Untargeted metabolomics analysis of *Ralstonia eutropha* during plant oil cultivations reveals the presence of a fucose salvage pathway

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Process engineering of biotechnological productions can benefit greatly from comprehensive analysis of microbial physiology and metabolism. *Ralstonia eutropha* (syn. *Cupriavidus necator*) is one of the best studied organisms for the synthesis of biodegradable polyhydroxyalkanoate (PHA). A comprehensive metabolomic study during bioreactor cultivations with the wild-type (H16) and an engineered (Re2058/pCB113) *R. eutropha* strain for short- and or medium-chain-length PHA synthesis has been carried out. PHA production from plant oil was triggered through nitrogen limitation. Sample quenching allowed to conserve the metabolic states of the cells for subsequent untargeted metabolomic analysis, which consisted of GC–MS and LC–MS analysis. Multivariate data analysis resulted in identification of significant changes in concentrations of oxidative stress-related metabolites and a subsequent accumulation of antioxidative compounds. Moreover, metabolites involved in the de novo synthesis of GDP-L-fucose as well as the fucose salvage pathway were identified. The related formation of fucose-containing exopolysaccharides potentially supports the emulsion-based growth of *R. eutropha* on plant oils.

*Ralstonia eutropha* (also known as *Cupriavidus necator*) is one of the most studied organisms for polyhydroxyalkanoate (PHA) homeostasis. PHAs have been shown to be biodegradable in soil and aqueous environments, which makes them promising “green” alternatives to conventional plastic1–3. After the genome of *R. eutropha* strain H16 was completely sequenced4, multiple “big data” studies were carried out, ranging from transcriptomics5–9 and proteomics9–12 to metabolomics13–15. In general, there are two main types of metabolomic studies: targeted metabolomics, where only specific, known metabolites are quantified and untargeted metabolomics, where compounds that were hitherto unknown or unidentified in an organism can be identified. Untargeted metabolic profiling has the potential for identifying novel pathways or biomarkers16–19. For example, Fukui et al. performed metabolomic analysis on *R. eutropha* using labeled glucose and observed the presence of ribulose 1,5-bisphosphate, suggesting that the Calvin-Benson-Bassham cycle is active in *R. eutropha* even during heterotrophic growth13. Alagesan et al. confirmed this by examining metabolic flux of *R. eutropha* on fructose and glycerol as carbon sources and have shown that the Calvin-Benson-Bassham cycle is active under heterotrophic conditions15.

The wild-type strain *R. eutropha* H16 synthesizes polymers containing solely short chain length (scl) monomers (i.e., monomers with less than five carbon atoms). To synthesize a more flexible thermoplastic polymer, many strains have been engineered to integrate medium chain length (mcl, 5 < C < 15) monomers into the polymers. Particularly, strain construction efforts of several research groups have focused on the production of poly(hydroxybutyrate-co-hydroxyhexanoate) [P(HB-co-HHx)]20–22. This kind of copolymer is more flexible, tougher, less crystalline and has lower melting temperatures compared to scl-PHA, which facilitates a wide range of applications23. A strain that has been studied extensively for the production of P(HB-co-HHx) from oleaginous feedstocks is the engineered *R. eutropha* strain Re2058/pCB11320,29–35. *R. eutropha* possesses the metabolic capabilities to metabolize oleaginous feedstocks, such as refined plant oils like palm oil (PO), soybean oil or canola oil, as well as waste oils and animal fats. These feedstocks have proven to be excellent substrates for efficient PHA production21,23,32,36–38. This effective production is facilitated in *R. eutropha* cultures by the secretion of lipases.
which mediate the hydrolyzation of the triacylglycerols into free fatty acids, monoacylglycerols, diacylglycerols and glycerol and consequently allow the formation of a natural emulsion 39.

In this study, the metabolic characteristics of *R. eutropha* H16, a scl-PHA producer, and Re2058/pCB113, a *scl-co-mcl*-PHA producer, were examined during bioreactor cultivations under growth and nitrogen limitation (PHA synthesis) conditions using plant oil as the main carbon source. Both strains were evaluated by non-targeted metabolite profiling. Since the two strains produce different PHA polymer products, it was assumed that the metabolite profiles of the strains would exhibit significant differences, which were observed in this study. In addition, the metabolite profiling yielded the identification of the de novo and salvage pathway synthesis of GDP-l-fucose, a component of exopolysaccharide/lipopolysaccharide (EPS/LPS), as well as oxidative stress-related metabolite changes.

**Results**

In this study, *R. eutropha* H16 and the engineered strain *R. eutropha* Re2058/pCB113 were grown in biological triplicate bioreactor cultivations to perform non-targeted metabolism analysis under controlled conditions for a further elucidation of the metabolic activities during growth and PHA formation under nitrogen limitation on plant oils.

