De novo biosynthesis of paracetamol from glucose by metabolically engineered Escherichia coli

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Research

Abstract

Background: Paracetamol is among the most commonly used of all medications, widely accepted as a safe and effective analgesic/antipyretic for mild-to-moderate pain and fever. The biosynthesis of paracetamol from renewable sugars has not been reported so far, due to the lack of natural biosynthetic pathways.

Results: In this study, we demonstrated for the first time the development of an *E. coli* cell factory for production of paracetamol from glucose. First, p-aminobenzoic acid, an intermediate of folic acid in microorganism, is selected as precursor substrates for the production of paracetamol. Second, a monooxygenase MNX1 from *Candida parapsilosis* CBS604 that can efficiently catalyze the decarboxylation and hydroxylation of p-aminobenzoic acid into corresponding p-aminophenol and another N-acetyltransferase PANAT from *Pseudomonas aeruginosa* that can efficiently catalyze the esterification of acetyl-CoA and p-aminophenol to form paracetamol were discovered. Finally, an engineered *E. coli* that allows production of paracetamol from simple carbon sources was established.

Conclusions: The present study opens up a new direction for engineering microbial production of paracetamol from cheap and readily-available renewable raw materials such as sugars and cellulose in the future.

Background

Paracetamol is among the most commonly used of all medications, widely accepted as a safe and effective analgesic/antipyretic for mild-to-moderate pain and fever [1]. Paracetamol was first used clinically in 1893 by von Mering, but it was not used commercially until 1950 [2, 3]. Now it is a first-line drug molecule for pain and fever management. Its antipyretic and analgesic effects are similar to aspirin. However, It lacks the inhibitory effect on platelet aggregation and gastrointestinal toxicity, making it an excellent choice for patients at risk of gastrointestinal bleeding, such as those with chronic liver disease[1, 4]. Traditionally it is synthesized chemically. However, the biosynthesis of paracetamol from renewable sugars can reduce the dependence on petrochemical resources. Thus, bioproduction of paracetamol by microbial cell factories is considered as a promising alternative. However, the biosynthesis of paracetamol from renewable sugars has not been reported so far, due to the lack of natural biosynthetic pathways.

In this study, we report the development of an *E. coli* cell factory for the production of paracetamol from glucose (Fig. 1). First, p-aminobenzoic acid (PABA), an intermediate of folic acid in microorganism, is selected as precursor substrate for production of paracetamol. Second, a monooxygenase MNX1 that can efficiently catalyze the decarboxylationnd hydroxylation of p-aminobenzoic acid into corresponding p-aminophenol and another N-acetyltransferase that can efficiently catalyze the esterification of acetyl-CoA and p-aminophenol to form paracetamol were discovered. Finally, biosynthetic pathway of p-aminophenol from glucose was constructed in engineered *E. coli.*

Results

Designing biosynthetic pathway for paracetamol production

At present, there is no biosynthetic pathway of paracetamol found in nature. Thus, we proposed two artificial paracetamol biosynthetic pathways in this study. P-aminobenzoic acid, an intermediate of folic acid in microorganism, is selected as precursor substrates for production of paracetamol. There are two possible biosynthetic pathways for the biosynthesis of paracetamol from p-aminobenzoic acid. The first biosynthetic pathway: decarboxylation and hydroxylation of p-aminobenzoic acid to p-aminophenol and subsequent acetylation of p-aminophenol into paracetamol. The second biosynthetic pathway: acetylation of p-aminobenzoic acid to p-acetylaminobenzoic acid and subsequent decarboxylation and hydroxylation of p-acetylaminobenzoic acid into paracetamol.

