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Master of Science HES-SO in Life Sciences

Biosynthesis of unusual polyhydroxyalkanoates in recombinant *Escherichia coli* strains

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APPLIED BIOSCIENCES

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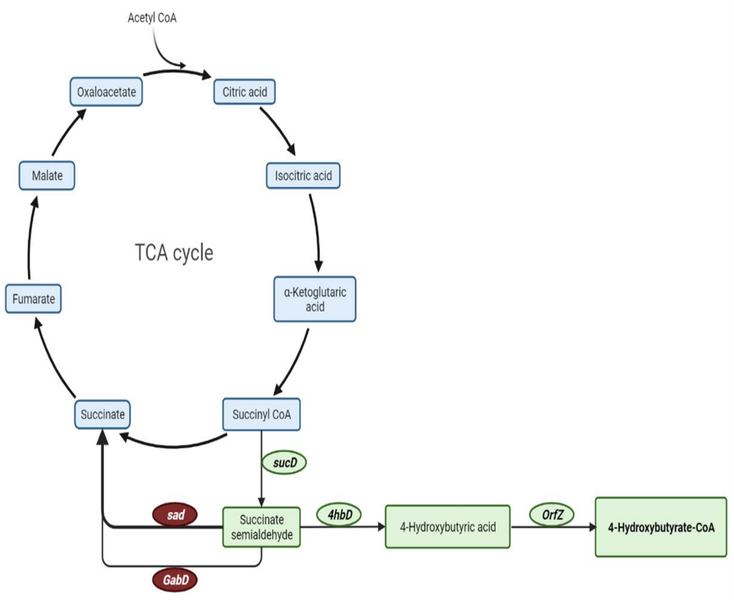
DESCRIPTION

The biopolymer family of polyhydroxyalkanoate (PHA) is getting more and more attraction in the ongoing environmental crisis. They attract attention due to their natural production by microorganisms and the variety of synthesized monomers, that can result in polymers with different properties. One monomer is of particular interest, the 4-hydroxybutyrate, because it can be polymerized into poly(4-hydroxybutyrate) (P4HB), which is the only PHA approved by the FDA for medical use. This is explained by its biocompatibility and release of non-toxic compounds after assimilation in the body. The production of this polymer is usually realized by supplying directly the 4-hydroxybutyrate precursor to a culture of a recombinant strain harboring the polymerizing enzymes PhaC. Nonetheless, one of the challenges with this polymer production is in regards of law regulation. Since the 4-hydroxybutyric acid is also known as the GHB drug, it is not commercially available in the 4-hydroxybutyric acid form and it needs to be synthesized by alkaline hydrolysis of the corresponding lactone, the γ-butyrolactone.

Another way to synthesize this monomer could be to use the pathway of a naturally producing organism, such as *Clostridium kluyveri,* and to insert it in a recombinant *Escherichia coli* strain. This pathway allows the production of the 4-hydroxybutyric acid from unrelated carbon source, such as glucose or xylose. The use of a recombinant strain is advantageous in the fact that it does not possess the ability to metabolize the substrate, thus it is theoretically improving the yield of the product formation. *E. coli* being one of the most studied organism, it a first choice when working with synthetic biology, but the bacterium also possesses two enzymes that are redirecting the carbon flux toward the TCA cycle.



4HB production pathway

OBJECTIVES

The main objective of this study is to produce, directly *in vivo*, the 4-hydroxybutyrate monomer, from unrelated carbon source, such as xylose. This monomer would then be polymerized *in vivo* in a biopolymer that is approved by the FDA for medical use. To do so, a plasmid construct (pS4HB) is designed, containing two enzymes from *Clostridium kluyveri* and two gene deletions are performed in the host strain to ensure proper carbon flux toward the desired product. The deletions are realized with the Lambda red method. This gene deletion is performed on different strains of *E. coli* in order to later identify the best host strain for P4HB production.

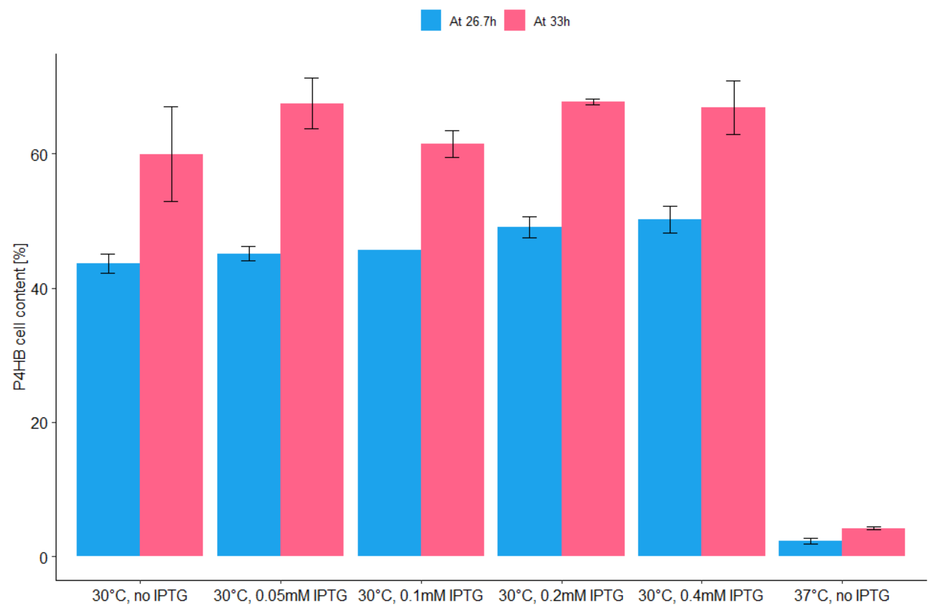
The effects of parameters such as the level of induction and temperature can be assessed to determine the best conditions. Because the promoter of the plasmid is the *LacZ* operon, the induction by IPTG (isopropyl β-D-1-thiogalactopyranoside) is an essential parameter for a better control of the polymer biosynthesis. Since temperature affects the copy number of the plasmids, this is also a parameter to consider.