**Bioreactor cultivations.** The cultivation samples mentioned above were analyzed by gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS) for non-targeted metabolite profiling in a range of 50–1700 g mol⁻¹. In total, 1,054 substances were identified and annotated (Fig. S1). The first two principal components (PCs) of the *R. eutropha* H16 samples can only be differentiated between wild-type and engineered strain. The substances found in the samples of each cultivation were analyzed by principal component analysis (PCA), which is an unsupervised multivariate data analysis method. After the projection of the high-dimensional data into the 2D space by PCA, the samples were grouped by their sampling time point (Fig. S1). The first two principal components (PCs) of the *R. eutropha* H16 samples depict 54.7% of the variance in the sample set (Fig. 1d). ANOVA analysis showed that 88 metabolites change significantly (Fig. 1e; Table S3). The clear separation of the metabolic profiles of the samples before and after nitrogen limitation for both strains led to the conclusion to investigate the two phases further by clustering the respective samples of each phase.

After clustering all samples into the corresponding nitrogen availability phase, the dataset was further analyzed (Fig. 1f). The first two PCs explain 45.2% of the variance and allow a good separation of the classes along both PCs. Using partial least square discriminant analysis (PLS-DA) the classes are clearly separated, but only 656 of them could be annotated. Among the metabolites identified, 87 were shown to be di-, tri- and tetrapeptides. The substances found in the samples of each cultivation were analyzed by principal component analysis (PCA), which is an unsupervised multivariate data analysis method. After the projection of the high-dimensional data into the 2D space by PCA, the samples were grouped by their sampling time point (Fig. S1). The first two principal components (PCs) of the *R. eutropha* H16 samples depict 54.7% of the variance in the sample set (Fig. 1d). ANOVA analysis showed that 88 metabolites change significantly (Fig. 1e; Table S3). The clear separation of the metabolic profiles of the samples before and after nitrogen limitation for both strains led to the conclusion to investigate the two phases further by clustering the respective samples of each phase.

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**Tricarboxylic acid cycle and glycerol pathway analysis.** The tricarboxylic acid (TCA) cycle is the central pathway for aerobic growth in all organisms. During growth of *R. eutropha* on plant oil, TCA cycle precursors are supplied from the β-oxidation pathway in form of acetyl-CoA or through the catabolism of glycerol. The glycerol levels in both strains decreased during the cultivation to a minimum observed at the last time point (Fig. 2). In contrast, the levels of glycerol-3-phosphate, which is a direct product of glycerol phosphorylation did
not decrease. Interestingly, the levels of acetyl-CoA in both strains clearly decreased after nitrogen limitation. Within the TCA cycle, overall higher levels of succinate and fumarate were detected, whereas citrate had similar levels in both strains not considering the 12 h timepoint of the wild-type strain. A decrease of cis-aconitate throughout the whole cultivation was detected. The 2-oxoglutarate concentration in both strains was the lowest at the first time point, the highest at the second time point and then decreased. Even though the trend was the same, higher 2-oxoglutarate levels were observed in the wild-type strain.

**Polyhydroxyalkanoate pathway analysis.** Our main purpose of cultivating *R. eutropha* is the production of PHA bioplastic. Whereas the wild-type strain can only synthesize scl-PHA, the engineered strain is able to produce the copolymer P(HB-co-HHx). A decrease of acetyl-CoA was observed for both strains after nitrogen limitation, whereas the acetoacetyl-CoA levels did not show a significant trend in either strain (Fig. 3). The metabolic profiling method did not differentiate between (R)- and (S)-hydroxyacyl-CoAs, which are either...
methionine, which indicates oxidative stress. High levels of methionine sulfoxide were detected in the beginning of both cultivations, which then significantly decreased towards the end, whereas a significant increase of the amino acid derivat 5-methyltetrahydropteroyltri-L-glutamate was detected. The antioxidants δ-tocopherol, β-tocopherol, octyl gallate significantly increased, but other substances with antioxidative effects like epigallotechin sulfate and lipoic acid did not show any trends. Additionally, D-sedoheptulose 7-phosphate did not exhibit any noticeable trends throughout the cultivations similar to methionine sulfoxide. In contrast, cysteine glutathione disulfide, which is also formed upon oxidative stress, did not show any significant trends.

**Discussion**

Fucose is a 6-deoxyhexose, which is found in LPS or EPS. Fucose-containing LPS or EPS are synthesized from guanosine diphosphate fucose (GDP-l-fucose), which can be synthesized via two pathways: The de novo pathway produces GDP-l-fucose from GDP-mannose or the salvage pathway, where GDP-l-fucose is synthesized from GDP-4-oxo-6-deoxy-mannose (EC 1.1.1.271) nor the enzymes for the GDP-L-fucose synthesis pathways were identified: d-mannose and l-fucose 1-phosphate (Fig. 4). In this context, neither the enzymes for the de novo synthesis from GDP-4-oxo-6-deoxy-mannose (EC 1.1.1.271) nor the enzymes for the GDP-L-fucose salvage pathway (EC 2.7.7.30, EC 2.7.1.52) are known in prokaryotes. During analysis of metabolic changes induced by nitrogen depletion, l-fucose was identified as a significantly increasing metabolite in both strains (Table 1), whereas GDP-l-fucose and GDP-d-mannose were identified as significantly decreasing metabolites (Table 1). Additionally, further metabolites from both GDP-L-fucose synthesis pathways were identified: p-mannose and l-fucose 1-phosphate (Fig. 4). In this context, neither the enzymes for the de novo synthesis from GDP-4-oxo-6-deoxy-mannose (EC 1.1.1.271) nor the enzymes for the GDP-L-fucose salvage pathway (EC 2.7.7.30, EC 2.7.1.52) are known in R. eutropha and also not in other Ralstonia or Cupriavidus species (data not shown).

