoculated to an initial OD_{600} of 0.4 and incubated at 45 °C and 200 rpm. Fermentations were halted after 72 h and samples were taken to determine culture density and PHA titre and composition. To optimise GBL addition, feed concentrations of 5, 10 and 15 g/L were assessed, as were feed times of 0, 24, or 48 h post inoculation. All flask fermentations were performed in duplicate.

2.5. Fed-batch bioreactor fermentation

All bioreactor fermentations were performed in 3 L cylindrical bioreactors (Applikon Biotechnology, 1 Rushton turbine, 3 baffles, 2.2 H/D ratio) with a working volume of 1.2 L. Temperature was maintained at 37 °C, pH was maintained at 6.8 using 3 M HCl, and dissolved oxygen was maintained at 20 % via aeration at 0.75 vvm and variable stirrer speed ranging between 200 and 800 rpm. Cultivations were performed in MSM media containing 0.2 μg/mL novobiocin, and strain Hfx_*Nm*CAT was inoculated to an initial OD_{600} of 0.4. 10 g/L glucose was supplemented into the initial culture broth with intermittent feeding when culture concentration dropped below 3 g/L. 10 g/L of GBL was added after an initial 48 h of fermentation. Fermentations were performed in duplicate and terminated after 216 h. Culture samples were taken at 24 h intervals to determine culture density, glucose concentration, and PHA titre and composition.

2.6. PHA quantification and compositional analysis

GC-FID was used to determine the biopolymer composition, with the amount of each monomer in the terpolymer being quantified. To prepare the sample for analysis 1 mL of culture was centrifuged at 13,000 rpm for 5 min, and the cell pellet washed three times with 0.1 % SDS before a final wash with distilled water. The washed pellet was then transferred to a pressure tube and dried at 110 °C. Once dried, the pellet was

resuspended in 4 mL of acidic methanol-chloroform solution ($CHCl_3$: CH_3OH:H_2SO_4 ratio of 10:7:3) and incubated at 95 °C for 140 min. Following this the reaction mixture was washed with 1 mL of distilled water and an aliquot of the organic fraction removed for analysis by a GC-FID equipped with a 30 m × 0.25 mm × 0.25 μm SGE BPX70 capillary column under the following conditions: 1 μL injection volume, column temperature of 100 °C for 3 min followed by a 20 °C/min ramp to 200 °C and then a 30 °C/min ramp to 220 °C before being held for 2 min. Helium was used as the carrier gas. Product identification and quantification was carried out using relevant standards.

C NMR was used to determine the terpolymer structure, 1 mL of culture was washed and dried as described above. The dried pellet was then homogenized in chloroform while being magnetically stirred for 48 h. The solution was then filtered to remove cellular debris and the solution was added dropwise to chilled methanol under continuous stirring to a 1:10 (v/v) ratio. Purified PHA was then dissolved in $CDCl_3$ and ^{13}C NMR was recorded at room temperature in a B500 MHz Avance II+ (Brucker) to analyse the terpolymer.

3. Results and discussion

3.1. Evaluation of heterologous 4HB-CoA transferases/synthetases expression upon terpolymer production in H. mediterranei

H. mediterranei is capable of producing a P[(3HB)-*co*-(3HV)-*co*-4(HB)] terpolymer. While the requisite 3HB-CoA and 3HV-CoA substrates are endogenously produced, 4HB-CoA production requires exogenous supplementation with a precursor (such as GBL) which upon entry into the cell is converted to 4HB (Fig. 1) [13,17]. Endogenous activation of 4HB to 4HB-CoA in *H. mediterranei* is low [13,14], suggesting the requisite 4HB-CoA transferase or synthetase is repressed or that the observed activation is the result of promiscuous substrate specificity by other enzymes.

