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Citation: Fox, Kevin J. and Kristala L. J. Prather et al. "Production of d-Glyceric acid from d-Galacturonate in Escherichia coli." Journal of Industrial Microbiology and Biotechnology 47, 12 (October 2020): 1075–1081 © 2020 Society for Industrial Microbiology and Biotechnology

As Published: https://doi.org/10.1007/s10295-020-02323-2

Publisher: Oxford University Press (OUP)

Persistent URL: https://hdl.handle.net/1721.1/128964

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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Cite this article as: Kevin J. Fox and Kristala L. J. Prather, Production of D-Glyceric acid from D-Galacturonate in *Escherichia coli*, Journal of Industrial Microbiology & Biotechnology https://doi.org/10.1007/s10295-020-02323-2

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Production of D-Glyceric Acid from D-Galacturonate in *Escherichia coli*

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Abstract

A microbial production platform has been developed in *Escherichia coli* to synthesize D-glyceric acid from D-galacturonate. The expression of uronate dehydrogenase (*udh*) from *Pseudomonas syringae* and galactarolactone isomerase (*gli*) from *Agrobacterium fabrum*, along with the inactivation of *garK*, encoding for glycerate kinase, enables D-glyceric acid accumulation by utilizing the endogenous expression of galactarate dehydratase (*garD*), 5-keto-4-deoxy-D-glucarate aldolase (*garL*), and 2-hydroxy-3-oxopropionate reductase (*garR*). Optimization of carbon flux through the elimination of competing metabolic pathways led to the development of a Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC* mutant strain that produced 4.8 g/l of D-glyceric acid from D-galacturonate, with an 83% molar yield. Cultivation in a minimal medium produced similar yields and demonstrated that galactose or glycerol serve as possible carbon co-feeds for industrial production. This novel platform represents an alternative for the production of D-glyceric acid, an industrially relevant chemical, that addresses current challenges in using acetic acid bacteria for its synthesis: increasing yield, enantio-purity and biological stability.

Keywords: metabolic engineering, *Escherichia coli*, D-glyceric acid

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1. Introduction

Production of commodity chemicals is currently highly-dependent on fossil fuels, a non-renewable and environmentally detrimental feedstock [1]. Finding alternative, renewable starting materials to synthesize these products allows for a more secure production outlook. Renewable resources bring new challenges to chemical production, however, including logistical challenges due to their often de-centralized production as well as the increased complexity of the material itself [2]. Food waste, which is rich in diverse carbon sources and is at a surplus in many parts of the world, is seen as a promising renewable feedstock [3-5]. Food wastes contain a diverse set of sugars naturally structured into pectin, hemicellulose or cellulose that are valuable resources after chemical or enzymatic pretreatment [6, 7]. Bioprocessing, specifically using metabolically engineered microbes such as *Escherichia coli*, is an attractive method to create value-added products from these diverse sugar feeds [8, 9]. Using *E.coli* as a host organism is attractive due to the relatively high degree of understanding of many of its cellular processes as well as the many natural sugar catabolism and transporter proteins that are present in its proteome that enable easier utilization of these feeds. The fermentation of glucose, xylose and other sugars present in food waste by engineered *E. coli* has enabled the production of many industrially relevant organic acids and alcohols such as succinic acid and 1,4-butanediol [10, 11].

Racemic glyceric acid is an industrially-relevant organic acid that has applications in medicine, polymer synthesis, and in surfactants as a base material [12]. Optically pure D-glyceric acid, however, has a large amount of untapped potential in medicine, due to its bioactivity, as well as in its material properties, as when included in solvents [13]. Currently, racemic glyceric acid and D-glyceric acid are predominately synthesized from glycerol, both biologically and chemically. For biological production, acetic acid bacteria *Gluconobacter frateurii* and *Acetobacter tropicalis* naturally produce glyceric acid at high titers from glycerol [14, 15]. These methods, however, utilize the glycerol substrate relatively poorly, resulting in yields around or below 50%. The optimization of culture conditions led to higher yields, near 60%, but these results still fall short of optimal and require an intensified culture strategy [16]. Additionally, a majority of acetic acid bacteria produce both enantiomers of glyceric acid, eliminating the opportunity that producing an enantio-pure product presents. The primary counterexample of this is an isolated strain of *Acetobacter tropicalis* that produces 99% enantiomeric excess (ee) D-glyceric acid [17].

