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Metabolic Engineering of *Bacillus subtilis* for the Production of Poly-γ-Glutamic Acid from Glycerol Feedstock

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Abstract: Poly-γ-glutamic acid (γ-PGA) is an attractive biopolymer for medical, agri-food, and environmental applications. Although microbial synthesis by *Bacilli* fed on waste streams has been widely adopted, the obtainment of efficient sustainable production processes is still under investigation by bioprocess and metabolic engineering approaches. The abundant glycerol-rich waste generated in the biodiesel industry can be used as a carbon source for γ-PGA production. Here, we studied fermentation performance in different engineered *Bacillus subtilis* strains in glycerol-based media, considering a *swrA*$^{+}$ *degU32*^{Hy} mutant as the initial producer strain and glucose-based media for comparison. Modifications included engineering the biosynthetic *pgs* operon regulation (replacing its native promoter with $P_{hyspank}$), precursor accumulation (*sucCD* or *odhAB* deletion), and enhanced glutamate racemization (*racE* overexpression), predicted as crucial reactions by genome-scale model simulations. All interventions increased productivity in glucose-based media, with $P_{hyspank}$-*pgs* Δ*sucCD* showing the highest γ-PGA titer (52 g/L). Weaker effects were observed in glycerol-based media: Δ*sucCD* and $P_{hyspank}$-*pgs* led to slight improvements under low- and high-glutamate conditions, respectively, reaching ~22 g/L γ-PGA (26% increase). No performance decrease was detected by replacing pure glycerol with crude glycerol waste from a biodiesel plant, and by a 30-fold scale-up. These results may be relevant for improving industrial γ-PGA production efficiency and process sustainability using waste feedstock. The performance differences observed between glucose and glycerol media also motivate additional computational and experimental studies to design metabolically optimized strains.

Keywords: fermentation; biodiesel industry waste; genome-scale metabolic model; metabolic engineering; *sucCD*; *racE*; *odhAB*

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1. Introduction

Poly-γ-glutamic acid (γ-PGA) is a biopolymer composed of D- and/or L-glutamic acid monomers connected by amide linkages between α-amino and γ-carboxyl groups. It has emerged as a multifaceted compound with a wide range of applications across various industries. γ-PGA's attractiveness is due to its biodegradability, biocompatibility, water solubility, and high hygroscopicity, with the ability to absorb moisture up to 5000 times its own weight [1]. These remarkable features make this polymer a relevant tool for applications in the health, agriculture, cosmetic, and food fields [2,3]. Products based on γ-PGA such as thickening gels, dietary supplements, cream humectants, soil additives, metal flocculants, bioplastics, drug carriers, and biomaterials for medical devices have been developed, with many of them already available as commercial products [4–7].

The main strategy for γ-PGA bioproduction is microbial fermentation using strains from different *Bacillus* species, e.g., *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. velezensis*, and *B. methylotrophicus* [5,8,9]. Many *Bacillus* strains are considered safe because of

their long track record of safe use in human foods, e.g., to produce enzymes used for food processing or as microbial strains used for the production of fermented foods [10]. This safe status of different natural γ-PGA producer strains facilitates their industrial use in terms of downstream processing for the purification of the polymer [11]. Although naturally occurring strains have been successfully adopted for high-level polymer production, the cost-effectiveness of the process is still a challenge, mainly due to the trade-off between productivity and the cost of fermentation medium substrates [12–14].

Improvements in bioprocess were reported, optimizing fermentation parameters such as pH and aeration, and using diverse strains [14–16]. Several non-canonical substrates were also reported to support γ-PGA biosynthesis, in an effort to lower production costs [17,18]. For example, agricultural residues, such as rice bran, can serve as cost-effective and sustainable substrates for γ-PGA production [19]. This approach not only reduces the environmental impact associated with waste disposal but also transforms by-products into value-added biocommodities. Additionally, the use of renewable feedstock aligns with the principles of a circular economy, promoting a closed-loop system in which resources are reused and recycled to minimize waste. Similarly, industrial waste streams can enhance the sustainability of fermentation processes [14]. Crude glycerol is a major by-product of biodiesel production plants, representing about 10% *w*/*w* of the total product [20,21]. Despite impurities that may pose challenges in its cost-effective utilization, it has been considered a convenient carbon source for polymer production [14,22]. In fact, purified glycerol, a known substrate for the growth of microbes such as *B. subtilis* and *B. amyloliquefaciens*, has been previously adopted in γ-PGA fermentation processes as an additive to glucose-based media to enhance polymer production [23,24] or as the main carbon source [12,25,26]; however, glycerol utilization is less efficient than that of glucose and biopolymer production needs to be improved.

Previous works indicated that metabolic engineering interventions in different hosts were able to enhance γ-PGA yield and increase the sustainability of polymer production. The expression of a completely heterologous γ-PGA synthesis pathway has been shown to provide polymer production capability to *Escherichia coli* [27] and *Corynebacterium glutamicum* [28,29], the latter reaching γ-PGA titers of about 50 g/L, which is a competitive production level, comparable with natural producers. However, most reports describe the genetic optimization of natural γ-PGA producers such as *B. subtilis* and *B. amyloliquefaciens*. The most common strategies include the deletion of pathways competing with γ-PGA precursor accumulation [30–33], the removal of polymer-degrading enzymes [34–36], the enhancement of oxygen availability by the *Vitreoscilla* hemoglobin gene [35,37], the improvement of glutamate metabolism [38–40], and the tuning of the *pgs* biosynthetic operon regulatory network by engineering its promoter or by mutating the *degU* response regulator [30,31,41,42]. Some of these interventions were conceived by rational model-based design, in which modifications were suggested by mathematical models [32]. Metabolic engineering aimed at improving glycerol catabolism to enhance the carbon flux towards γ-PGA in *B. licheniformis* resulted in up to a 70% improvement in polymer production using purified or crude glycerol [31,43,44]. Other strain design strategies were also developed, aimed at tuning the stereochemical composition and the molecular weight of the produced biopolymer, by swapping glutamate racemase and/or the γ-PGA synthesis genes with variants from different species [40,45,46]. The above strategies led to improvements in polymer production, typically by 2–4-fold by deleting competing pathways, by up to 2-fold by removing degrading enzymes, by up to 1.3-fold by enhancing oxygen availability, and by up to 1.7-fold by improving glutamate metabolism, while modifying *pgs* regulation allowed γ-PGA production in *Bacillus* strains that did not exhibit polymer production. However, it is worth mentioning that these strategies were applied to a wide array of species or strains, for which performances were described under diverse growth conditions and fermentation times, making it hard to fairly compare the calculated indexes. Also, the success of the above strategies often turns out to be strain- and/or media-dependent, leading to inconsistent results among different works, as the endogenous regulations leading