**Antioxidants and antioxidant-related metabolites.** Methionine sulfoxide is the oxidized form of methionine, which indicates oxidative stress. High levels of methionine sulfoxide were detected in the beginning of both cultivations, which then significantly decreased towards the end, whereas a significant increase of different antioxidants was detected (Fig. 5, Table S2, Table S3). The oxidized form of glutathione decreased in concentration throughout the cultivations similar to methionine sulfoxide. In contrast, cysteine glutathione disulfide, which is also formed upon oxidative stress, did not exhibit any noticeable trends throughout the cultivations, but ethenodeoxyadenosine, a product derived from oxidative stress, increased and a significant increase of the amino acid derivat 5-methyltetrahydropteroyltri-L-glutamate was detected. The antioxidants δ-tocopherol, β-tocopherol, octyl gallate significantly increased, but other substances with antioxidative effects like epigallotechin sulfate and lipoic acid did not show any trends. Additionally, D-sedoheptulose 7-phosphate and thiamine pyrophosphate, which both play a role in stress response mechanisms, were identified, but did not show any significant trends.
Figure 2. Metabolites identified in the TCA and glycerol degradation pathways during batch cultivations of *R. eutropha* H16 and *R. eutropha* Re2058/pCB113 with nitrogen limitation occurring after 24 h and 30 h, respectively. Identified metabolites are highlighted by green boxes, whereas metabolites that were not identified in this analysis are surrounded by red boxes. Concentration profiles are shown as boxplot diagrams (n = 3) showing the median (solid line), the standard error (box), the mean value (yellow square) and the minimum and maximum values (whiskers). Different colors are indicating different sample time points.
production of novel engineered strains with an increased fucose-EPS production for an enhanced emulsion formation. This could lead to strains with a reduced lag phase on plant oil or hard to emulsify solid waste animal fats, as it was shown for cultures with a chemical pre-emulsified PO or strains overexpressing an extracellular lipase. The fucose-related metabolites in our study were identified by the annotation and subsequent statistical analysis of 656 metabolites, which are at least sixfold more annotated metabolites than in other comprehensive metabolomic studies with \textit{R. eutropha}. Genes for synthesis of GDP-l-fucose via both pathways have not been discovered or characterized in \textit{R. eutropha} so far. The decrease of GDP-d-mannose and GDP-l-fucose throughout our cultivations suggests, that the de novo synthesis pathway is highly active in the beginning to build up EPS (Fig. 4). In contrast, the significant increase of l-fucose throughout the cultivations indicates, that the salvage pathway is more active during the later phase of the cultivation. The fucose salvage pathway was considered to be only present in eukaryotes until a bifunctional l-fucokinase/fucose-1-phosphate guanylyltransferase enzyme in the mammalian gut commensal bacterium \textit{B. fragilis} was identified. A BLAST search for a similar enzyme in \textit{R. eutropha} did not result in a positive match (data not shown).

High levels of methionine sulfoxide were detected in the beginning of the cultivations and these levels significantly decreased towards later time points (Fig. 5). Methionine sulfoxide is a product of methionine oxidation due to the presence of reactive oxidative intermediates, which are typically present in an aerobic bioprocess. Although \textit{R. eutropha} cells, which are exposed to oxidative stress, typically produce protective proteins, DNA...
damage occurs\(^{10,11}\). The increase in ethenodeoxyadenosine in our cultures can also be interpreted as an indication of such DNA damage (Fig. 5). Additionally, oxidative stress in *R. eutropha* is counteracted by antioxidative molecules\(^{52}\). In this study, we detected a significant increase of octyl gallate and two tocopherols throughout the cultivations (Fig. 5). The latter are hydrophobic antioxidants, which are present in high concentrations in PO\(^{53}\). It is most likely that *R. eutropha* is able to absorb these compounds, from the PO containing media, to cope with oxidative stress.