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However, this strain has been reported to suffer from stability problems that likely reduce its industrial applicability. Chemically, glyceric acid has also been produced from glycerol at high yields using various selective oxidation reactions [18, 19]. However, these processes also result in a racemic product. Attempts to perform cell-free enzymatic reactions have resulted in the production of enantio-pure D-glyceric acid from glycerol, but these processes are limited by low conversion [20].

The limitations in current D-glyceric acid production methods, including low yield, ee and stability, can be addressed through the metabolic engineering of alternative microbial hosts. We have developed a pathway in *E. coli* for the conversion of D-galacturonate to D-glyceric acid (Figure 1). D-Galacturonate is a sugar that is a main component of pectin and is of interest as a pathway substrate due to its high content in many agriculture waste streams [21]. Additionally, the high volume of these waste streams—for example, 250×10^6 metric tons of sugar beets are produced annually worldwide—further encourages biotechnological development for their use [22]. The price of D-galacturonate is not currently comparable with waste glycerol, with the cost of the latter having decreased greatly due to the increase in biofuel manufacturing [23]. However, the potential production available for the sugar makes it still an attractive substrate for value-added product synthesis. The pathway is composed of two exogenous enzymes, uronate dehydrogenase from *Pseudomonas syringae* and galactarolactone isomerase from *Agrobacterium fabrum*, followed by three endogenous enzymes that are a part of the galactarate degradation pathway. Extensive knowledge of the sugar utilization networks in *E. coli* enables the optimized direction of carbon flux to product through genetic engineering, increasing molar yield. Additionally, the utilization of D-galacturonate as a starting material dictates that only D-glyceric acid is generated based on previous activity measurements demonstrating that GarR was only active on the D enantiomer [24]. Finally, *E. coli* is known to be a stable microbe that is commonly used in the industrial production of many chemicals.

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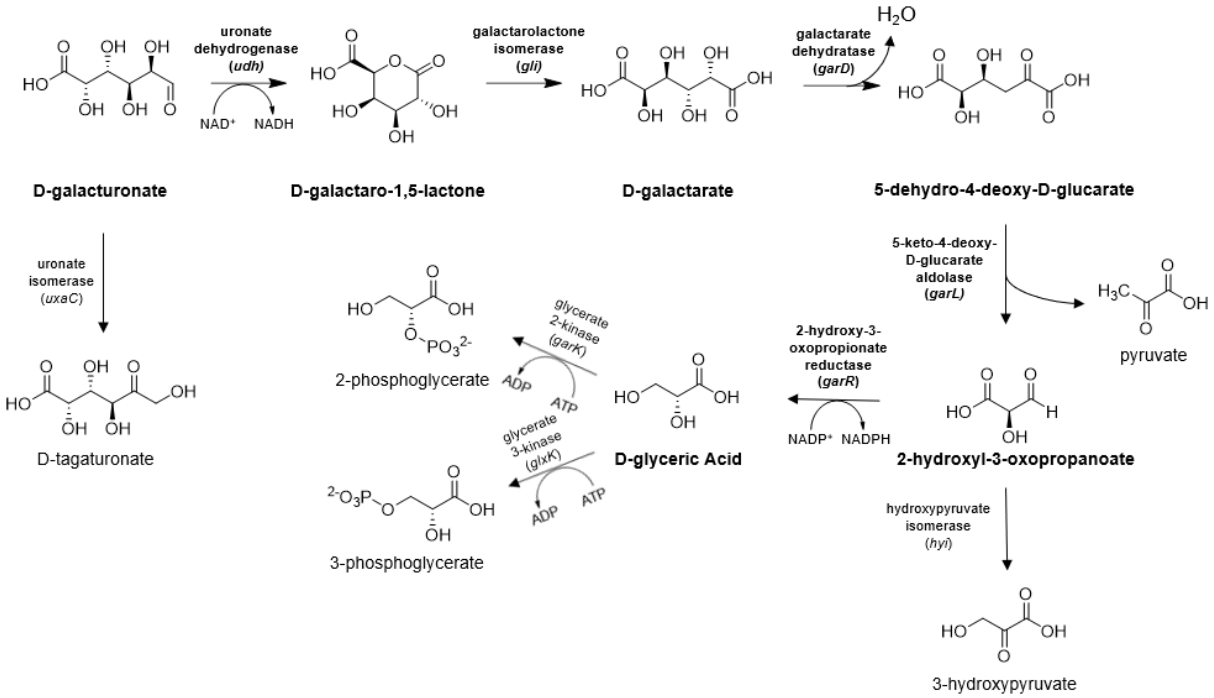