To improve 4HB incorporation into the PHA terpolymer a panel of four 4HB-CoA transferases/synthetases was evaluated. Included were: OrfZ from *C. kluyveri*, a 4HB-CoA transferase involved in the reduction of succinate to butyrate, and which has previously been demonstrated to improve 4HB incorporation into the PHA polymer of a number of microbial hosts [1,18]. AbfT from *C. aminobutyricum*, a 4HB-CoA transferase involved in the fermentation of 4-aminobutyrate [18]. *Cn*CAT from *C. necator* N-1, a putative 4HB-CoA transferase. *C. necator* is capable of significant 4HB incorporation into the PHA polymer upon GBL supplementation, suggesting it possesses an active 4HB-CoA transferase or synthetase [3]. *Cn*CAT possesses 35 % sequence identity to both OrfZ and AbfT, indicating it likely possesses similar activity (See supplementary materials), however its native role in *C. nector* N-1 is currently unknown. This CAT appears to be conserved across other P[(3HB)-*co*-(4HB)] copolymer producing *Cupriavidus* sp., namely *C. necator* H16 and *C. malaysiensis*, where it is annotated as an acetyl-CoA hydrolase/transferase, and possesses 97.5 % and 85.2 % amino acid sequence identity to the *Cn*CAT, respectively (See supplementary materials). *Nm*CAT from marine archaea *N. maritimus*, a 4HB-CoA synthetase involved in the thaumarchaeal hydroxyproprionate/hydroxybutyrate (HP/HB) carbon fixation cycle (Fig. 1) [19]. With the exception of OrfZ, none of the panel of 4HB-CoA transferases/synthetases have been previously assessed for their ability to promote 4HB incorporation into a PHA polymer.