to γ-PGA production may differ across *Bacillus* species or even among strains of the same species [45,47].

In summary, the design of low-cost media and the enhancement of microbial productivity of γ-PGA are acknowledged as key factors limiting the industrial-scale production and use of this polymer. This work aims to investigate γ-PGA production in glycerol-based media using engineered *B. subtilis* strains bearing novel combinations of genomic modifications.

2. Materials and Methods

2.1. Strains

All the strains used in this work are derivatives of the JH642 domesticated *B. subtilis* strain [48] and are listed in Table 1. Briefly, PB5383 carries the *swrA*$^{+}$ *degU32*^{Hy} double mutation that confers γ-PGA production capability [42]. PB5716 and PB5691 are derivatives of PB5383, into which the *sucCD* and *odhAB* knockouts were introduced, respectively, to enhance γ-PGA flux by blocking the TCA cycle at the succinyl-CoA to succinate reaction and the 2-oxoglutarate to succinyl-CoA reaction steps, to promote γ-PGA precursor accumulation (Figure 1). These deletion strains were previously constructed based on the predictions of a genome-scale model and tested in glucose-based media [32]. Conversely, PB5741 does not have the *degU32*^{Hy} mutation, but the *pgs* operon expression depends on a transcriptional fusion of the IPTG-inducible $P_{hyspank}$ promoter with the *pgsB* gene. This strain was previously constructed to study the regulation of *pgs* [47], and here, the modification was leveraged to test γ-PGA production, not characterized before, without relying on the endogenous transcriptional regulations occurring in the native P_{pgs} promoter. The new strains constructed in this work are derivatives of PB5741 and contain either the *sucCD* knockout or the *racE* overexpression (encoding a glutamate racemase), or both modifications.

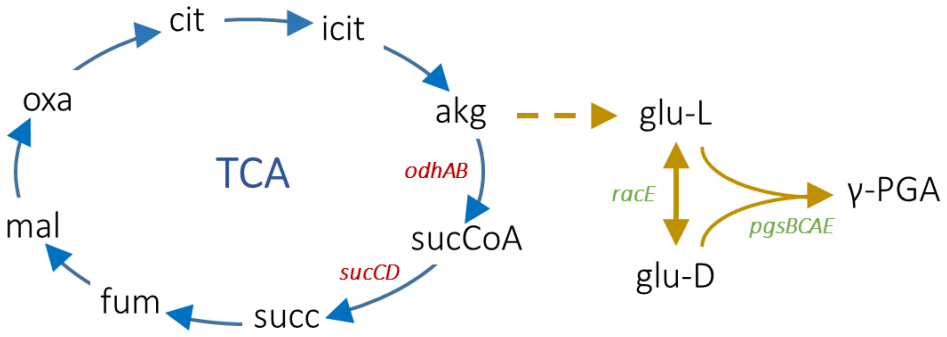

Figure 1. Main metabolic reactions leading to γ-PGA production in *B. subtilis*. The tricarboxylic acid (TCA) cycle is shown with blue arrows, and the γ-PGA biosynthesis reactions are shown with yellow arrows. The dashed arrow indicates a set of reactions leading to L-glutamate production from 2-oxoglutarate (reported in [32]). Abbreviations: citrate (*cit*), isocitrate (*icit*), 2-oxoglutarate (*akg*), succinyl-CoA (*sucCoA*), succinate (*succ*), fumarate (*fum*), malate (*mal*), oxalacetate (*oxa*), L-glutamate (*glu-L*), and D-glutamate (*glu-D*). The relevant genes investigated in this work are also reported with red (deleted genes) and green (overexpressed genes) text. Reactions and molecules have been retrieved from the *B. subtilis* iYO844_ec genome-scale metabolic model [32].

Table 1. Strains used in this work.

Strain	Relevant Genotype [1]	Reference
PB5383	*swrA*$^{+}$ *degU32*^{Hy} (Sm^{r})	[42]
PB5691	*swrA*$^{+}$ *degU32*^{Hy} (Sm^{r}) Δ*odhAB* (Cat^{r})	[32]
PB5716	*swrA*$^{+}$ *degU32*^{Hy} (Sm^{r}) Δ*sucCD* (Tet^{r})	[32]
PB5741	*swrA*$^{+}$ *ywsC::RBS*$_{spoVG}$*:sfGFP_P*$_{hyspank}$*::ywsC*; (Em^{r})	[47]
PB5763	*swrA*$^{+}$ *ywsC::RBS*$_{spoVG}$*:sfGFP_P*$_{hyspank}$*::ywsC*; (Em^{r}) *racE::P*$_{spac}$*::racE* (Cat^{r})	This study
PB5765	*swrA*$^{+}$ *ywsC::RBS*$_{spoVG}$*:sfGFP_P*$_{hyspank}$*::ywsC*; (Em^{r}) Δ*sucCD* (Tet^{r})	This study
PB5764	*swrA*$^{+}$ *ywsC::RBS*$_{spoVG}$*:sfGFP_P*$_{hyspank}$*::ywsC*; (Em^{r}) *racE::P*$_{spac}$*::racE* (Cat^{r}) Δ*sucCD* (Tet^{r})	This study

[1] All strains have the *trpC2 pheA1* mutations, causing tryptophane and phenylalanine auxotrophies.