Figure 1. Designed pathway for the production of D-glyceric acid from D-galacturonate in *E. coli*. The pathway requires heterologous enzymes uronate dehydrogenase (encoded by *udh*) and galactarolactone isomerase (encoded by *gli*) and endogenous enzymes galactarate dehydratase (encoded by *garD*), 5-keto-4-deoxy-D-glucarate aldolase (encoded by *garL*), and 2-hydroxy-3-oxopropionate reductase (encoded by *garR*). Also shown are endogenous enzymes uronate isomerase (encoded by *uxaC*), hydroxypyruvate isomerase (encoded by *hyi*), glycerate 2-kinase (encoded by *garK*), and glycerate 3-kinase (encoded by *glxK*) which represent carbon flux diversions from the pathway. Bolded enzyme names and compounds represent the intended direction of flux to increase D-glyceric acid titer and yield.

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2. Materials and Methods

2.1. E. coli strain and plasmid construction

E.coli DH5α was used as a cloning strain for all plasmid manipulations. Cultures were propagated in Luria-Bertani (LB) broth (BD, Franklin Lakes, NJ) and all biological manipulations were done in accordance with standard practices [25]. Primers used in this study are included in Table S.1. The Zyppy Plasmid Miniprep kit was used for all plasmid isolations (Zymo Research, Irvine, CA) and New England Biolabs restriction enzymes were used for all digestions (Ipswich, MA). The *gli* gene, encoding for galactarolactone isomerase from *Agrobacterium fabrum*, was purchased as a gblock from Integrated DNA Technologies (Coralville, IA) (Table S.2). The pRSFDuet-1 vector with two IPTG-inducible T7 promoters preceding cloning sites was used as a backbone for the expression of these genes. A pRSFDuet vector with the *udh* gene from *Pseudomonas syringae* in the second cloning site was previously constructed in our group [26]. The kanamycin resistance gene was replaced with beta lactamase using circular polymerase extension cloning (CPEC) [27] to enable carbenicillin resistance. A Golden Gate cloning procedure [28] was then followed to insert the *gli* gene into the first cloning site using primers Gli_F and Gli_R (Table S.1).

Mutations were made in host strain MG1655(DE3), carrying the DE3 lysogen encoding the T7 polymerase. Chromosomal inactivation of *garK*, *glxK*, *hyi*, and *uxaC* was completed using the procedure as described by Datsenko and Wanner [29]. Homology region lengths of at least 100 bp, employing two rounds of nested PCR (primers in Table S.1), were used for the inactivation procedure (e.g., primer pairs dglxK_F_1 + dglxK_R_1 and dglxK_F_2 + dglxK_R_2 to inactivate gene *glxK*). Primers used to verify successful gene deletion are given in Table S.1.

2.2. Culture and analysis conditions for D-glyceric acid production

Cultures were grown in LB or MOPS minimal medium. All chemicals used for medium formulations and analytic standards were purchased from Sigma-Aldrich (St. Louis, Mo). The MOPS minimal medium was prepared from a 10x stock that contained 0.4 M MOPS, 0.04 M tricine, 0.1 mM $FeSO_4$, 95 mM NH_4Cl, 2.76 mM K_2SO_4, 0.005 mM $CaCl_2$, 3 mM $MgCl_2$, 500 mM NaCl, 0.03 µM $(NH_4)_6Mo_7O_{24}$, 4 µM H_3BO_3, 0.55 µM $CoCl_2$, 0.15 µM $CuSO_4$, 1.27 µM $MnCl_2$ and 0.17 µM $ZnSO_4$. This stock was diluted, supplemented with 1.3 mM K_2HPO_4 and

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titrated with NaOH to achieve a final pH of 7.2. For production experiments, the LB medium was supplemented with 10 g/l of D-galacturonate and the MOPS medium was supplemented with 5 g/l of D-galacturonate as well as 5 g/l of an additional carbon source as indicated in the Results section. LB cultures were inoculated at an OD_{600} of 0.05 and induced with 0.1 mM of IPTG at inoculation. Tubes containing 10 mL of culture were incubated at 30°C with agitation at 250 RPM. MOPS cultures were inoculated at an OD_{600} of 0.05. Tubes containing 10 mL of culture were incubated at 37°C with agitation at 250 RPM until exponential phase. Then, the incubation temperature was decreased to 30°C and the cultures were induced with 0.1 mM of IPTG.