The analysis of metabolites in the PHA homeostasis pathway revealed for both strains that the main precursor metabolite acetyl-CoA is available at higher levels prior to nitrogen limitation and the concentrations drastically decrease after the onset of nitrogen limitation (Fig. 3). It is known that \(\beta\)-ketothiolases (*phaAs*) are highly selective for acetyl-CoA and an enhanced expression of the *phaA* gene can yield in enhanced PHA accumulation\(^{54-56}\). However, *phaA* was found to be constitutively expressed in *R. eutropha* H16, which would not explain the lower levels of acetyl-CoA after the nitrogen depletion\(^{8,57}\). Multiple PHA mobilizing enzymes, namely PHA depolymerases PhaZ1–7 and oligomer hydrolases PhaY1–2, are responsible for the degradation of the intracellular PHA and finally supplying acetyl-CoA\(^{58-60}\). The high activity of the PHA mobilizing enzymes under growth conditions and downregulation under nutrient depleted conditions was previously shown, and can help to explain the acetyl-CoA pool size during growth in our experiments\(^{57}\). Another interesting finding about PHA metabolism is the absence of any HHx-CoA pools in the engineered strain Re2058/pCB113, but it is known that very high contents of HHx in the PHA are present during the growth phase\(^{20,32}\). Together with the fact that overall the HB-CoA levels are lower in the engineered strain compared to the wild-type strain, a very high activity of the PhaC\(_a\) can be assumed. A further engineering of the pathways supplying the acyl-CoA precursors for the PhaC\(_a\) could therefore potentially enhance the PHA productivity. The high polymerase activity could also explain why the engineered strain produces PHA with a significantly lower average molecular weight compared to the wild-type strain or a similar engineered strain harboring a low active PHA synthase, such as PhaCBP-M-CPF\(^{27}\). The comparison of the two strains resulted in the detection of a shortage of many amino acids in the engineered strain (Table S6). Due to the constant expression of the complete PHA operon, which is located on the plasmid pCB113, the engineered strain produces quantitatively more enzymes compared to the wild-type strain, which results in a PHA accumulation in the growth phase and could be the reason for the overall lower amino acid levels in the engineered strain.

In conclusion, our comprehensive analysis of data from untargeted metabolomics of *R. eutropha* cultivations facilitated the identification of the fucose salvage pathway, which was previously identified in just one other
bacterium. Even though R. eutropha is a well characterized organism, the presented approach allowed to add more understanding to the complexity of the microbial metabolic network. In detail, the findings presented here elucidate a potential additional emulsification mechanism for the efficient growth on oleaginous feedstocks. It can also be emphasized that diversity of fucose metabolism pathways in bacteria is poorly understood, suggesting that unknown enzymes involved in the fucose salvage pathway can be identified in future studies.

Experimental section

Bacterial strains. The experiments were performed with the R. eutropha wild-type strain H16 (DSM-428, Leibniz Institute DSMZ, Germany) or the engineered strain Re2058/pCB113 20. In contrast to the wild-type strain, the engineered strain is able to synthesize P(HB-co-HHx) when grown on fatty acid containing substrates.

Growth media and preculture conditions. The cultures were grown either in dextrose-free tryptic soy broth (TSB) media (Becton Dickinson, USA) or in minimal salt media (MSM) as described previously 55. All media contained 10 µg mL⁻¹ gentamycin sulfate. TSB media for R. eutropha Re2058/pCB113 cultivation additionally contained 200 µg mL⁻¹ kanamycin sulfate for plasmid stability. MSM used in this work contained PO (Arzeite de Dendê, Cepêra, Brasil) as the main carbon source and NH₄Cl as the sole nitrogen source. All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Carl Roth (Karlsruhe, Germany) unless stated otherwise.

Figure 5. Profiles of metabolites related to oxidative stress during batch cultivations of R. eutropha H16 and Re2058/pCB113, nitrogen limitation occurring after 24 h and 30 h, respectively. The following metabolites were identified: methionine sulfoxide (a), oxidized glutathione (b), β-tocopherol (c), δ-tocopherol (d), octyl galate (e), ethenodeoxyadenosine (f), 5-methyltetrahydropteroyltri-L-glutamate (g), epigallocatechin sulfate (h), lipoic acid (i), d-sedoheptulose 7-phosphate (j), cysteine glutathione disulfide (k), thiamine pyrophosphate (l). Concentration profiles are shown as boxplot diagrams (n = 3) showing the median (solid line), the standard error (box), the mean value (yellow square) and the minimum and maximum values (whiskers). Different colors are indicating different sample time points.
Initially, cultures were grown overnight in TSB media inoculated from a single colony until an optical density (OD<sub>600</sub>) ≥ 5. The overnight cultures were harvested by centrifugation, resuspended in 0.85% (w v<sup>−1</sup>) NaCl and used to inoculate the second preculture to an initial OD<sub>600</sub> of 0.05. A second preculture was grown in 50 mL MSM containing 0.4% (w v<sup>−1</sup>) NH₄Cl and 1% PO (w v<sup>−1</sup>) for 24 h in baffled glass flasks (250 mL, DURAN, Germany) at 200 rpm and 30 °C.

