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Optimization of Amorphadiene Synthesis in *Bacillus Subtilis* via Transcriptional, Translational and Media Modulation†

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Running title: Production of Isoprenoids in GRAS Bacillus

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Abstract

Genetically engineered microbes have been intensively investigated as a means for the cost-effective production of isoprenoids. *Bacillus subtilis* is a promising microbial host for this purpose because of its fast growth rate and GRAS (generally regarded as safe) status. To date, development of this host has been impaired by the lack of genetic tools for modulating the expression of multiple genes. In this study, we present a novel two-promoter system which can be used to independently control the expression levels of two gene cassettes over a large dynamic range. Coupled with protein translation engineering and systematic media optimization, ~20mg/L amorphadiene was produced in shake flask scale, a 40 fold improvement over the highest reported isoprenoid product yield in *B. subtilis.* As the tools and strategies developed here can be extended to the overproduction of other valuable metabolites, this proof-of-concept study lays the foundation for high level heterologous production of isoprenoids in *Bacillus*.

Key words

Isoprenoids, *Bacillus subtilis*, Deoxyxylulose phosphate pathway, Metabolic engineering, Multiple gene expression

Manuscript

To date, more than 55,000 isoprenoid compounds have been discovered, many of which are therapeutic molecules such as paclitaxel (anti-tumor (Ajikumar et al. 2010)) and artemisinin (anti-malaria (Xia et al. 2012)). Presently, commercial production of these isoprenoid pharmaceuticals heavily relies on plant resources, from which the compounds are directly extracted or their precursor metabolites are purified and chemically converted into products. These plant-based processes are inadequate to meet market needs of many isoprenoids simply because most isoprenoids are present in extremely low amounts in plants (often <1% of the total carbon (Kolewe et al. 2008)) and production is subject to unpredictable factors (such as weather conditions).

To address this challenge, microbial production of isoprenoids has been extensively studied in the past decade. *Escherichia coli* is one of the most commonly used hosts due to its fast growth rate on a variety of carbon sources and the availability of abundant genetic tools. Research with this organism has yielded more than one gram per liter production levels for certain isoprenoid precursors (for example taxadiene, (Ajikumar et al. 2010)). Besides *E. coli*, *Saccharomyces cerevisiae* has been used for microbial production of pharmaceuticals due to its GRAS status and availability of a very advanced set of tools for genetic transformation. However, *S. cerevisiae* grows at half the rate as *E. coli*, thus limiting its isoprenoid productivity (Xia et al. 2012). Considering also the non-GRAS status of *E. coli*, it is of great commercial interest to engineer a fast growing GRAS bacterium for high level production of pharmaceutical isoprenoids. *Bacillus subtilis* is a good candidate because it is a GRAS bacterium with rapid growth rate (Phan et al. 2012) and has yielded satisfactory performance in the production of proteins and valuable metabolites from economical carbon feed stocks (Sivy et al. 2011; Xue and Ahring 2011). To date, there is only one report on producing heterologous isoprenoids in *B. subtilis*, where Yoshida et al. demonstrated the synthesis of a carotenoid with no quantification data on the product yield (Yoshida et al. 2009). The challenge for producing isoprenoid pharmaceuticals in *B. subtilis* is the lack of well characterized genetic tools for fine-tuning multiple gene expression that is essential for high level heterologous production of isoprenoids.

Recently, a promoter of *Bacillus megaterium* gene *xylA* (PxylA) was demonstrated to be a strong and inducible (by xylose) promoter for protein expression in *B. subtilis* (Yamashiro et al. 2011). If another controllable promoter that is compatible with PxylA in *B. subtilis* could be identified, then it could be combined with PxylA to independently control expression of two gene cassettes. Pgrac, an IPTG inducible hybrid promoter containing an *E. coli* lac operator (Maeda 2012), was selected on the hypothesis that promoters from different species were unlikely to interact. To test the two promoter system, PxylA and Pgrac were employed to express genes for the synthesis of amorphadiene, a critical precursor of the antimalarial drug artemisinin (Martin et al. 2003). The genes overexpressed here were *ads* and *dxs-idi*; *ads* encodes the synthase to cyclize farnesyl diphosphate into amorphadiene; *dxs* and *idi* were two genes in the deoxyxylulose phosphate (DXP) pathway and were found to control the availability of farnesyl diphosphate in *E. coli* (Yuan et al. 2006). The gene, *ads,* was inserted downstream of Pgrac on plasmid pHT01 (termed as pHT-ads, Figure 1A and B), and *dxs-idi* were cloned under the control of PxylA on plasmid pWH1520 (termed as pWH-DI Figure 1A and B). Transcription quantification showed that PxylA and Pgrac were inducible by xylose and IPTG respectively when used together in *B. subtilis* (Figure 1C). Transcriptions of *dxs-idi* and *ads* were well controlled over a wide dynamic range (10^4 to 10^6 a.u.), and maximal induction of PxylA and Pgrac was attained with 0.1% xylose and 0.1 mM IPTG respectively.