For analysis, samples were taken from the cultures at 24 hour intervals. OD measurements were taken and the supernatant was analyzed by high-performance liquid chromatography (HPLC), using a 1200 Series Agilent Technologies instrument (Santa Clara, CA) with an Aminex HPX-87H Ion Exclusion Column (Bio-Rad Laboratories, Hercules, CA) and refractive index detector. The column temperature was maintained at 65°C and detector temperature was 35°C, with an isocratic mobile phase of 5 mM sulfuric acid run at 0.6 ml/min. This enabled baseline-level separation of elution peaks corresponding to each of the carbon sources tested as well as D-glyceric acid. A 22 minute method was used, with approximate elution times as follows: glucose (9.1 minutes), glycerol (13.6 minutes), galactose (9.75 minutes), arabinose (10.7 minutes), D-galacturonate (8.6 minutes), and D-glyceric acid (11 minutes).

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3. Results and Discussion

3.1. D-glyceric acid production using novel pathway and strain optimization for titer and molar yield from D-galacturonate

Our pathway for the synthesis of D-glyceric acid from D-galacturonate in *E. coli* uses two heterologous proteins, uronate dehydrogenase (Udh), encoded by *udh*, from *Pseudomonas syringae* and galactarolactone isomerase (GLI), encoded by *gli*, from *Agrobacterium fabrum*, to convert D-galacturonate to D-galactarate. The *udh* gene has been utilized previously in our group for the production of glucaric acid and therefore was known to be functionally expressed in *E.coli* [30]. Endogenous enzymes galactarate dehydratase (encoded by *garD)*, 5-keto-4-deoxy-D-glucarate aldolase (encoded by *garL*), and 2-hydroxy-3-oxopropionate reductase (encoded by *garR*) then convert D-galactarate to D-glyceric acid (Figure 1). We expressed *udh* and *gli* from a high-copy-number pRSFDuet vector. Induced expression of these genes alone does not allow for significant accumulation of D-glyceric acid in the culture (Figure 2). Additionally, the deletion of the most active glycerate kinase, glycerate 2-kinase (encoded by *garK*), does not enable detectable amounts of D-glyceric acid to accumulate without expression of the exogenous genes. However, a Δ*garK* strain with induced expression of *udh* and *gli* was able to synthesize of 3.5 g/l product with a molar yield of 60% after 48 hours.

To increase the carbon flux from D-galacturonate to D-glyceric acid, we engineered the strain to minimize loss to unwanted byproducts by eliminating competing pathways [10]. Uronate isomerase, encoded by *uxaC*, converts D-galacturonate to tagaturonate and is the first committed step of hexauronate catabolism in *E. coli*. This gene was not deleted initially since the competing enzyme (Udh) was being overexpressed. However, it was surprising that the deletion of *uxaC* led to only negligible increases in both titer and molar yield since it is a major pathway in the utilization of the feedstock for biomass. In fact, the titer at 24 hours in the Δ*uxaC* strain is actually lower than that produced by the Δ*garK* strain, suggesting that there could be competition for cellular resources at early time points. The similar titer and yield produced by this mutant strain suggests that the recombinant enzyme flux is large enough to outcompete the catabolic pathway for the D-galacturonate substrate, but competing reactions in the downstream (endogenous) portion of the pathway direct carbon flux away from the target product (Figure 1). The *uxaC* knockout was maintained in strains moving forward, however, since it was anticipated

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that as the medium complexity changed there could be larger relative loss from the upstream portion of the pathway. Hydroxypyruvate isomerase, encoded by *hyi*, competes with product formation by converting 2-hydroxyl-3-oxopropaoate to 3-hydroxypyruvate [31]. Deletion of *hyi* increased yield in comparison to the Δ*garK*Δ*uxaC* strain (Figure 2, bars 4 and 5). Another glyceric acid kinase, *glxK*, is present in the genome of *E. coli* in addition to *garK*. These two proteins differ based on their thermolability, pH dependence and substrate binding affinity [32]. The mutation of both kinases increased the production of D-glyceric acid, with the more active GarK having a larger effect. The best productivity was observed by the Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC* and Δ*garK*Δ*glxK*Δ*uxaC* mutant strains, with values of 0.101 ± 0.002 g/l/hr and 0.098 ± 0.001 g/l/hr, respectively (Table 1). This suggests that while adding the Δ*hyi* mutation to the Δ*garK*Δ*uxaC* strain increased both titer and yield, its importance is highly diminished when a Δ*glxK* mutation is also present. The MG1655(DE3)Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC* mutant on average produced the most product, 4.8 g/l of D-glyceric acid, with a molar yield of 83%.