Validation Of The Activity Of MNX1 And Panat

A flavoprotein monooxygenase MNX1 from *C. parapsilosis* CBS604 could catalyze the decarboxylation and hydroxylation of 4-hydroxybenzoate into corresponding 4-hydroxyphenol[5] and another salicylate monooxygenase NahG from *P. aeruginosa* could catalyze the decarboxylation and hydroxylation of salicylate into corresponding catechol[6]. We assessed whether the substrate of the MNX1 or NahG could extend to p-aminobenzoic acid or p-acetylaminobenzoic acid. To evaluate the activity of MNX1, plasmid pDG46 for expression MNX1 was introduced into *E. coli* BL21 to yield strain DG001. The strain DG001 was cultivated in M9 medium containing 1 g/L p-aminobenzoic acid or p-acetylaminobenzoic acid. 645 ± 32.5 mg/L p-aminophenol was produced within 12 hours at the expense of 1 g/L p-aminobenzoic acid with a molar conversion yield of 81% (Table 1). However, there is no paracetamol production at the expense of 1 g/L p-acetylaminobenzoic acid. These results indicated that MNX1 had catalytic activity for p-aminobenzoic acid but not for p-acetylaminobenzoic acid. To evaluate the activity of NahG, plasmid pDG47 for expression NahG was transferred into *E. coli* BL21. However, the results indicated that NahG had no catalytic activity on either p-aminobenzoic acid or p-acetylaminobenzoic acid.

Table 1
p-Aminophenol, p-acetylaminobenzoic acid and paracetamol production by cell catalysis. All experiments were performed in triplicate and SD is indicated.

Strains	Production	Titer(mg/L)
DG001 + 1 g /L of p-aminobenzoic acid	p-aminophenol	645 ± 32.5
DG002 + 1 g /L of p-aminobenzoic acid	p-acetylaminobenzoic acid	1263 ± 66.8
DG002 + 1 g /L of p-aminophenol	Paracetamol	1296 ± 68.7

P. aeruginosa arylamine N-acetyltransferase PANAT and *E. coli* arylamine N-acetyltransferase ECNAT could catalyse the transfer of an acetyl group from acetyl-CoA to arylamines, arylhydrazines and hydroxyarylamines[7]. We assessed whether the substrate of the PANAT or ECNAT could extend to p-aminobenzoic acid or p-aminophenol. To evaluate the activity of PANAT, plasmid pDG48 for expression

PANAT was introduced into *E. coli* BL21 to yield strain DG002. The strain DG002 was cultivated in M9 medium containing 1 g/L p-aminobenzoic acid or p-aminophenol. 1263 ± 66.8 mg/L p-acetylaminobenzoic acid from p-aminobenzoic acid with a molar conversion yield of 97% or 1296 ± 68.7 mg/L paracetamol from p-aminophenol with a molar conversion yield of 94% were produced, respectively (Table 1). These results indicated that PANAT had catalytic activity on both p-aminobenzoic acid and p-aminophenol. For ECNAT, the results showed that it had no catalytic activity on both p-aminobenzoic acid and p-aminophenol.

Production Of P-aminophenol From Glucose

Next, we assembled a p-aminophenol biosynthetic pathway from glucose in *E. coli* BL21. This biosynthetic pathway consists of five enzymes: AroG^{fbr} for the efficient overproduction of chorismate, PabA, PabB and PabC from *E. coli* for the conversion of chorismate to p-aminobenzoic acid, MNX1 for the conversion of p-aminobenzoic acid to p-aminophenol. The resulting strain DG003 was grown in M9 medium with 20 g/L of glucose. The fermentation products were extracted by ethyl acetate and analyzed by GC−MS (Fig. 2 and Figure S1). 412 ± 19.8 mg/L p-aminophenol was produced by the recombinant strains DG003 for co-expression of AroG^{fbr}, PabA, PabB, PabC and MNX1 after 28 h of IPTG induction (Table 2).

Table 2
Paracetamol and p-aminophenol production in engineered *E. coli* strains. All experiments were performed in triplicate and SD is indicated.