2.2. Media and Reagents

The strain construction steps were carried out in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar). Pre-cultures for fermentation experiments were grown in Penassay broth (Difco Antibiotic Medium 3) with 0.5% glucose. Fermentations were carried out in E medium [49]: 1.2% citric acid, 0.7% NH_4Cl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.004% $FeCl_3 \cdot 6H_2O$, 0.015% $CaCl_2 \cdot 2H_2O$, and 0.0104% $MnSO_4 \cdot H_2O$. In this medium, tryptophan and phenylalanine (both at 50 µg/mL) were always added to complement the auxotrophies of the strains. L-glutamic acid was added at the indicated concentrations, in the 0–4% *w*/*v* range. Finally, glucose or glycerol were added as main carbon sources at the 8% *w*/*v* concentration (glucose) or in the 2–8% *w*/*v* range (glycerol). The 8% glycerol concentration was chosen according to the original E medium formulation [49], and the 2% and 4% concentrations were selected as 4- and 2-fold dilutions to test lower glycerol levels. When indicated, laboratory-grade glycerol was replaced by crude glycerol from a biodiesel plant (Oxem S.p.A., Mezzana Bigli, PV, Italy). Crude glycerol was the by-product of an industrial process that used animal fats and accounted for 83% *w*/*v* of glycerol with no detectable heavy metals or methanol, based on the analyses carried out by Oxem. Crude glycerol was autoclaved and then added to the fermentation medium at the concentrations indicated in Section 3. To facilitate comparisons between crude and laboratory-grade glycerol as fermentation feedstock, the crude glycerol concentration reported for the media refers to the sole glycerol content.

Enzymes and molecular biology kits were from New England Biolabs and were used according to the manufacturer's instructions. Antibiotics were added during the cloning steps and for debugging purposes to assess the stability of engineered strains: erythromycin (Em, 50 µg/mL) was used to select PB5741 and its derivatives; tetracycline (Tet, 20 µg/mL) was used to select for *sucCD* knockout; chloramphenicol (Cat, 5 µg/mL) was used to select for *odhAB* knockout, or the *racE* overexpression; and spectinomycin (Sm, 60 µg/mL) was used to select the strains with the *degU32*^{Hy} mutation.

When required, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to fermentation medium at 1 mM final concentration to trigger the expression of the *pgs* operon; antifoam (#A5757, Sigma Aldrich, St. Louis, MI, USA) was used in bioreactor experiments by injecting a 4-fold-diluted emulsion, prepared with deionized water.

2.3. Strain Construction

The pHCMC05 expression vector [50], containing the IPTG-inducible P_{spac} promoter, was digested with *XbaI*. The *racE* gene was amplified from *B. subtilis* 168 genomic DNA with primers ForRacE-05 (5′-agcttaaggaggtgaggatcctatgttggaacaaccaataggagtc-3′) and RevRacE-05 (5′-ctgccccggggacgtcgactctatcttttaatcggttcttgcag-3′). Finally, pHCMRacE was obtained by assembling digested pHCMC05 and *racE* via Gibson Assembly [51] and amplifying the resulting plasmid using competent DH5α *Escherichia coli* cells. The pHCMRacE was then digested with *BglII* in order to remove the replication origin of *B. subtilis* to allow for chromosomal integration, obtaining the pRACE plasmid.

The PB5763 strain was constructed by transforming the pRACE plasmid into PB5741 and selecting with chloramphenicol. The PB5764 and PB5765 strains were obtained by using the genomic DNA of strain GP791 (Δ*sucC-sucD::Tet*) (Jörg Stülke's Lab, Gottingen, Germany) for PB5763 and PB5741 transformation, respectively, and selecting for tetracycline resistance. *B. subtilis* transformation was carried out using the protocol described in [52]. The correct integration of pRACE and *sucCD* deletion were confirmed via colony PCR with the primers pairs PspacFOR (5′-ttgactttatctacaaggtgtggc-3′)—PraceREV (5′-aggttttcaaccttcaaatcaggg-3′) and SucCDForCheck (5′-gattttgcatcgaactgtagac-3′)—TetRRevCheck (5′-gtcgtaaattcgattgtgaa-3′), respectively.

2.4. Fermentation

Strains from a streaked LB agar plate were grown for about 7 h in Penassay broth at 37 °C, at 250 rpm, to an optical density at 600 nm (OD_{600}) of at least 2. For shake flask experiments, cultures were diluted to an OD_{600} of 0.1 in 20 mL of E medium and incubated as above for about 74 h. For bioreactor experiments, strains were diluted to an OD_{600} of 0.1 in 600 mL of E medium and incubated in a glass thermo-jacketed 3 L vessel (Applikon, Ghaziabad, Uttar Pradesh, India), with temperature control at 37 °C, agitation at 480 rpm, and 1.25 L/min aeration. At the beginning of fermentation, 600 µL antifoam was added. When required, antifoam was added again during the experiment, to a maximum of 960 µL.