The molar yields of D-glyceric acid obtained in this production system are higher than those currently reported from the fermentation of glycerol by acetic acid bacteria [12]. Additionally, using this production platform allows for only D-glyceric acid to be produced, resulting in complete enantio-purity.

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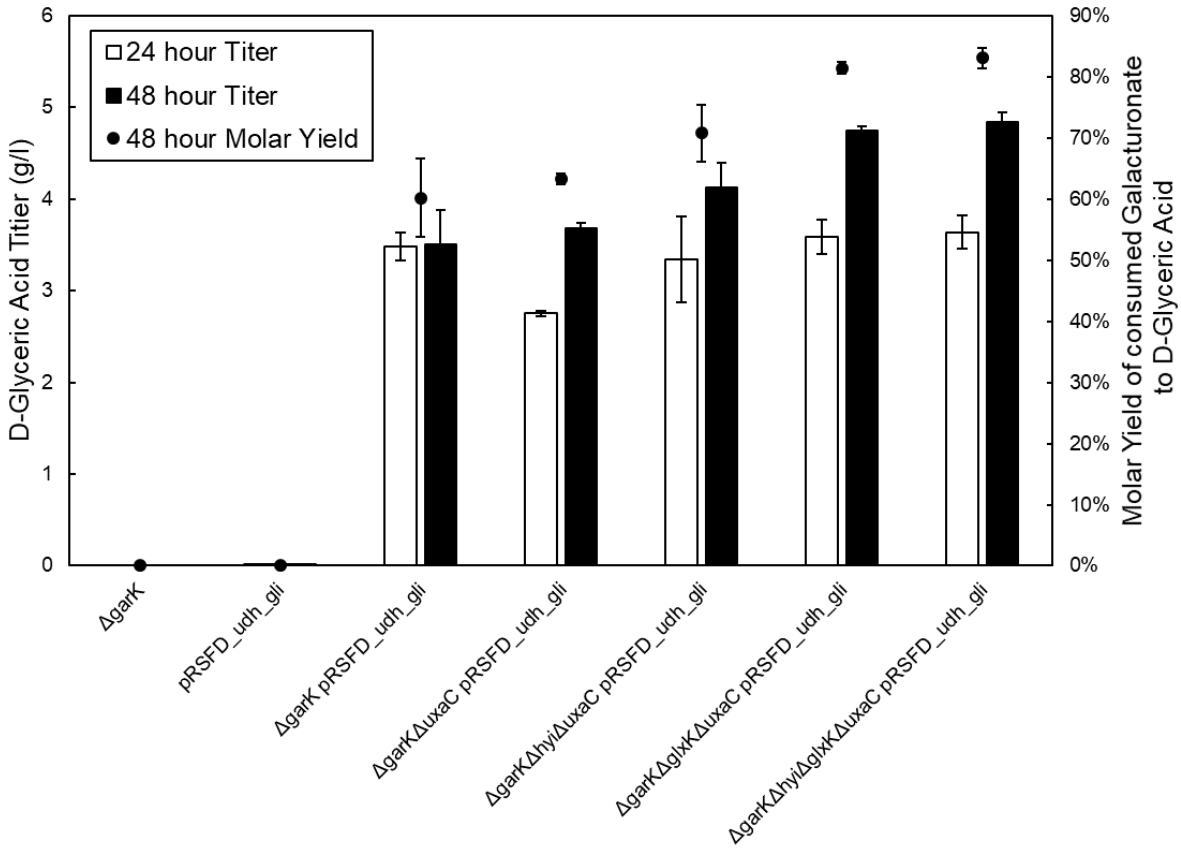

Figure 2. Production of D-glyceric acid by different MG1655(DE3) mutant strains. Cultures were grown in LB medium supplemented with 10 g/l of D-galacturonate and 0.1 mM IPTG at 30°C with a shaking rate of 250 RPM. Error bars represent standard deviation of biological triplicates. Molar yield is defined as mol D-glyceric acid produced per mol D-galacturonate consumed in the culture.