**Bioreactor cultivations.** Bioreactor cultivations were performed in 6.6-L bioreactors equipped with two six-blade Rushton impellers (BIOSTAT Aplus, Sartorius AG, Germany). The experiments were operated as batch cultivations with an initial working volume of 2.5 L MSM containing 3% (w v<sup>−1</sup>) PO and 0.4% (w v<sup>−1</sup>) NH₄Cl. The temperature was maintained at 30 °C and the pH was controlled at 6.8 ± 0.1 by addition of 2 M NaOH and 1 M H₃PO₄. The aeration rate was set to 0.5vvm and the dissolved oxygen concentration was kept above 40% through an automated stirrer cascade ranging from 300 to 1350 rpm. Two pairs of cable ties were mounted in the head space at the stirrer shift to mechanically break the foam.

**Analytical methods.** The CDW was determined by harvesting 5–15 mL of the culture suspension in pre-weighed test tubes by centrifugation (4000×g, 10–20 min, 4 °C). The pellets were washed with a mixture of 2 mL cold n-hexane and 5 mL cold water to remove residual lipids. The washed pellet was resuspended in 1–2 mL cold water, frozen at −80 °C, dried for 24 h by lyophilization, and the CDW was determined by weighing the test tubes. The PHA content in the dried cells was determined using a methanolysis protocol described previously. The nitrogen content was determined by clarifying the supernatant through a 0.2 µm cellulose acetate filter and subsequently using an ammonium test kit (Spectroquant, Merck KGaA, Germany) according to the manufacturer’s instructions.

**Metabolomic and statistical analysis.** To conserve the metabolic state of the cells in each sample, all preparations were carried out as quickly as possible, the equipment was kept cool, and work was carried out over a dry ice/ethanol mixture. The quenching solution consisted of 60% (v v<sup>−1</sup>) methanol in water (Milli-Q). Prior to the sampling procedure, 15-mL tubes, the quenching solution and a centrifuge rotor were pre-cooled to −40 °C. A 2 mL aliquot of the culture was removed from the bioreactor and added immediately to a tube containing 10 mL of the quenching solution. After mixing, the samples were centrifuged (15000×g, 5 min), the supernatant discarded, and the sample was washed with 10 mL quenching solution (−40 °C). The supernatant was discarded, the pellet was immediately frozen in liquid nitrogen and stored at −80 °C until shipping. The samples were shipped on dry ice to Metabolon GmbH (Munich, Germany), which carried out the metabolomic analysis and annotation. The LC separation was performed using hydrophilic interaction chromatography with a ZIC-HILIC column (3.5 µm, 200 Å, Merck Sequant, Umeå, Sweden), operated by an Agilent 1290 UPLC system (Agilent, Santa Clara, CA, USA). The LC mobile phase was a linear gradient from 90% to 70% acetonitrile over 15 min, followed by a linear gradient from 70 to 10% acetonitrile over 1 min, 3 min wash with 10% and 3 min reequilibration with 90% acetonitrile. The flow rate was 400 µL min<sup>−1</sup>, the injection volume was 1 µL. The mass spectrometry was performed using a 6540 QTOF/MS Detector (Agilent, Santa Clara, CA, USA).

The metabolomic analysis consisted of 12 samples for each strain (4 time points, 3 biological triplicates). Non-targeted metabolite profiling comprising analysis by GC–MS and LC–MS was described previously. Metabolites were analyzed in a range of 50–1700 g mol<sup>−1</sup> with an accuracy up to 1–2 ppm and a resolution of mass/∆mass < 0.00001. The dimensionless metabolite concentration is shown through an internal standard normalization. Metabolites that were not annotated are listed according to their mass and retention time in minutes (e.g. 731.5113–1.55).

The generated data was explored by multivariate data analysis using the MetaboAnalyst 4.0 platform. First, the data was normalized by autoscaling (mean centering and division by standard deviation) and a PCA was performed. PCA is used as a technique for dimensionality reduction and data visualization. It is an orthogonal projection of the high dimensional data onto a lower dimensional linear space, such that the variance of the projected data is maximized. In this lower dimensional space, the eigenvector having the largest eigenvalue is known as the first PC. A one-way analysis of variance (ANOVA) was performed based on false discovery rate (FDR) adjusted p values ≤ 0.05. In addition, a Fisher’s significant difference test was conducted. When differences of two sample classes were investigated, a t-test with FDR adjusted p values ≤ 0.05 was performed and a fold change analysis based on the ratio of mean concentrations with a threshold of ≥ 2 was conducted.

**Data availability**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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**References**

1. Kourmentza, C. et al. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering* 4, 55 (2017).
2. Chen, G. Q., Hajnal, I., Wu, H., Lv, L. & Ye, J. Engineering biosynthesis mechanisms for diversifying polyhydroxyalkanoates. *Trends Biotechnol.* 33, 565–574 (2015).
3. Riedel, S. L. & Brigham, C. J. Inexpensive and waste raw materials for PHA production. In *The Handbook of Polyhydroxyalkanoates* (ed. Koller, M.) 203–221 (CRC Press, 2020).
8. Peplinski, K. et al. Genome-wide transcriptome analyses of the ‘Knallgas’ bacterium *Ralstonia eutropha* H16 with regard to poly-3-hydroxyalkanoate metabolism. Microbiology 156, 2136–2152 (2010).