2.5. Quantification of Cell Density and γ-PGA

Culture samples were withdrawn at the indicated time points during fermentations, to measure OD_{600}. Samples were also collected to quantify γ-PGA: 500 µL culture broth was centrifuged (16,000 rpm, 4 °C, 20 min) and three volumes of cold methanol were added to the supernatant to precipitate γ-PGA. Samples were kept at −20 °C for at least 12 h. γ-PGA was recovered by centrifugation (14,000 rpm, 4 °C, 15 min), and the supernatant was discarded. A vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany) was used to dry the γ-PGA pellet, which was finally dissolved in deionized water. Polymer quantification was carried out spectrophotometrically at 216 nm [53] in quartz cuvettes (NanoPhotometer UV/Vis spectrophotometer, Implen, Schatzbogen, München, Germany). Deionized water was used as a blank, and purified γ-PGA (#G1049, Sigma Aldrich) dissolved in deionized water was used to construct a calibration curve.

For the 600 mL bioreactor experiments, γ-PGA concentration was corrected to consider water loss over time in the culture due to evaporation, by multiplying the polymer quantification by *Volume(measured, in mL)/600*.

γ-PGA titer was calculated as the maximum polymer concentration reached in the time course, expressed as g/L. γ-PGA production rate was calculated as the maximum numeric time derivative value of the polymer concentration time series, expressed as g/L/h [54].

2.6. Genome-Scale Metabolic Modeling

To further improve the production performance of γ-PGA, the enzyme-constrained model of *B. subtilis* (ec_iYO844 [32]), with the addition of the synthesis reaction reported in the genome-scale metabolic model of *B. licheniformis* (0.77 glu-D + 0.23 glu-L → γ-pga [55]), was analyzed to find the candidate genes to be overexpressed, using the concept of Flux Scanning based on Enforced Objective Flux (FSEOF [56]). The FSEOF method identifies the reaction fluxes that show an increase for increasing flux towards target production. In particular, the fluxes of selected reactions obtained from wild-type configuration and under enforced γ-PGA production flux were normalized with respect to growth rate. The fold changes were computed (Equation (1)) for each *i*-th selected reaction and the average

values through the *j*-th enforced steps and were used as final scores to support the selection of overexpression targets.

$$FSEOF\ score\ (i-th\ reaction) = mean\left(\frac{\frac{flux_{enforced_{ij}}}{OD_{enforced_{ij}}}\cdot\frac{flux_{wt_{ij}}}{OD_{wtij}}}{\frac{flux_{wt_{ij}}}{OD_{wtij}}}\right) \quad (1)$$

Flux Variability Analysis (FVA, [57]) was finally applied to assess the robustness of results. The algorithm was implemented via MathWorks MATLAB R2012a and run with the COBRA toolbox [58].

2.7. Statistical Analysis and Visualization

Differences in γ-PGA titers were evaluated between the *swrA*$^+$ *degU32*^{Hy} and $P_{hyspank}$-*pgs* strains via two-sided *t*-tests for independent samples. Two-way ANOVA was used to evaluate the effect of genetic modifications (knockouts and/or *racE* overexpression) in the *swrA*$^+$ *degU32*^{Hy} or the $P_{hyspank}$-*pgs* strains. The Dunnett method was used to perform multiple comparisons. A *p*-value (*p*) cutoff of 0.05 was adopted to evaluate statistical significance. GraphPad Prism 8.3.0 was used for statistical analysis and data visualization.

3. Results

3.1. Evaluation of γ-PGA Production from Glycerol Using Strains with Engineered Accumulation of Polymer Precursors in the swrA$^+$ *degU32*^{Hy} *Background*

We previously constructed a γ-PGA hyper-producing strain by joining the *swrA*$^+$ and *degU32*^{Hy} mutations [42], herein used as a reference strain, and its derivatives with the Δ*sucCD* and Δ*odhAB* deletions [32]. Both deletions affect the TCA cycle, promoting γ-PGA precursor accumulation by removing competing pathways. In shake flask fermentations in glucose-based medium, both deletions positively affected γ-PGA production [32]. Here, we aimed to assess whether their productivity could be maintained using glycerol as a carbon source; in addition, we explored the possibility of improving process sustainability by deriving the carbon source from glycerol-rich industrial waste.

3.1.1. Selection of Glycerol Concentration in Fermentation Media

To screen the optimal glycerol concentration for fermentation, different concentrations (2, 4, and 8% *w*/*v*) were used in the γ-PGA production medium (E medium) with 4% L-glutamic acid in flask fermentations. For all the above strains, γ-PGA titer followed a similar trend as a function of the glycerol level in the fermentation medium (Figure 2a). However, the highest γ-PGA titer did not correspond to the highest glycerol concentration. In each case, the optimal glycerol level was 4%, for which the parent and the Δ*sucCD* strains reached significantly higher polymer levels than the Δ*odhAB* strain ($p < 0.05$; ANOVA). The 2% glycerol concentration showed a slightly lower γ-PGA titer than the 4% concentration for the three strains. Finally, the 8% glycerol condition showed a drop in γ-PGA titer for all the strains compared with the other glycerol levels tested, although the parent strain reached a slightly higher polymer titer than the deleted strains. Considering the optimal glycerol concentration (4%), the parent and Δ*sucCD* strains performed similarly in terms of polymer titer. However, the cell growth performances, herein measured in terms of maximum cell density attained (Figure 2b), indicated that both the Δ*sucCD* and Δ*odhAB* strains reached a significantly lower OD_{600} than the parent strain ($p < 0.05$, ANOVA). This growth defect, more pronounced for the Δ*odhAB* strain, was probably due to the perturbation of the TCA cycle due to the two knockouts, even though no growth decrease was previously detected in E medium with glucose as the main carbon source [32].