In order to ensure a sufficient carbon flux toward the product, two genes were identified as negatively impacting the production rates. They are expressed into two enzymes that use the succinate semialdehyde and thus are responsible for diminishing product formation. Those two enzymes will be removed using the Lambda red method based on homologous deletion.

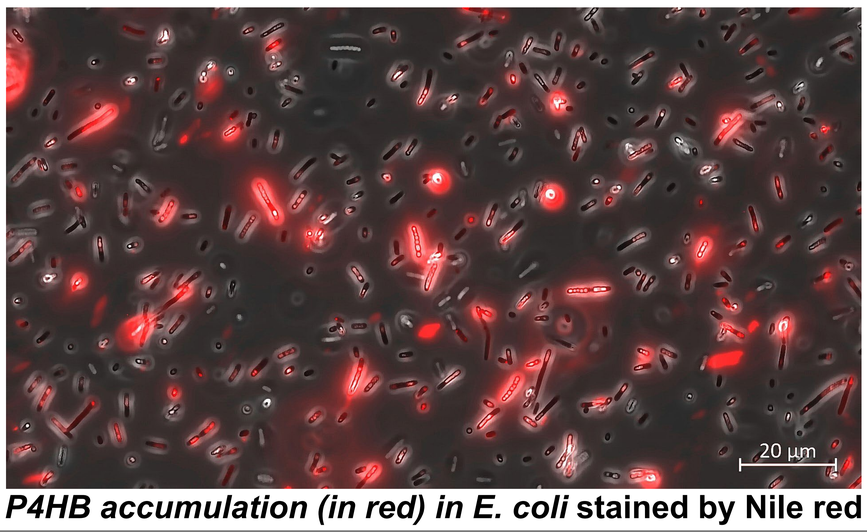
RESULTS

First, the deletion of the *sad* and *gabD* gene was conducted in different strains of *E. coli* and resulted in the generation of multiple clones lacking one or both genes (JW5247Δ*gabD*, MDS42Δ*sad*, MG1655Δ*sad*). The strain JW5247 already lacking the gene for the Sad enzyme was modified to now also lack the *gabD gene.*

Also, a plasmid was designed (pS4HB) in order to insert the 4-HB production from unrelated carbon source in *E. coli* and was transformed in the newly generated strain JW5247Δ*gabD.* This transformant was then used to evaluate the production of the 4-hydroxybutyrate and was cotransformed with a plasmid (pKSSE5.3) harboring the polymerase PhaC from *C. necator.* A series of shake flask experiments was conducted with different IPTG levels of induction and with two growth temperatures. Two sampling times were compared for the different conditions in terms of 4HB and P4HB productions. A visible augmentation of 4HB and P4HB accumulation was observed between both timepoints with a greater accumulation after a longer time, as expected. A constancy was observed for the conditions at 30°C but at 37°C almost no accumulation was observed after both 26,7h and 33h. The temperature clearly plays a role in the P4HB accumulation and overall, at 30°C the percentage of the biomass composed of P4HB is between 60wt% and up to 70wt% (30°C, 0.05mM of IPTG) compared to the maximal value of 4.3wt% at 37°C.



P4HB accumulation at different induction levels and temperatures



P4HB accumulation (in red) in E. coli **stained by Nile red**

CONCLUSION

The plasmid designed in this study, pS4HB, harboring the pathway to produce 4-hydroxybutyric acid was able to produce significant amount of monomer, up to 1200 mg/l after 33h of growth on a defined medium. Also, the co-transformation with the plasmid pKSSE5.3 allowed to polymerize the monomer and accumulation of PHA granules in the cells was of about 60-70wt% at 30°C. The different induction levels had no effect on the productivity either of the 4HB or the P4HB and the only parameter identified as negatively impacting the production was the temperature. At 37°C no accumulation was observed.

Further research on the optimization of the plasmid pS4HB expression could be conducted to optimize the P4HB production. To do so, the range of growth temperature could be changed to define more precisely this parameter, and this could also be done on the induction levels and time of induction. Also, a protein analysis by western blot and Bradford could show if one enzyme is not produced sufficiently during the process. This should allow identification of potential bottlenecks in the inserted pathway. Finally, the system could be conducted in a fed-batch bioreactor to assess feasibility at larger scale.

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