Table 1. Productivity of D-glyceric acid from *E. coli* strains tested. Cultures were grown in LB medium supplemented with 10 g/l of D-galacturonate and induced with 0.1 mM IPTG.

Strain	**D-Glyceric Acid Productivity (g/l/hr)**
Δ*garK*	0
pRSF_*udh_gli*	0.000113 ± 0.000003
Δ*garK* (pRSFD_*udh_gli*)	0.073 ± 0.008
Δ*garK*Δ*uxaC* (pRSFD_*udh_gli*)	0.077 ± 0.001
Δ*garK*Δ*glxK*Δ*uxaC* (pRSF_*udh_gli*)	0.098 ± 0.001
Δ*garK*Δ*hyi*Δ*uxaC* (pRSF_*udh_gli*)	0.086 ± 0.006
Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC* (pRSF_*udh_gli*)	0.101 ± 0.002

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3.2. Carbon source co-feed analysis

Production in minimal medium is preferred for commercial purposes as it lessens purification burden and reduces fermentation inconsistencies caused by rich medium. To transition the production into a minimal medium, it was anticipated that the feeding of an additional carbon source, other than D-galacturonate, would be necessary for robust growth due to the modification of the hexuronate utilization pathway in the optimal production strain (Δ*uxaC*). Secondary sugars commonly found in pectin, such as galactose and arabinose, were tested since they would likely be found in natural mixtures with D-galacturonate [21]. In addition, common co-feeds such as glucose and glycerol were tested. The MG1655(DE3)Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC* strain that gave the optimal results in LB medium was used in this experiment. The combination of the high-copy-number vector with induction at inoculation produced a long lag period with delayed production if the same procedure used for the LB medium was used for the MOPS minimal medium. Therefore, we chose to first culture the cells at 37°C until exponential growth was observed, then induce the expression of *udh* and *gli* and reduce the incubation temperature to 30°C.

All combinations of secondary sugars produced some amount of D-glyceric acid, with varying success, and all maintained approximately the same molar yield that was observed in the strain optimization study (Figure 3). A pure feed of D-galacturonate was able to produce product since the reaction catalyzed by GarL produces a mol of pyruvate for each mol of D-galacturonate consumed. However, this flux appeared to be too little to enable robust growth and an extended lag period in comparison to the other conditions was observed (Figure S.1). Galactose and arabinose, both components of pectin along with D-galacturonate, showed differing success when used as a secondary sugar. Feeding galactose led to high titers of D-glyceric acid while adding arabinose was observed to be detrimental to production. This could be due to the different utilization networks for each of these substrates with galactose entering glycolysis from the Leloir Pathway and arabinose using the non-oxidative section of the pentose phosphate pathway (PPP). By entering in the non-oxidative part of the PPP, arabinose skips crucial NADPH production which is a necessary co-factor for GarR and could explain the lower D-glyceric acid titer with this co-feed. Unsurprisingly, the glucose co-feed produced the poorest results when used as the co-substrate with D-galacturonate. This is likely due to both inducer exclusion and

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transcriptional regulation of the galacturonate transporter gene *exuT*, effects brought on by carbon catabolite repression that prevent the usage of some sugars when co-fed with glucose [33]. A glycerol co-feed produced yields that were similar to those seen in LB medium. However, secondary carbon source utilization was lowest with glycerol (41% usage) compared to complete utilization in all other co-feeds tested (Table S.3). In all feeding strategies, the molar yield remained high (often higher than in the LB medium case) showing that the co-feeding strategy did not activate other pathways that would reduce efficiency. This system therefore enables a majority of the D-galacturonate fed to be directed toward product synthesis and all of the additional carbon source to be utilized for growth, similar to the parallel metabolic pathway engineering approach recently described [34]. Further analysis into the effect of arabinose on the transport of D-galacturonate and the usage of it in the designed pathway is necessary to enable this feeding strategy. Additionally, application of a carbon catabolite repression relaxation methods would be beneficial for more complete usage of the primary contents of pectin.