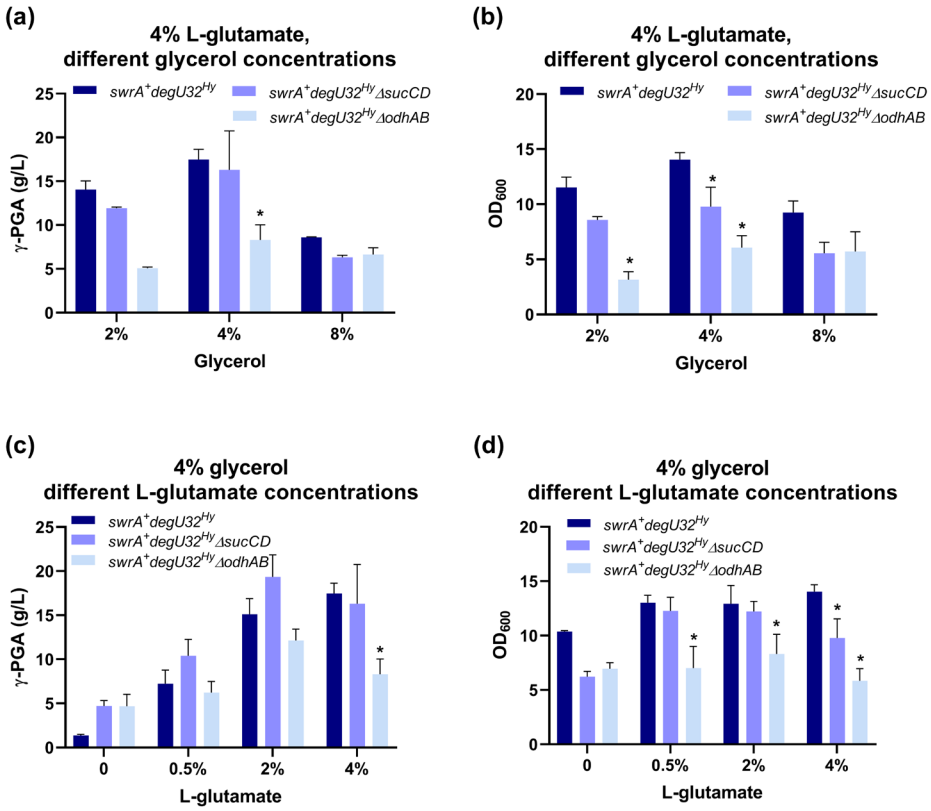

Figure 2. Characterization of the *swrA*$^+$ *degU32*^{Hy} strain (PB5383) and its two derivatives (PB5691 and PB5716) in glycerol media. (**a**) Maximum γ-PGA concentrations reached in shake flask fermentations for the indicated strains as a function of glycerol concentration. (**b**) Maximum OD_{600} reached in the experiments of panel (**a**). (**c**) Maximum γ-PGA concentrations reached in shake flask fermentations in 4% glycerol at different L-glutamate levels. (**d**) Maximum OD_{600} reached in the experiments of panel (**c**). Bars represent the mean of at least two independent replicates, and error bars indicate the standard error of the mean. For each glycerol (panels (**a**,**b**)) or glutamate (panels (**c**,**d**)) condition, asterisks indicate statistically significant differences from the *swrA*$^+$ *degU32*^{Hy} strain.

These data demonstrated that variations in the carbon source had dramatic effects on growth and γ-PGA production, resulting in non-monotonic trends; in addition, the ranking of the strains in terms of γ-PGA titer differed when fermentation was carried out in glycerol (herein tested) or glucose (previously characterized [32]). The 8% glycerol concentration was excluded from the subsequent experiments due to poor growth and low polymer production. Most of the further experiments were conducted in E medium containing 4% glycerol as optimal concentration.

3.1.2. Fermentation of Crude Glycerol

The fermentation capabilities of the reference strain (*swrA*$^+$ *degU32*^{Hy}) were tested in E medium containing 4% L-glutamic acid and 2% or 4% crude glycerol derived from a waste stock of an industrial biodiesel plant. The time course of γ-PGA production was similar in growth media containing laboratory-grade purified glycerol or crude glycerol (Figure 3). In fact, both glycerol concentrations reached the same γ-PGA titer at the end of the experiment. In the 2% glycerol condition, the profile of γ-PGA production was also highly similar between purified and crude glycerol (Figure 3a). In fermentations with 4% glycerol, only a slight delay was observed in the crude glycerol condition compared with the purified glycerol (Figure 3b). Overall, these data demonstrate that no relevant inhibitors of fermentation are present in the crude glycerol used in this work, which thus represents a convenient feedstock to replace purified glycerol.

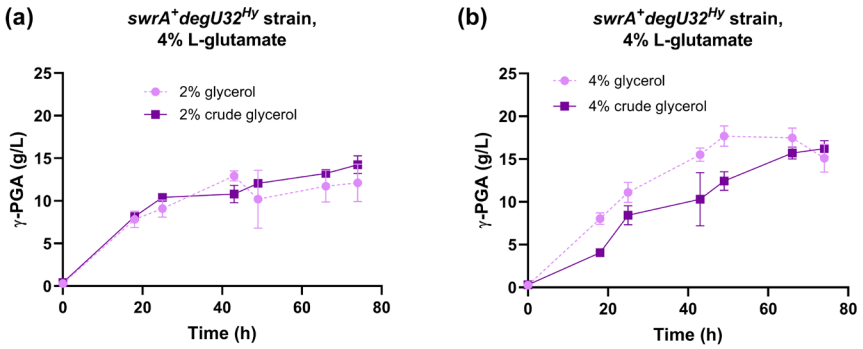

Figure 3. Characterization of the *swrA*$^{+}$ *degU32*^{Hy} strain (PB5383) in medium with crude glycerol. Time course of γ-PGA concentration in shake flask fermentations for the *swrA*$^{+}$ *degU32*^{Hy} strain, with 2% (**a**) or 4% (**b**) glycerol content. The time course obtained with laboratory-grade glycerol is shown for comparison. Data points represent the mean of at least two independent replicates, and error bars indicate the standard error of the mean.