Strains	Production	Titer(mg/L)
DG003	p-aminophenol	412 ± 19.8
DG004	p-aminophenol	1138 ± 57.3
DG005	Paracetamol	738 ± 24.6

Improving P-aminophenol Biosynthesis

p-Aminobenzoic acid is the precursor for the biosynthesis of p-aminophenol, and increasing its concentration would be expected to enhance the synthesis of p-aminophenol. Recently, several studies have shown that CCpabAB from *Corynebacterium callunae* and XBpabC from *Xenorhabdus bovienii* are ideal candidates for efficient p-aminobenzoic acid biosynthesis[8]. Thus, by replacing of *E. coli* pabABC with CCpabAB and XBpabC, it is possible to further improve the production of p-aminophenol. In this study, using CCpabAB and XBpabC instead of *E. coli* pabABC was evaluated to improve the production of p-aminophenol. The resulting strain DG004 produced 1138 ± 57.3 mg/L p-aminophenol after 28 h of IPTG induction (Table 2). This result proves that replacing of *E. coli* pabABC with CCpabAB and XBpabC can effectively improve p-aminophenol production.

Finally, we constructed a biosynthetic pathway for direct biosynthesis of paracetamol from glucose. The order of reaction of the designed pathway is as follows: first glucose is converted to p-aminobenzoic acid via the upstream enzymes, then p-aminobenzoic acid is converted to p-aminophenol via MNX1, and finally p-aminophenol is converted to paracetamol via PANAT. Considering that PANAT has catalytic activity on both p-aminobenzoic acid and p-aminophenol, we expressed the PANAT gene on the heat-inducible expression plasmid pBV220. Plasmid pBV220 contains the lambda pRpL promoters, and the cI857 gene encoding the temperature-sensitive repressor which provides tight control over protein expression[9, 10]. The resulting strain DG005 was grown in M9 medium with 20 g/L of glucose at 30°C. After 20 h of isopropyl β-D-thiogalactoside induction for the biosynthesis of p-aminophenol, PANAT expression was induced by increasing the culture temperature from 30°C to 40°C to catalyze the acetylation of p-aminophenol into paracetamol. The fermentation products were extracted by ethyl acetate and analyzed by GC−MS (Figure S2). 738 ± 24.6 mg/L paracetamol was produced after 8 h of heat induction (Table 2).

Discussion

With the developments of biotechnology, significant advances have been made on engineered microorganism for the biosynthesis of a variety of chemicals and drugs from renewable resources[11−15]. Paracetamol is one of the most commonly used drugs and is widely regarded as a safe and effective drug for the treatment of mild to moderate pain and fever. Chemical synthesis processes have been developed to produce paracetamol. However, chemical methods usually were accompanied with some inevitable problems such as generation of byproducts, use of hazardous chemicals, and high temperature and pressure conditions[16]. One enzymatic method has been reported for the preparation of paracetamol from p-aminophenol and acetate by a bacterial aryl acylamidase[17]. However, there is no report of the biosynthesis of paracetamol from renewable resources.

In this study, we demonstrated for the first time the development of an *E. coli* cell factory that allows production of paracetamol simple carbon sources. We evaluated candidate enzymes and discovered *C. parapsilosis* MNX1 could catalyze the decarboxylation and hydroxylation of p-aminobenzoic acid into corresponding p-aminophenol and *P. aeruginosa* PANAT could catalyse the acetylation of p-aminobenzoic acid or p-aminophenol into p-acetylaminobenzoic acid and paracetamol, respectively. Next, we assembled a p-aminophenol biosynthetic pathway from glucose in *E. coli*. We further improved the p-aminophenol production to 1138 ± 57.3 mg/L by replacing of *E. coli* pabABC with *C. callunae* pabAB and *X.bovienii* pabC. Finally, we have demonstrated the de novo biosynthesis of paracetamol from glucose by further heat-inducible expression of N-acetyltransferase PANAT.

Conclusions

In this study, PANAT is active on both p-aminobenzoic acid and p-aminophenol, we successfully circumvented this problem by using a two-step induction strategy. Although additional work is needed to further improve the yield of paracetamol, this study opens up a way for the production of paracetamol from renewable sugars by engineered microorganisms and showed that this artificial biosynthetic approach has good potential.