9. Raberg, M. et al. Proteomic and transcriptomic elucidation of the mutant *Ralstonia eutropha* Gr-1 with regard to glucose utilization. Appl. Environ. Microbiol. 77, 2058–2070 (2011).

10. Sharma, P. K. et al. Global changes in the proteome of *Cupriavidus necator* H16 during poly-(3-hydroxybutyrate) synthesis from various biodiesel by-product substrates. AMBExpress 6, 1–16 (2016).

11. Schwartz, E. et al. A proteomic view of the facultatively chemolithoautotrophic lifestyle of *Ralstonia eutropha* H16. Proteomics 9, 5132–5142 (2009).

12. Kohlmann, Y. et al. Analyses of soluble and membrane proteomes of *Ralstonia eutropha* H16 reveal major changes in the protein complement in adaptation to lithoautotrophy. J. Proteome Res. 10, 2767–2776 (2011).

13. Fukui, T. et al. Metabolite profiles of poly(3-hydroxyalkanoate)-producing *Ralstonia eutropha* H16. Metabolomics 10, 190–202 (2014).

14. Im, D. K., Yun, S. H., Jung, J. Y., Lee, J. & Oh, M. K. Comparison of metabolite profiling of *Ralstonia eutropha* H16 phaBCA mutants grown on different carbon sources. Korean J. Chem. Eng. 34, 797–805 (2017).

15. Alagesan, S., Minton, N. P. & Malys, N. 13C-assisted metabolic flux analysis to investigate heterotrophic and mixotrophic metabolic pathways of *Cupriavidus necator* H16. Metabolomics 14, 1–10 (2018).

16. Tautenhahn, R. et al. An accelerated workflow for untargeted metabolomics using the METLIN database. Nat. Biotechnol. 30, 826–828 (2012).

17. Alonso, A., Marsal, S. & Julià, A. Analytical methods in untargeted metabolomics: State of the art in 2015. Front. Bioeng. Biotechnol. 3, 1–20 (2015).

18. Dunn, W. B. et al. Mass: metabolite identification in mass spectrometry-focused untargeted metabolomics. Metabolomics 9, 44–66 (2013).

19. Schirrme-Runledge, A. C., Codreanu, S. G., Sherrod, S. D. & McLean, J. A. Untargeted metabolomics approaches—Challenges and emerging directions. J. Am. Soc. Mass Spectrom. 27, 1897–1905 (2016).

20. Budde, C. F., Riedel, S. L., Willis, L. B., Rha, C. & Sinskey, A. J. Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. Appl. Environ. Microbiol. 77, 2847–2854 (2011).

21. Kahar, P., Tsuge, T., Taguchi, K. & Doi, Y. High yield production of poly(3-hydroxyalkanoate) from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polym. Degrad. Stab. 83, 79–86 (2004).

22. Tsuge, T. et al. Combination of N149S and D171G mutations in Aeromonas caviae poly(3-hydroxyalkanoate) synthase and impact on poly(3-hydroxyalkanoate) biosynthesis. FEMS Microbiol. Lett. 277, 217–222 (2007).

23. Sato, S., Maruyama, H., Fujiki, T. & Matsumoto, K. Regulation of 3-hydroxyalkanoate composition in PBBH synthesized by recombinant *Cupriavidus necator* H16 from palm oil by using butyrate as a co-substrate. J. Biosci. Bioeng. 120, 246–251 (2015).

24. Sato, S., Fujiki, T. & Matsumoto, K. Construction of a stable plasmid vector for industrial production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by a recombinant *Cupriavidus necator* H16 strain. J. Biosci. Bioeng. 116, 677–681 (2013).

25. Wong, Y.-M.-M., Brigham, C. J., Rha, C. K., Sinskey, A. J. & Sudesh, K. Biosynthesis and characterization of poly(3-hydroxyalkanoate) containing high 3-hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *Cupriavidus necator*. Bioresour. Technol. 121, 320–327 (2012).

26. Bhubalan, K. et al. Controlled biosynthesis and characterization of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) from mixtures of palm kernel oil and 3H3-precursors. Polym. Degrad. Stab. 93, 17–23 (2008).

27. Tan, H. T. et al. Evaluation of BP-M-CFP4 poly(3-hydroxyalkanoate) (PHA) syntheses on the production of poly(3-hydroxyalkanoate-co-3-hydroxyhexanoate) from palm oil using *Cupriavidus necator* transformants. Int. J. Biol. Macromol. 159, 250–257 (2020).

28. Noda, L., Green, P. R., Satkowski, M. M. & Schechtman, L. A. Preparation and properties of a novel class of poly(3-hydroxyalkanoate) copolymers. Biomacromol. 6, 580–586 (2005).