3.1.3. Fermentations in Glycerol-Based Media with Different Glutamate Concentrations

Previous characterization of the three strains in glucose media showed that lowering the L-glutamate concentration in the fermentation medium resulted in lower γ-PGA production. This outcome was expected since glutamic acid is a precursor of γ-PGA. However, in low-glutamate conditions, the Δ*sucCD* and Δ*odhAB* strains reached higher polymer titers than the parent strain [32]. Here, we tested different glutamic acid levels in E medium with 4% glycerol as the carbon source to investigate if the knockout strains could outperform the parent strain in such precursor-poor conditions (Figure 2c).

Consistent with expectation, when no glutamic acid was added, both Δ*sucCD* and Δ*odhAB* strains showed a higher γ-PGA than the parent strain. γ-PGA titer increased, as expected, with increasing L-glutamate concentrations. However, the Δ*odhAB* strain produced significantly less polymer than the other two strains for L-glutamate concentrations greater than zero (statistically significant for the 4% glutamate concentration; $p < 0.05$, ANOVA). This behavior was not expected, but it was consistent with the low performance of the Δ*odhAB* strain grown in 4% glycerol, even in the presence of L-glutamate (Section 3.1.1). As expected, the parent strain showed a monotonic increase in γ-PGA production as a function of L-glutamate. The Δ*sucCD* strain showed a slightly better production performance than the parent strain at intermediate glutamate concentrations (although the difference was not statistically significant—$p > 0.05$; ANOVA). In other words, data suggest that the *sucCD* deletion decreased L-glutamate requirement, consistent with previous data obtained with glucose [32].

An analysis of cell growth (Figure 2d) showed that the OD_{600} in the Δ*sucCD* strain was always lower than or equal to the cell density of the parent strain (statistically significant difference for the 4% glutamate concentration; $p < 0.05$, ANOVA). The poor growth in the Δ*sucCD* strain was consistent with the data described in Section 3.1.1 and indicates that this deletion strain was able to reach γ-PGA titers equal to (4% glutamate condition) or greater than (0 to 2% glutamate) the parent strain, even with a lower amount of cells.

3.2. Engineering the γ-PGA Biosynthetic Operon Regulation Using a Synthetic Promoter

To investigate the impact of different carbon sources on γ-PGA production excluding confounding factors linked to their effects on the *pgs* promoter modulation, we replaced the native P_{pgs} promoter with a new one. Promoter replacement is an alternative strategy to guarantee the expression of the *pgs* operon genes without modifying the transcriptional regulation pathway of P_{pgs} (e.g., occurring with the *swrA*$^{+}$ *degU32*^{Hy} mutations). A reporter strain, PB5741, was recently constructed in our lab to study the regulation of the *pgs* promoter [47]. PB5741 contains an IPTG-inducible $P_{hyspank}$ promoter [59] upstream of the *pgs* operon genes, enabling user-defined tuning of operon expression and removing all the existing regulations. PB5741 has a strong IPTG-inducible mucoid phenotype, qualitatively

indicating γ-PGA production [60]. This behavior motivated us to quantitatively characterize γ-PGA production in this strain under different conditions. Conflicting results have been reported about the replacement of the *pgs* operon promoter with synthetic promoters, producing either strong or mild effects compared with other metabolic engineering strategies, and even being detrimental to γ-PGA production performance [30,33,61,62]. This lack of consensus is likely due to the different strains, promoters, and fermentation conditions used in the previous works.

3.2.1. γ-PGA Production in Glucose-Based Media

As expected, γ-PGA production in the $P_{hyspank}$-*pgs* strain was IPTG-inducible (Figure 4a): in the absence of L-glutamate and the presence of glucose and IPTG, the strain performed significantly better than the previously characterized *swrA*$^{+}$ *degU32*^{Hy} derivatives. PB5741 almost reached 13 g/L γ-PGA, while under the same condition, the *swrA*$^{+}$ *degU32*^{Hy} strain and its derivatives reached 3 to 7 g/L [32]. In the presence of L-glutamate, the $P_{hyspank}$-*pgs* strain showed a performance (39 g/L γ-PGA, Figure 4b) similar to that of the *swrA*$^{+}$ *degU32*^{Hy} strain and its derivatives (32 to 43 g/L γ-PGA [32]). Due to the strong positive effect on γ-PGA production, the $P_{hyspank}$-*pgs* strain was cultured in the presence of IPTG for the subsequent experiments.

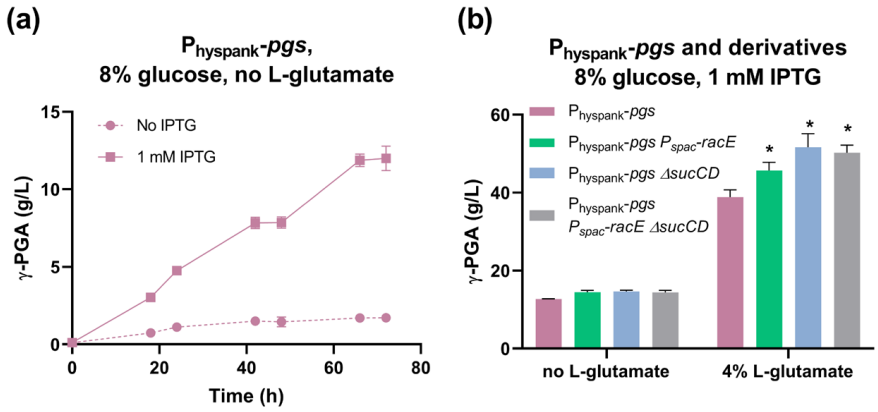

Figure 4. Characterization of the $P_{hyspank}$-*pgs* strain (PB5741) and its derivatives (PB5763, PB5765, and PB5764) in glucose media. (**a**) γ-PGA concentration over time for $P_{hyspank}$-*pgs* strain in glutamate-free media without IPTG and with induction by IPTG in shake flask fermentations. (**b**) Maximum γ-PGA titer reached for the indicated strains in shake flask fermentations, with 1 mM IPTG. Bars and data points represent the mean of at least three independent replicates, and error bars indicate the standard error of the mean. For each glutamate concentration in the bar plot, asterisks indicate statistically significant differences from the $P_{hyspank}$-*pgs* strain.