Methods

Plasmid and strains

The pabA, pabB and pabC (GenBank NC_000913.3) genes were amplified from *E. coli* genomic DNA using primers pabA-XbaI/ pabA-SpeI-BamHI, pabB-XbaI/pabB-SpeI-BamHI and pabC-XbaI/pabC-SpeI-BamHI, and ligated into pET28a(+) to generate pDG40, pDG41 and pDG42. The XbaI-XhoI fragment of pabB from pDG41 was ligated into SpeI and XhoI sites of pDG40 to generate pDG43. The XbaI-XhoI fragment of pabC from pDG42 was ligated into SpeI and XhoI sites of pDG43 to generate pDG44. The XbaI-XhoI fragment of pabA, pabB and pabC from pDG44 was ligated into XbaI and XhoI sites of pBBRMCS1 to generate pDG45.

Flavoprotein monooxygenase MNX1 (GenBank XP_036663424.1) from *Candida parapsilosis* CBS604, salicylate monooxygenase NahG (GenBank WP_014861933.1) and arylamine N-acetyltransferase PANAT (GenBank WP_003110506.1) from *Pseudomonas aeruginosa* were synthesized and amplified using primers MNX1 – XbaI/MNX1 – SpeI – BamHI, NahG – XbaI/NahG – SpeI – BamHI and PANAT – XbaI/PANAT – SpeI – BamHI, and ligated into pET28a(+) to generate pDG46, pDG47 and pDG48. The arylamine N-acetyltransferase ECNAT (GenBank CP072054.1) gene was amplified from *E. coli* genomic DNA using primers ECNAT -XbaI/ECNAT-SpeI-BamHI, and inserted into pET28a(+) to give pDG49. Plasmid pDG11 for expression the feedback resistant mutant of 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase AroG^{fbr} (D146N) was constructed in our previous study[18]. The XbaI-SalI fragment of MNX1 from pDG46 was inserted into SpeI and SalI sites of pDG11 to give pDG50. The XbaI-SalI fragment of AroG^{fbr} and MNX1 from pDG50 was ligated into XbaI and XhoI sites of pDG45 to generate pDG51.

CCpabAB (GenBank WP_015650817.1) from *Corynebacterium callunae* and XBpabC (GenBank CDG97586.1) from *Xenorhabdus bovienii* were synthesized and amplified by PCR using primers CCpabAB – XbaI/CCpabAB – SpeI – BamHI and XBpabC – XbaI/XBpabC – SpeI – BamHI, and ligated into pET28a(+) to generate plasmid pDG52 and pDG53. The XbaI-XhoI fragment of XBpabC from pDG53 was inserted into SpeI and XhoI sites of pDG52 to give pDG54. the XbaI-XhoI fragment of CCpabAB and XBpabC from pDG54 was ligated into XbaI and XhoI sites of pBBRMCS1 to generate plasmid pDG55. The XbaI-SalI fragment of AroG^{fbr} and MNX1 from pDG50 was ligated into SpeI and XhoI sites of pDG45 to generate pDG56. PANAT from *Pseudomonas aeruginosa* was further amplified using primers PANAT – EcoRI and PANAT-BamHI, and ligated into pBV220 to give pDG57. The strains, primers and plasmids used in this study are summarized in Table 1S and Table 2S.

Shake Flask Cultures

Strains were cultured in 100 mL M9 medium with 2% glucose at 30°C as previously described by Guo[18]. Appropriate antibiotics (ampicillin, kanamycin or chloromycetin) were added to M9 medium. When the medium OD_{600} reached about 0.8, isopropyl β-D-thiogalactoside was added to a concentration of 0.1 mM.

Analytical Methods

Cultures samples (5 ml) were transferred to 20-ml glass tubes. Glass beads (0.1 mm) were used for breaking the cells by vigorous vortexing for 5 minutes. Ethyl acetate (5 mL) is used to extract fermentation products. After centrifuging the extract, a 1 μL of the ethyl acetate phase was analysed by GC-MS as previously described by Guo[18].

Declarations

Authors' contributions

D Guo and H Pan designed the research, supervised the project, and wrote the manuscript. D Guo, X Fu, S Kong and Y Sun performed experiments and analyzed the data.