29. Thiniagaran, L. & Sudesh, K. Evaluation of sludge palm oil as feedstock and development of efficient method for its utilization to produce poly(3-hydroxyalkanoate). Waste Biomass Valoriz. https://doi.org/10.1007/s12649-017-0078-8 (2017).

30. Purama, R. K., Al-Sahabi, J. N. & Sudesh, K. Evaluation of date seed oil and date molasses as novel carbon sources for the production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by *Cupriavidus necator* H16 Re2058/pCB113. Ind. Crops Prod. 119, 83–92 (2018).

31. Murugan, P. et al. Production of P(3HB-co-3HHx) with Controlled Compositions by Recombinant *Cupriavidus necator* Re2058/pCB113 from Renewable Resources. Clean: Soil, Air, Water 44, 1234–1241 (2016).

32. Riedel, S. L. et al. Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by *Ralstonia eutropha* in high cell density palm oil fermentations. Biotechnol. Bioeng. 109, 74–82 (2013).

33. Ong, S. Y. et al. An integrative study on biologically recovered polyhydroxyalkanoates (PHAs) and simultaneous assessment of gut microbiome in yellow mealworm. J. Biotechnol. 265, 31–39 (2017).

34. Murugan, P., Gan, C. & Sudesh, K. Biosynthesis of P(3HB-co-3HHx) with improved molecular weights from a mixture of palm olein and fructose by *Cupriavidus necator* Re2058/pCB113. Int. J. Biol. Macromol. 102, 1112–1119 (2017).

35. Riedel, S. L. et al. Polyhydroxyalkanoates production with *Ralstonia eutropha* from low quality waste animal fats. J. Biotechnol. 214, 119–127 (2015).

36. Gutschmann, B. et al. In-line monitoring of polyhydroxyalkanoate (PHA) production during high-cell-density plant oil cultivations using photon density wave spectroscopy. Bioengineering 6, 85 (2019).

37. Arikawa, H. & Matsumoto, K. Evaluation of gene expression cassettes and production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) with a fine modulated monomer composition by using it in *Cupriavidus necator*. Microb. Cell Fact. 15, 1–11 (2016).

38. Zainab-L, I. & Sudesh, K. High cell density cultures of *Cupriavidus necator* H16 and improved biological recovery of polyhydroxyalkanoates using mealworms. J. Biotechnol. 305, 35–42 (2019).

39. Lu, J., Brigham, C. J., Rha, C. & Sinskey, A. J. Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. Appl. Microbiol. Biotechnol. 97, 2443–2454 (2013).

40. Vanhooren, P. T. & Vandamme, E. J. 1-Fucose: Occurrence, physiological role, chemical, enzymatic and microbial synthesis. J. Chem. Technol. Biotechnol. 74, 479–497 (1999).

41. Miki, M. & Renkonen, R. Biosynthesis of 6-deoxyhexose glycans in bacteria. Glycobiology 14, 1–15 (2004).

42. Coyne, M. J., Reinap, B., Lee, M. M. & Comstock, L. E. Human symbionts use a host-like pathway for surface fucosylation. Science (80-) 307, 1778–1781 (2005).
43. St. John, G. et al. Peptide methionine sulfoxide reductase from Escherichia coli and Mycobacterium tuberculosis protects bacteria against oxidative damage from reactive nitrogen intermediates. Proc. Natl. Acad. Sci. USA 98, 9901–9906 (2001).

44. Chin, Y. W., See, N., Kim, J. H. & See, J. H. Metabolic engineering of Escherichia coli to produce 2'-fucosyllactose via salvage pathway of guanosine 5’-diphosphate (GDP)-t-fucose. Biotechnol. Bioeng. 113, 2443–2452 (2016).

45. Freitas, F. et al. Fucose-containing exopolysaccharide produced by the newly isolated Enterobacter strain A47 DSM 23139. Carbohydr. Polym. 83, 159–165 (2011).

46. Freitas, F. et al. Controlled production of exopolysaccharides from enterobacter A47 as a function of carbon source with demonstration of their film and emulsifying abilities. Appl. Biochem. Biotechnol. 172, 641–657 (2014).

47. Neu, T. R. & Poralla, K. Emulsifying agents from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. 32, 521–525 (1990).

48. Ribeiro, P. L. L., Campos, M. I. & Druzan, J. I. Novel extracellular polymeric substances produced by Cupriavidus necator IPT 027 grown on glucose and crude glycerol originated from biodiesel. Polym. Adv. Technol. 28, 549–556 (2017).

49. Radzuan, M. N., Banat, I. M. & Winterburn, J. Production and characterization of rhamnolipid using palm oil agricultural refinery waste. Bioresour. Technol. 225, 99–105 (2017).

50. Budde, C. F. et al. Growth and polyhydroxybutyrate production by Ralstonia eutropha in emulsified plant oil medium. Appl. Microbiol. Biotechnol. 89, 1611–1619 (2011).