With the validated promoter system, amorphadiene synthesis was systematically optimized by fine tuning the expression of the DXP genes (*dxs* and *idi*) and *ads*. This system was able to elicit a wide range of expression of the two gene cassettes by applying different concentration of inducers xylose and IPTG. An optimal expression condition was identified where ~2mg/L amorphadiene was produced (Figure 2). Amorphadiene production increased gradually with both IPTG and xylose induction until the above inducers reached concentrations of 0.1mM and 0.1% respectively. As maximal transcription levels were achieved at similar inducer concentrations, it seemed plausible that amorphadiene synthesis was controlled by the availability of ADS, DXS and IDI enzymes. Furthermore, considering the strength of Pgrac was generally weaker than that of PxylA (Figure 1C), it was plausible that

production of amorphadiene was limited by *ads* even though *ads* transcription (under control of Pgrac) was maximally induced.

Once a limiting step has been identified in a pathway (such as the step catalyzed by ads), it is typical to overcome such limitations by overexpressing the corresponding gene through, for example, the use of a stronger promoter. However, replacement of Pgrac with a stronger promoter would require re-characterization of the new promoter in the presence of PxylA to identify conditions of optimal functioning of the combined system. As an alternative approach, we opted instead to optimize *ads* expression by employing N terminal fusion tags which could increase the efficiency of translation (Panavas et al. 2009), a critical step in protein production. Two highly charged tags, six-arginine tag (6xR, positive charge (Kim et al. 2007)) and six-aspartic acid tag (6xD, negative charge (Kim et al. 2007)), and one protein stabilizing tag trxA (Dyson et al. 2004) were examined. Abundance of ADS protein but not *ads* mRNA was significantly increased by use of the 6xR tag (Figure 3 A), indicative that translation efficiency of ADS was indeed enhanced by fusing the 6xR tag to N terminus of *ads*. In line with the hypothesis that more ADS protein would enhance amorphadiene production, amorphadiene production by cells expressing the 6xR tagged *ads* increased by 2.5 fold over the untagged *ads* transcription (Figure 3 B).

Besides genetic modulations, we also systematically optimized the growth medium to further increase amorphadiene productivity. Pyruvate and dipotassium phosphate were chosen as supplements, because pyruvate is a substrate of the DXP pathway and dipotassium phosphate has recently been found to be beneficial for isoprenoid production in *E. coli* (Zhang et al. manuscript under review). Both chemicals were confirmed to increase amorphadiene production when added to growth medium individually (Supplementary file S1). Simultaneous addition of both compounds was subsequently optimized by examining the combination of the compounds over wide range of concentrations, which finally increased the yield to 17mg/L (Figure 4 A). To gain an insight into the molecular mechanism underlying this semi-empirical optimization strategy, we measured transcription and intermediate metabolite concentrations in the cells harvested at three representative conditions (No chemical

addition, addition of 8g/L pyruvate and addition of 8g/L pyruvate as well as 32g/L dipotassium phosphate, Figure 4 A). Transcription of *dxs-idi* and *ads* were not significantly altered in the conditions (Figure 4 B), suggesting that factors other than genetic expression improved productivity. Consistent with the observed increase in amorphadiene production, metabolic intermediates in the DXP pathway were generally increased upon the chemical addition (Figure 4 C), indicative of the enhanced DXP pathway flux. A further non-targeted metabolite analysis revealed that co-factor (ATP and CTP) and precursor (PEP) of the DXP pathway were significantly up-regulated upon chemical addition (Figure 4 D, Supplementary file S2). The data suggested a plausible hypothesis that peripheral metabolism to the DXP pathway was enhanced by adding pyruvate and dipotassium phosphate, which kinetically drove the reactions in the DXP pathway towards isoprenoid synthesis.

Although the yield achieved in our proof-of-concept study (~20mg/L) is considerably lower than that achieved in *E. coli* (~700mg/L amorphadiene in shake flask scale (Ma et al. 2011)), it has significantly superseded the highest yield reported for isoprenoid production in *B. subtilis* (~0.5mg/L isoprene by (Xue and Ahring 2011)). To further increase amorphadiene productivity, the use of computationally guided competing pathway knockout (Alper et al. 2005) and global transcription machinery engineering (Alper et al. 2006) will be explored. Further improvements can be expected using higher biomass concentrations and controlled bioreactor conditions. Considering its distinct advantages in simplifying downstream processing (Xue and Ahring 2011), the GRAS *B. subtilis* strain demonstrated in this study is indeed a promising microbial host for overproduction of isoprenoid pharmaceuticals.