Funding

Availability of data and materials

All data generated or analyzed during this study are included in this published

article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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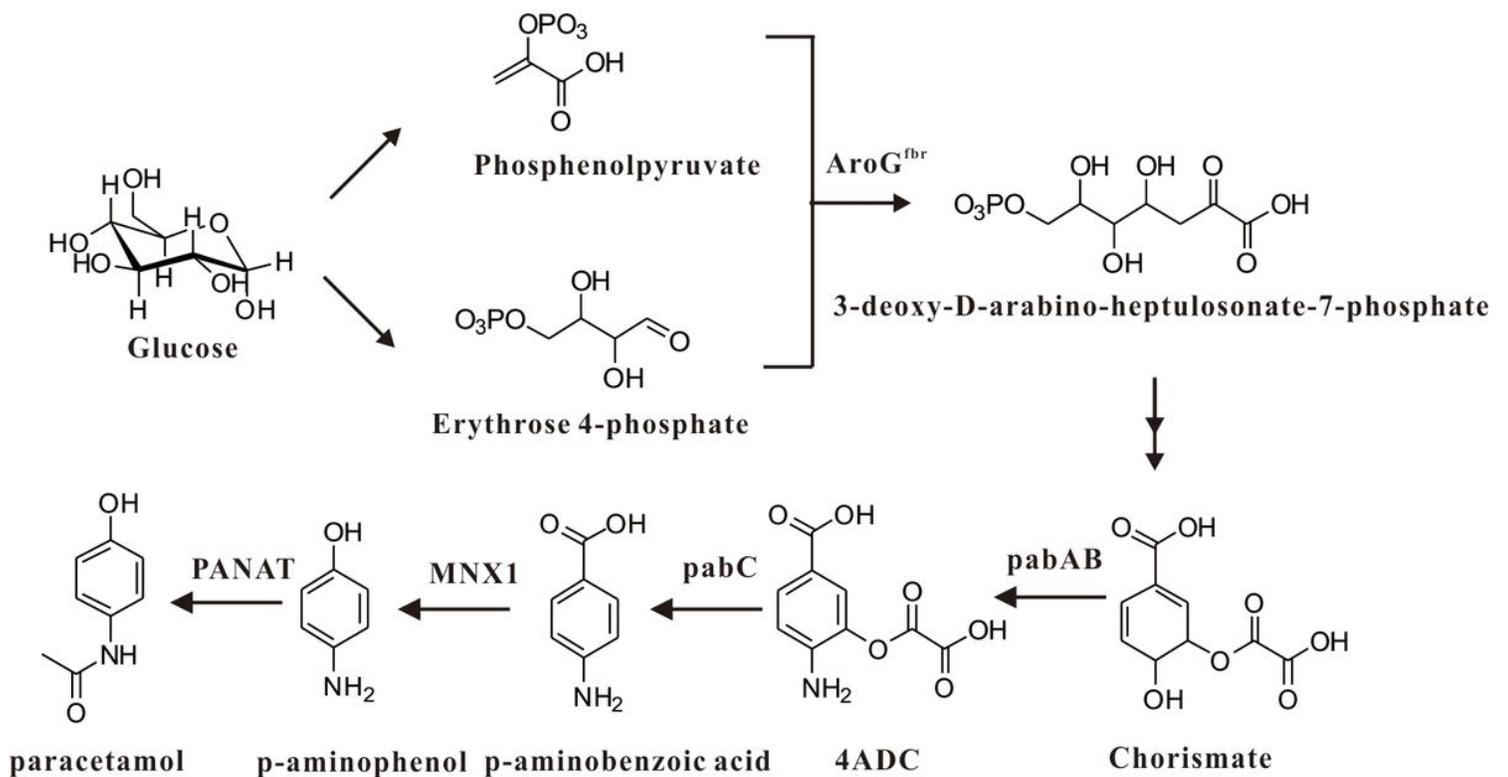
Figures