51. Chiang, S. M. & Schellhorn, H. E. Regulators of oxidative stress response genes in Escherichia coli and their functional conservation in bacteria. Arch. Biochem. Biophys. 525, 161–169 (2012).

52. Obruca, S., Marova, I., Svoboda, Z. & Mikulikova, R. Use of controlled exogenous stress for improvement of poly(3-hydroxybutyrate) production in Cupriavidus necator, Folia Microbiol. (Praga) 55, 17–22 (2010).

53. Azlan, A. et al. Comparison of fatty acids, vitamin E and physicochemical properties of Canarium odontophyllum Miq. (dabai), olive and palm oils. J. Food Compos. Anal. 23, 772–779 (2010).

54. Slater, S. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in Ralstonia eutropha. J. Bacteriol. 180, 1979–1987 (1998).

55. Budde, C. F., Mahan, A. E., Lu, J., Rha, C. & Sinskey, A. J. Roles of multiple acetocetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in Ralstonia eutropha H16. J. Bacteriol. 192, 5319–5328 (2010).

56. Jo, S., Matsumoto, K. & Leong, C. R. Improvement of Poly(3-Hydroxybutyrate) [P(3HB)] biosynthetic genes related to poly-(R)-3-hydroxybutyrate homeostasis during batch fermentation. Appl. Microbiol. Biotechnol. 68, 663–672 (2005).

57. Lawrence, A. G. et al. Transcriptional analysis of Ralstonia eutropha genes related to poly-(R)-3-hydroxybutyrate homeostasis against oxidative damage from reactive nitrogen intermediates. Proc. Natl. Acad. Sci. USA 98, 9901–9906 (2001).

58. Riberio, P. L. L., Campos, M. I. & Druzan, J. I. Novel extracellular polymeric substances produced by Cupriavidus necator IPT 027 grown on glucose and crude glycerol originated from biodiesel. Polym. Adv. Technol. 28, 549–556 (2017).

59. Radzuan, M. N., Banat, I. M. & Winterburn, J. Production and characterization of rhamnolipid using palm oil agricultural refinery waste. Bioresour. Technol. 225, 99–105 (2017).

60. Obruca, S., Marova, I., Svoboda, Z. & Mikulikova, R. Use of controlled exogenous stress for improvement of poly(3-hydroxybutyrate) production in Cupriavidus necator, Folia Microbiol. (Praga) 55, 17–22 (2010).

61. Azlan, A. et al. Comparison of fatty acids, vitamin E and physicochemical properties of Canarium odontophyllum Miq. (dabai), olive and palm oils. J. Food Compos. Anal. 23, 772–779 (2010).

62. Slater, S. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in Ralstonia eutropha. J. Bacteriol. 180, 1979–1987 (1998).

63. Budde, C. F., Mahan, A. E., Lu, J., Rha, C. & Sinskey, A. J. Roles of multiple acetocetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in Ralstonia eutropha H16. J. Bacteriol. 192, 5319–5328 (2010).

64. Jo, S., Matsumoto, K. & Leong, C. R. Improvement of Poly(3-Hydroxybutyrate) [P(3HB)] biosynthetic genes related to poly-(R)-3-hydroxybutyrate homeostasis during batch fermentation. Appl. Microbiol. Biotechnol. 68, 663–672 (2005).

65. Lawrence, A. G. et al. Transcriptional analysis of Ralstonia eutropha genes related to poly-(R)-3-hydroxybutyrate homeostasis against oxidative damage from reactive nitrogen intermediates. Proc. Natl. Acad. Sci. USA 98, 9901–9906 (2001).

66. Riberio, P. L. L., Campos, M. I. & Druzan, J. I. Novel extracellular polymeric substances produced by Cupriavidus necator IPT 027 grown on glucose and crude glycerol originated from biodiesel. Polym. Adv. Technol. 28, 549–556 (2017).

67. Radzuan, M. N., Banat, I. M. & Winterburn, J. Production and characterization of rhamnolipid using palm oil agricultural refinery waste. Bioresour. Technol. 225, 99–105 (2017).

68. Obruca, S., Marova, I., Svoboda, Z. & Mikulikova, R. Use of controlled exogenous stress for improvement of poly(3-hydroxybutyrate) production in Cupriavidus necator, Folia Microbiol. (Praga) 55, 17–22 (2010).

69. Azlan, A. et al. Comparison of fatty acids, vitamin E and physicochemical properties of Canarium odontophyllum Miq. (dabai), olive and palm oils. J. Food Compos. Anal. 23, 772–779 (2010).

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S.L.R. conceived and designed the experiments; S.L.R., M.B. and S.J. performed the experiments; B.G. analyzed the metabolomic raw data; B.G., C.J.B. and S.L.R. analyzed the data and wrote the first draft of the manuscript; B.G., C.J.B., S.L.R. and P.N. edited the manuscript; all authors have given approval to the final version of the manuscript.

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