Each 4HB-CoA transferase/synthetase was codon optimized to the

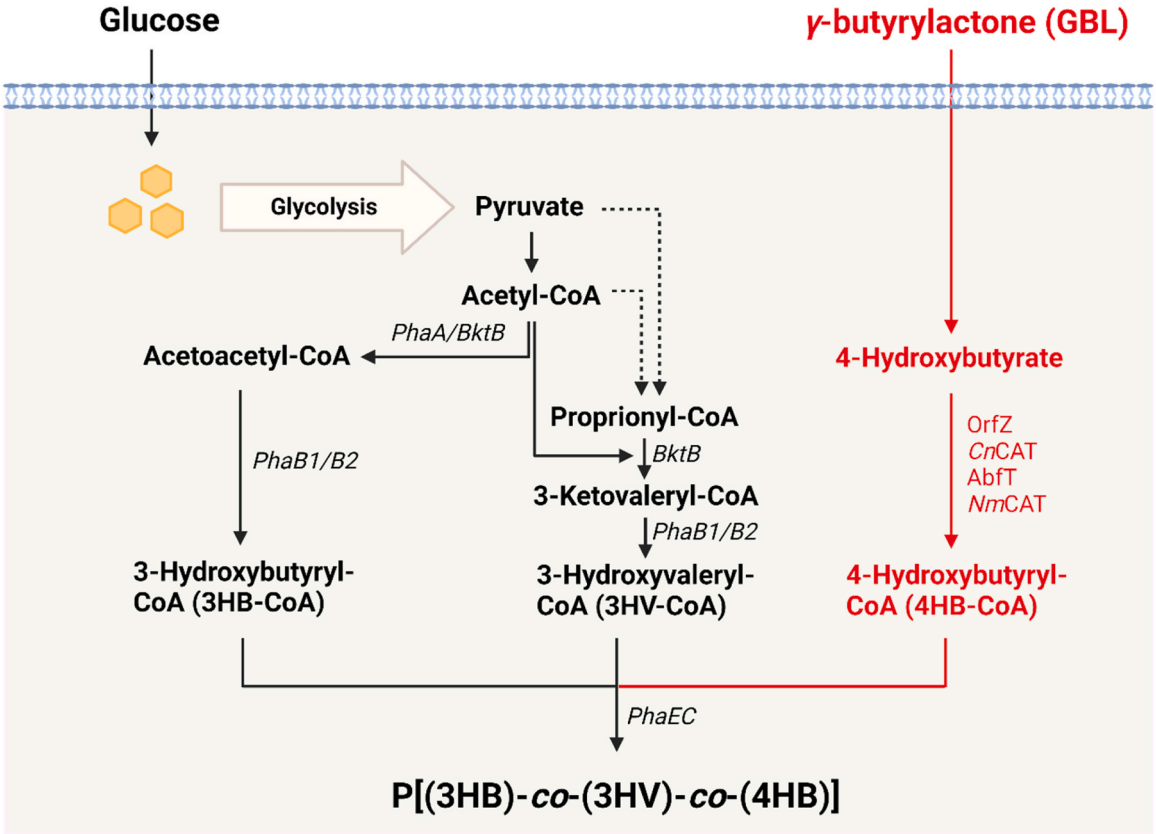

Fig. 1. Illustration of the metabolic steps towards enhanced terpolymer production in *H. mediterranei*. Pyruvate and acetyl-CoA derived from glucose produce 3HB-CoA and 3HV-CoA via the endogenous activity of PhaA/BktB and PhaB1/B2. 4HB-CoA synthesis is achieved via the heterologous expression of a 4HB-CoA transferase/synthetase and exogenously supplied γ-butyrylactone (GBL). Here, several candidates were assessed, including: 4HB-CoA transferases from *C. kluyveri* (OrfZ), *C. necator* (*Cn*CAT), *C. aminobutyricum* (AbfT), and a 4HB-CoA synthetase from *N. maritimus* (*Nm*CAT). The three monomers are then incorporated into a P[(3HB)-co-(3HV)-co-(4HB)] terpolymer via a PHA synthetase, PhaEC.

genome of *H. mediterranei*, which, like many halophiles, has a high GC content in order to increase DNA stability under high salt concentrations [20]. Each gene was then inserted into a pTA231-p.Syn plasmid for constitutive archaeal expression, and transformed into *H. mediterranei* to generate strains Hfx_OrfZ, Hfx_*Cn*CAT, Hfx_AbfT, and Hfx_*Nm*CAT. Each strain was then cultivated in 20 mL of MSM media supplemented with 10 g/L glucose and 10 g/L GBL in shake-flask for 72 h before analysis of PHA terpolymer content and composition (Table 1). All strains produced similar PHA titres, between 83 and 106 mg/L, with the exception of Hfx_OrfZ which demonstrated significantly reduced growth and no PHA accumulation. This was unexpected as it has been previously successfully expressed in a number of microbial hosts, including the halophile *H. bluephagenesis* TD01 [1,21]. Further, wild type *H. mediterranei* produced only a P[(3HB)-*co*-(3HV)] copolymer despite the GBL co-feed. Hfx_*Cn*CAT, Hfx_AbfT, and Hfx_*Nm*CAT all successfully incorporated 4HB into a PHA terpolymer, as confirmed by 500-MHz ^{13}C NMR spectroscopy (See supplementary materials) – validating the novel application of these 4HB-CoA transferases/synthetases in PHA terpolymer production. Of these strains, Hfx_*Nm*CAT incorporated 18.9 mol% 4HB into its terpolymer, 3.7-fold more than previously reported for *H. mediterranei* [14], while Hfx_AbfT incorporated the least, with 4.8 mol% (Table 1). Interestingly, despite *Nm*CAT being a 4HB-CoA synthetase, which requires ATP for catalysis (unlike a CoA transferase), it did not appear to exert additional metabolic burden on the cell. The cultures of Hfx_*Cn*CAT, Hfx_AbfT, and Hfx_*Nm*CAT all accumulated to a higher cell density than the wild type *H. mediterranei*, which can likely be attributed to microbial toxicity associated with GBL, or the free 4HB acid to which it is hydrolysed *in vivo*. This toxicity will have been reduced in the heterologous strains as they sequestered this precursor/intermediate into the [P(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer – unlike the wild-type strain which did not successfully incorporate any exogenously fed GBL into its PHA polymer. Indeed, there is a positive correlation between mol% 4HB incorporation to the PHA polymer and culture density (Table 1).