3.2.2. γ-PGA Production in Glycerol-Based Media

The $P_{hyspank}$-*pgs* strain was then tested in a medium with 4% glycerol and 4% L-glutamate and showed a maximum γ-PGA titer of 22 g/L with a production rate of 0.94 g/L/h (Figure 5a). These data represent a statistically significant 26% and 68% increase in γ-PGA titer and production rate, respectively, compared with those of the *swrA*$^{+}$ *degU32*^{Hy} strain ($p < 0.05$, *t*-test). This suggests that, under these conditions, the $P_{hyspank}$ promoter replacement strategy outperformed the *degU*$^{(Hy)}$-based strategy for γ-PGA biosynthesis in both glucose and glycerol fermentations.

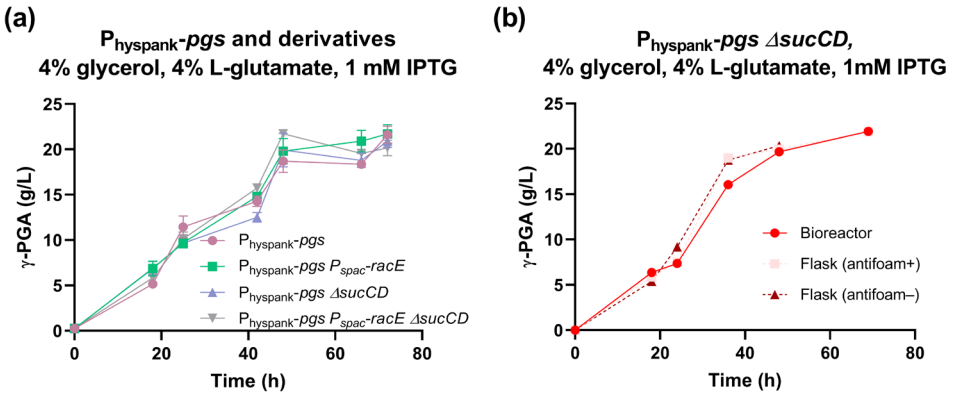

Figure 5. Characterization of the $P_{hyspank}$-*pgs* strain and its derivatives in glycerol media. (**a**) γ-PGA concentration over time for the indicated strains (PB5741, PB5763, PB5765, and PB5764) in shake flask fermentations with 4% glutamate and 1 mM IPTG. Data points represent the mean of at least three independent replicates, and error bars indicate the standard error of the mean. (**b**) γ-PGA concentration over time for the $P_{hyspank}$-*pgs* Δ*sucCD* strain (PB5765) with 4% glutamate and 1 mM IPTG, in flask or bioreactor conditions as specified in the graph. Single data points are shown from a parallel experiment, run on the same day.

3.3. Metabolic Engineering of the Inducible γ-PGA Production Strain to Remove Competing Pathways for Precursor Accumulation and Enhance Glutamate Metabolism

To remove competing pathways and enhance glutamate metabolism, new derivatives of the $P_{hyspank}$-*pgs* strain were constructed by integrating previous and new recommendations by the ec_iYO844 genome-scale metabolic model [32].

3.3.1. In Silico Identification of Overexpression Targets

New simulations were carried out to search for overexpression targets via the FSEOF method (see Section 2.6) using the ec_iYO844 model. This model was previously parametrized using omics data from glucose minimal media, for which extensive data of *B. subtilis* are available, and was used for identifying optimal gene knockouts [32]. For this reason, model recommendations may be less accurate when dealing with media containing different carbon sources. Since no extensive knowledge of *B. subtilis* physiology and proteomics (both required for model parametrization) in glycerol-based media is available, we decided to use the available model to gain insight into the recommended overexpression targets.

The *racE* gene, encoding for a glutamate racemase [63], was selected as the best target for upregulation. This gene product affects the L- to D-glutamate racemization, theoretically contributing to the supply of both forms of glutamate as precursors for γ-PGA biosynthesis. Previous experimental works focused on *racE* overexpression, including its effects on the γ-PGA stereochemistry, reported variable success rates for the improvement in polymer yield [38,40].

3.3.2. Introducing a *sucCD* Knockout and/or *racE* Overexpression in the $P_{hyspank}$-*pgs* Strain Background

Our first *racE* overexpression attempt exploited the pHCMRacE replicative plasmid, in which the *racE* gene was inserted under the control of the IPTG-inducible P_{spac} promoter. When this construct was inserted in a γ-PGA producer strain, a significant decrease in the γ-PGA titer was observed and the same decrease was detected even when inserting the pHCMC05 empty plasmid, used as a control. This behavior was probably due to the burden imposed by the presence of pHCMC05, which resulted in lower fermentation performances, as observed previously in other species engineered for bioproduction [54,64]. For this reason, we decided to integrate P_{spac}-*racE* into the chromosome of the $P_{hyspank}$-*pgs* strain, as described in Section 2.3. In addition, we chose the Δ*sucCD* mutation, described in Section 3.1, as a further candidate to try to enhance γ-PGA production in the $P_{hyspank}$-*pgs*

strain. Three strains were thus constructed in the $P_{hyspank}$-*pgs* background (P_{spac}-*racE*, Δ*sucCD*, and P_{spac}-*racE* Δ*sucCD*).