Figure 1

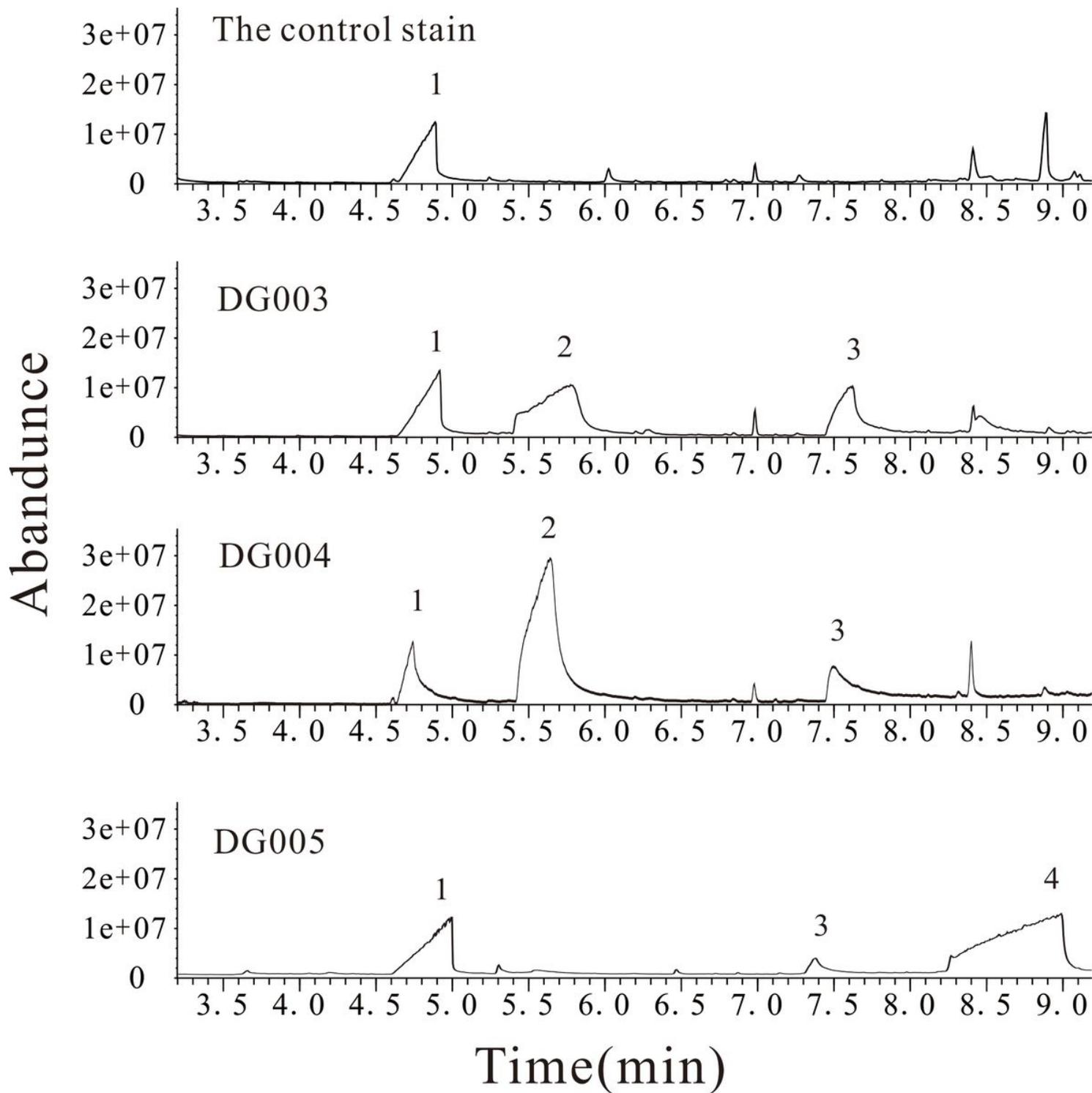Engineered pathways for production of paracetamol from glucose.

Figure 2

GC/MS analyses of paracetamol and p-aminophenol in engineered E. coli strains. Identified substances: 1, benzoic acid (internal standard); 2, p-aminophenol; 3, p-aminobenzoic acid; 4, paracetamol.

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