3.2. Investigation of GBL addition parameters for terpolymer production

Next, to optimise PHA titre and 4HB incorporation, the impact of GBL concentration and feed time was explored for Hfx_*Cn*CAT, Hfx_AbfT, and Hfx_*Nm*CAT. Here it was found that increasing GBL concentration from 5 to 10 g/L improved PHA production and 4HB molar fraction for all strains, while GBL concentrations of 15 g/L had a significantly negative impact on both metrics as well as culture density. At a constant GBL concentration (10 g/L), feed addition time also played a significant role in PHA titre/composition and optical density, with 24 h (mid-exponential phase) identified as optimal (Fig. 2). It is likely that cytotoxicity associated with the intracellular accumulation of GBL or free 4HB strongly influences the results observed for both parameters – with the tolerated dose-per-cell being exceeded at high feed concentrations (>10 g/L) and at times of low cell density, such as lag phase. Similar growth and production trends have been observed previously for PHA production by *Azotobacter vinelandii* fed sodium 4-hydroxybutyrate [22]. Following this, cultures of Hfx_*Cn*CAT, Hfx_AbfT, and Hfx_*Nm*CAT were cultivated in 200 mL of MSM media + 10 g/L glucose in shake-flask, with the 10 g/L GBL co-feed occurring after an initial 24-h incubation. An improved PHA titre and an increased 4HB molar fraction were observed for all strains, with Hfx_*Nm*CAT achieving 211 ± 12 mg/L of PHA terpolymer containing 34.8 ± 0.5 mol% 4HB (Fig. 2, see supplementary materials).

Table 1
Evaluation of 4-HB incorporation into the PHA terpolymer of *H. mediterranei* cultures expressing a 4-HB-CoA transferase or synthetase with GBL supplementation.

Strain	OD_{600}	Total PHA mg/L	4-HB mmol/L	4-HB/PHA (mol %)
Wildtype	7.3 (± 0.5)	106 (± 19)	0	0
Hfx_OrfZ	2.9 (± 0.2)	0	0	0
Hfx_*Cn*CAT	12.1 (± 0.4)	88 (± 10)	0.12 (± 0.05)	13.6 (± 0.5)
Hfx_AbfT	8.2 (± 0.3)	83 (± 12)	0.04 (± 0.02)	4.8 (± 0.5)
Hfx_*Nm*CAT	13.4 (± 0.6)	95 (± 24)	0.18 (± 0.04)	18.9 (± 0.4)

3.3. Fed-batch PHA terpolymer production by Hfx_NmCAT

A fed-batch fermentation was performed for P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer production by Hfx_*Nm*CAT. Cultures were grown in 1.2 L of MSM media initially supplemented with 10 g/L glucose and were pulse fed glucose intermittently (see supplementary materials). The addition of 10 g/L GBL was performed after 48 h, when the culture had reached mid-exponential growth phase (OD ≈22). PHA accumulation began after 24 h and continued until 216 h, reaching a final titre of 0.7 g/L. 4HB incorporation did not begin until 72 h, after the GBL co-feed. From 120 h onwards, 4HB was the greatest contributor to the increasing PHA titre, as the 3HB and 3HV constituents remained relatively constant (Fig. 3). At 216 h, a final yield of 7 % (CDW) PHA composed of P[(3HB)-*co*-(5 mol% 3HV)-*co*-(52 % 4HB)] was achieved – a 10-fold improvement on 4HB incorporation than what has been reported previously for *H. mediterranei* [14]. The considerable improvement in final 4HB molar fraction compared to the same strain, Hfx_*Nm*CAT, cultivated in shake-flask suggests that, in the latter condition, carbon limitation terminated PHA fermentation before 4HB incorporation was complete (Table 1; Fig. 3). This fermentation time dependent 4HB incorporation could be further exploited as a strategy to titrate terpolymer composition. As could the use of alternative carbon feedstocks, Ferre-Guell and Winterburn [23] demonstrated that the 3HV fraction of the P[(3HB)-*co*-(3HV)] copolymer of wild type *H. mediterranei* was proportional to the % C5:0 volatile fatty acid (VFA) present in the feedstock, and illustrated the production of PHA polymers with a 3HV molar fraction of up to an 87 %. The potential of engineered *H. mediterranei* to achieve high fractions of both 3HV and 4HB offers significant flexibility with regards to fine-tuning the polymer composition, and thereby the material properties. Toward this, Zhila and Shishatskaya [7] previously demonstrated that terpolymer, produced in a *Cupriavidus eutrophus* host, with compositions of P[(51.5 %mol 3HB)-*co*-(10.1 %mol 3HV)-*co*-(38.4 %mol 4HB)] and P[(27.4 %mol 3HB)-*co*-(17 %mol 3HV)-*co*-(55.6 %mol 4HB)] displayed considerably different degrees of crystallinity, elastic properties, and mechanical strength. Further, previous studies have demonstrated that the PHA titres of *H. mediterranei* can be improved considerably when grown on complex waste-derived carbon sources compared to glucose [9,24]. There is a requirement for further fermentation process development and intensification in order to improve overall productivity and yield of the fermentation, to move towards technoeconomically viable production processes.