In glucose media and the absence of L-glutamate, these modifications did not affect γ-PGA production compared with $P_{hyspank}$-*pgs* (Figure 4b). In the presence of 4% L-glutamate, we observed a statistically significant increase ($p < 0.05$, ANOVA) in γ-PGA production from 39 g/L ($P_{hyspank}$-*pgs*) to 46 g/L by overexpressing *racE* (+17%) and to 52 g/L by deleting *sucCD* (+33%) (Figure 4b). The P_{spac}-*racE* Δ*sucCD* double mutant showed a γ-PGA titer of 50 g/L (+29%), very similar to the Δ*sucCD* strain, demonstrating that the two mutations did not bring additive benefits.

In glycerol media, no relevant improvement in polymer production was observed (Figure 5a), except for a slight and not statistically significant increase in γ-PGA production rate to 1.2 g/L/h in the $P_{hyspank}$-*pgs* Δ*sucCD* strain, corresponding to a 31% improvement in this index.

3.3.3. Scale-Up in Bioreactor Experiments

Fermentation in glycerol-based media was carried out in a benchtop bioreactor with agitation and aeration in a 0.6 L volume, corresponding to a 30-fold scale-up with respect to the shake flask experiments (Figure 5b). We obtained γ-PGA production profiles very similar to those obtained by flask fermentations, reaching ~22 g/L of polymer. Although no improvement over flask experiments was detected, data showed that the process could be easily scaled up.

4. Discussion

Addressing the challenges of sustainable development requires a change from the currently dominant linear economy model to a circular economy. Due to the increasing popularity of the circular economy, the growing production of organic waste, and the need to manage it, the concept of biorefinery is becoming more and more popular. In this concept, waste and by-products constitute the basis for the production of compounds with added value. One of the compounds that can be biologically produced is γ-PGA, widely used in the food, pharmaceutical, polymer, and cosmetic industries. Various *Bacillus* species can produce this compound as a metabolite, but *B. subtilis* stands out among them due to its safe characteristics. Attempts are being made to increase the efficiency of γ-PGA bioproduction using various genetic modifications. We addressed this task by using a domesticated *B. subtilis* strain, for which a compendium of physiological and genomic information is available, making it amenable to several rational metabolic engineering interventions.

In this work, we showed that purified glycerol and glycerol-rich industrial waste can be used as feedstock for γ-PGA production with engineered *B. subtilis*, reaching γ-PGA titer and synthesis rate of up to 22 g/L and 1.2 g/L/h, respectively.

Among the strain engineering interventions we tested in glycerol fermentation in shake flasks, the expression of the *pgs* operon genes by the $P_{hyspank}$ synthetic promoter led to a 26% improvement in γ-PGA titer, compared with the initial producer (*swrA*$^+$ *degU32*^{Hy} strain). Also, *sucCD* deletion led to a slight (though not statistically significant) improvement in γ-PGA titer in the presence of 0.5% and 2% L-glutamate in the *swrA*$^+$ *degU32*^{Hy} strain background, and in the γ-PGA production rate with 4% L-glutamate in the $P_{hyspank}$-*pgs* strain background. A strain bearing these two genetic modifications was analyzed in a bioreactor test, resulting in performances identical to the shake flask experiments. Overall, the tested modifications pave the way to lowering L-glutamate demand and increasing the efficiency of γ-PGA production.

The metabolic engineering interventions herein tested include novel combinations of deletion and overexpression targets that had never been tested in glycerol-based fermentation media. This is especially relevant for the integration of $P_{hyspank}$, used to express the *pgs* operon genes, which highlighted the strong impact of the *pgs* operon transcription on γ-PGA production compared with the activated original promoter (by the *swrA*$^+$ *degU32*^{Hy}

mutation). The overexpression of the *pgs* operon genes through different promoters produced conflicting results in terms of polymer production performance [28,31,58,59]. The use of different promoters in different strains and the use of different carbon sources is likely to provide non-comparable outcomes.

In this context, the data herein presented highlight differences between glucose and glycerol media in terms of strain performance. In fact, some of the benefits of genomic modifications that have been analyzed here and reported in a previous work [32] in glucose media were not detected in glycerol media (e.g., the improved γ-PGA production in *odhAB* deletion strains detected in glucose-based media). Indeed, it has already been acknowledged that glycerol fermentation in *Bacillus* strains is more challenging than glucose fermentation [24,43]. Moreover, it is worth mentioning that the genome-scale mathematical model used to identify some of the deletion and overexpression targets was parametrized using physiological, proteomic, and metabolomic data of wild-type and mutant strains grown in glucose minimal media; for this reason, it is not surprising that some metabolic engineering interventions analyzed in this work, namely *sucCD* or *odhAB* deletion and *racE* overexpression, are more effective in glucose-based media than in glycerol-based media. The set of physiological and omics data available for *B. subtilis* in glycerol media is less extensive than those in glucose media; therefore, our genome-scale model could not be used to accurately simulate the growth and the reaction fluxes occurring in fermentations with glycerol as a carbon source. These aspects stress the importance of carrying out metabolic engineering strategies tailored for each carbon source, and the need to develop more accurate computational tools.

5. Conclusions

Thanks to the novel combinations of genetic interventions herein analyzed in glycerol media, this work significantly increments our knowledge toward the exploitation of a domesticated strain of *B. subtilis* for the sustainable production of γ-PGA by model-guided metabolic engineering. Using the metabolically engineered strains obtained, we anticipate that the scale-up of fermentation in bioreactors and the replacement of purified glycerol with crude glycerol, both successfully tested in this work without a decrease in fermentation performance, will provide useful insights into the design of efficient and sustainable microbial processes for the valorization of waste feedstock. Industrial-scale γ-PGA production processes may benefit from the findings of this work, due to the successfully demonstrated increase in polymer production titer, use of a waste material as a carbon source, and scale-up fermentation under bioreactor conditions.

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