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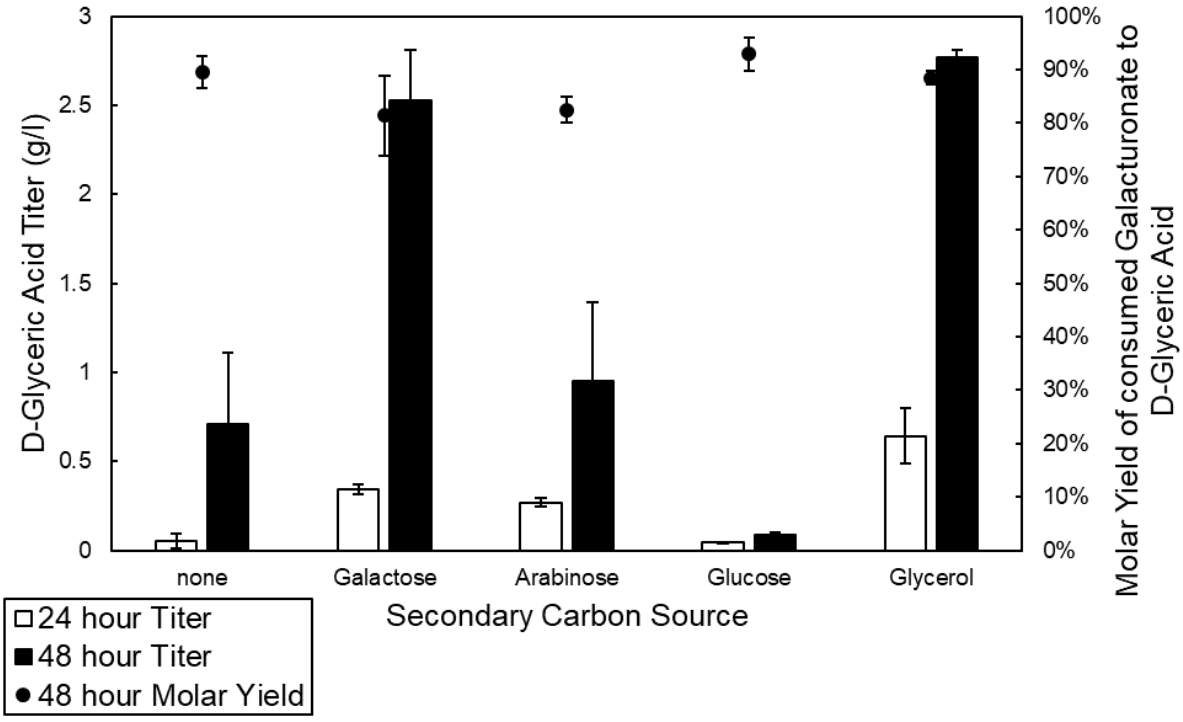

Figure 3. Production of D-glyceric acid from MG1655(DE3)Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC*(pRSFD_*udh_gli*) with various secondary sugar co-feeds. Cultures were grown in MOPS minimal medium supplemented with 5 g/l of D-galacturonate and 5 g/l of the listed secondary sugar. Cultures were grown at 37°C until exponential phase and then shifted to 30°C and induced with 0.1 mM IPTG. Error bars represent standard deviation of biological triplicates. Molar yield is defined as mol D-glyceric acid produced per mol D-galacturonate consumed in the culture.

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Conclusions

We have developed a partially synthetic pathway for the production of D-glyceric acid from D-galacturonate in *E. coli*. This pathway combines a heterologous upstream module consisting of enzymes Udh and GLI with a native downstream module consisting of GarD, GarL, and GarR. Through strain engineering we developed a MG1655(DE3)Δ*garK*Δ*hyi*Δ*glxK*Δ*uxaC*(pRSFD_*udh_gli*) production system that enabled 4.8 g/l of D-glyceric acid titer with a molar yield of 83%. While this molar yield is high, half of the substrate mass is lost as pyruvate in the pathway. Therefore, the yield results in this study compare favorably with those previously reported with the added benefit of an enantiomerically-

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pure D-glyceric acid product and a novel, renewable substrate. While we were unable to verify the enantiopurity of the product we generated, the galactarate dehydratase (GarD) homologue employed was previously demonstrated to exhibit stereospecificity for the desired enantiomer [24]. We have every reason to believe that utilization of this enzyme results in the synthesis of enantiopure material. Production in minimal medium was also achieved with varied success depending on the secondary sugar that was co-fed, with galactose and glycerol enabling the best results.

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Acknowledgments

This research was supported by the Institute for Collaborative Biotechnologies through grant W911NF-09-0001 from the U.S. Army Research Office. The content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

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