4. Conclusion

Four 4HB-CoA transferase/synthetase enzymes were evaluated for their ability to incorporate 4HB into the P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer of, *H. mediterranei*. Concentration and feed-time of GBL – the precursor to 4HB-CoA – was modified to improve the PHA incorporated 4HB fraction, and a strain expressing the most promising candidate gene, encoding a 4HB-CoA synthetase (*Nm*CAT) from *N. maritimus,* was carried forward into fed-batch fermentation. Here, a final PHA terpolymer composition of P[(3HB)-*co*-(5 mol% 3HV)-*co*-(52 mol% 4HB)] was observed, an order of magnitude improvement in 4HB molar incorporation over previous studies. This work expands the functionality of *H. mediterranei* for terpolymer production.

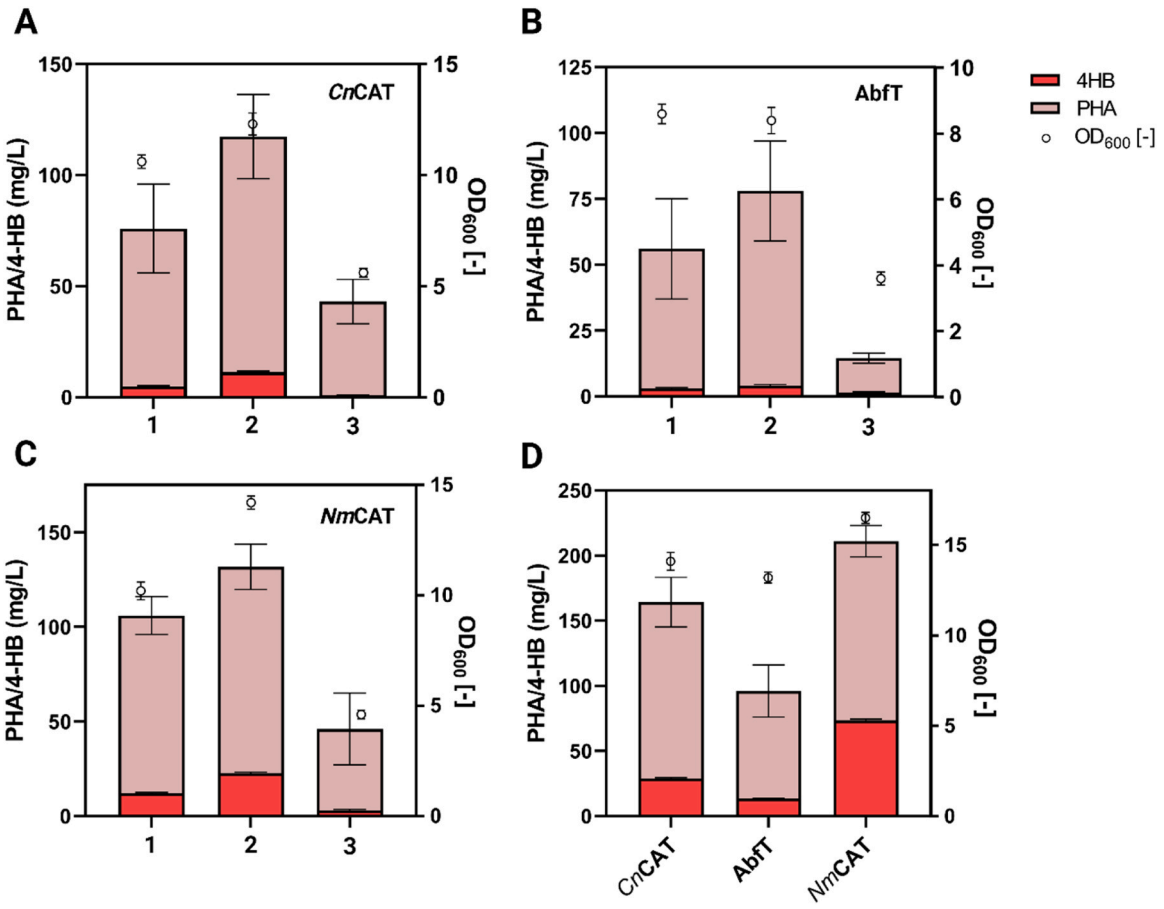

Fig. 2. Investigation of the GBL co-feed parameters for high PHA titre and 4HB incorporation by *H. mediterranei* constitutively expressing *Cn*CAT (A), AbfT (B), or *Nm*CAT (C). Condition 1= 5 g/L GBL at 24 h, 2= 10 g/l at 24 h, 3= 15 g/l at 24 h. (D) PHA production from each strain under improved GBL feed conditions, 10 g/L at 24 h, scaled up 200 mL cultures. Total PHA and polymer associated 4HB represented by bars; culture OD_{600} represented by filled circles. Data labels indicate the time of GBL addition, all cultures were harvested at 72 h. Data is the average of two biological replicates ± standard deviation.

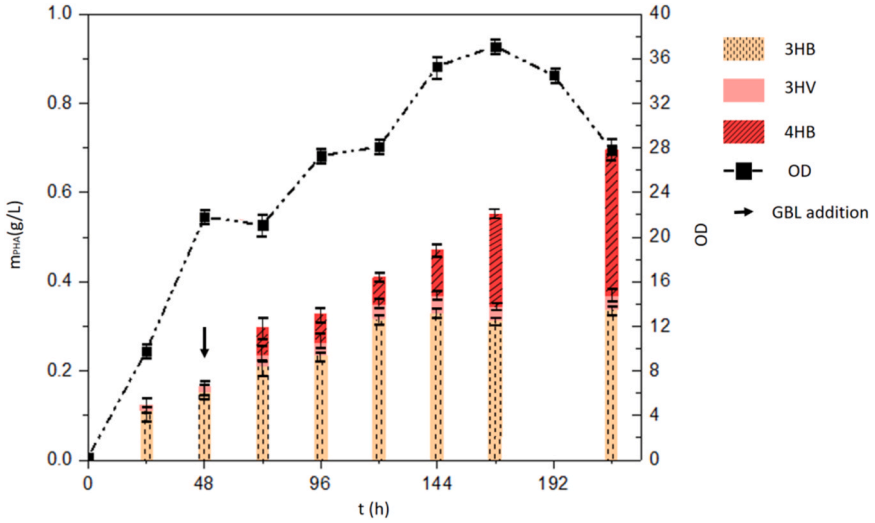

Fig. 3. Fed-batch fermentation of Hfx_*Nm*CAT for P[(3HB)-*co*-(3HV)-*co*-(4HB)] terpolymer production. Arrow used to illustrate the timing of the 10 g/L GBL co-feed. Glucose was pulse fed to maintain a culture concentration above 3 g/L. Data represents the average of two biological replicates ± standard deviation.

CRediT authorship contribution statement

Neil Dixon: Writing – review & editing, Supervision, Conceptualization. **Thorsten Allers:** Writing – review & editing, Methodology. **Micaela Chacón:** Writing – original draft, Methodology, Investigation, Conceptualization. **Xiangrui Zheng:** Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **James Winterburn:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109498.

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