Besides the focus on improving the amorphadiene productivity, our study also emphasized the importance of rational design (guided by transcription and metabolite analysis) in microbial isoprenoid production. Transcription analysis by quantitative PCR enabled us to thoroughly characterize the constructed promoter system and guided protein translation engineering. The mass spectrometry based metabolite analysis provided useful information on metabolic changes relating to the effects of media and suggested possible targets for future rational engineering and optimization (focusing on co-

factors and precursors of the DXP pathway). The method employed in this study should be applicable generally to metabolic engineering of microbes for metabolite overproduction.

Methods

Molecular cloning

Ads was synthesized with optimized codon (Genescript) based on amino acid sequence (AAF98444, NCBI) and subcloned into pHT01 (MoBiTec). Dxs and idi were PCR amplified from *B. subtilis* genomic DNA and subcloned into pWH1520 (MoBiTec). The 6xR and 6xD tag were added to N terminus of ads by PCR; the tag trxA was PCR amplified from *E. coli* genomic DNA and subcloned to N terminus of ads. The constructed plasmids were transformed into *B. subtilis* 1A1 (BGSC). Nucleotide sequence of synthetic ads is included in Supplementary file S3; the primers used in this study were summarized in Supplementary file S4.

Cell growth and induction

Single colony was picked up from agar plate, inoculated into 1mL 2xPY medium (20 g/L Peptone, 10 g/L Yeast extract, and 10 g/L NaCl, pH = 7) with proper antibiotics and incubated at 37ºC overnight. Pellets of overnight grown cells were resuspended in 1mL fresh medium to cell density 0.1 (OD595). The cells were incubated at 37ºC/300rpm until cell density reached 0.7 (OD595), and indicated concentration of IPTG and xylose were added to induce protein expression. 200μL dodecane was added to cell culture for in situ extraction of amorphadiene, and the cells were incubated at 28ºC/300rpm. The cells were cultured in 14mL falcon polypropylene tube (BD). Sodium pyruvate and dipotassium phosphate were used in medium optimization, and initial pH of media was adjusted to 7 (the same to 2xPY medium).

Protein analysis

Cell suspension equivalent to 1mL OD595=1.0 cells were sampled at 5h after induction, and cell pellets were lysed in 100μL 2% SDS. The cell lysates were separated by SDS-PAGE and ads protein was detected by western blot with Penta-His antibody (Qiagen).

Transcription/Metabolic intermediate analysis

The cells were sampled at 5h after induction; mRNA was quantified by reverse transcription quantitative PCR as described in (Zhou et al. 2011), and metabolites were quantified by UPLC-MS as described in (Zhou et al. 2012). The transcription results were normalized to cell number; the metabolite results were normalized to sample volume. The primers used for transcription quantification were summarized in Supplementary file S4. Transcript of dxs was quantified to represent dxs-idi cassette under control of PxylA. A control experiment was conducted to rule out that the unchanged transcription levels were due to high level genomic DNA contamination (Supplementary file S5).

Product analysis

Five micro-liter dodecane phase was sampled at indicated time and diluted with 45uL ethyl acetate, which was analyzed by GC-MS as described in (Tsuruta et al. 2009). In vitro synthetic amorphadiene standard was used to construct calibration curve for quantification.

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Figure caption

Figure 1 Characterization of the two promoter system

A: illustration of amorphadiene biosynthetic pathway in the engineered *B. subtilis*. B: illustration of the expression vector pHT-ads and pWH-DI. C: characterization of the expression vector pHT-ads (Pgrac) and pWH-DI (PxylA) by quantitative PCR; the cells transformed with both plasmids were induced with xylose or IPTG in this experiment. Dxs and idi were expressed as polycistron. The presented data were average of three replicates, and standard errors were plotted on the graph.

Figure 2 Optimization of gene expression by the two promoter system for amorphadiene production

IPTG controls Pgrac promoter for expression of ads; xylose controls PxylA promoter for expression of dxs and idi. Optimal expression levels of ads and dxs-idi for amorphadiene production was identified by using various combinations of IPTG and xylose. The amorphadiene titer was measured at 48h after induction. The experiment was repeated and the same trend was produced.

Figure 3 Optimization of protein translation by using N terminal tag

A: use of the six arginine tag (6xR) did not change transcription of dxs-idi and ads (6xR.ads), but significantly increased protein abundance of ADS; B: the *B. subtilis* expressing 6xR.ads produced more amorphadiene than that expressing ads. The presented data were average of three replicates, and standard errors were plotted on the graph. 1A1 pHT-ads: *B. subtilis* 1A1 harboring pWH-DI and pHT-ads; 1A1 pHT-6xR.ads: *B. subtilis* 1A1 harboring pWH-DI and pHT-6xR.ads. The cells were induced by 0.1mM IPTG and 0.1% xylose. The transcription was quantified by qPCR and protein expression was quantified by western blot with the antibody recognizing the six-histidine tag at C-terminus of ADS and 6xR.ADS. Because (to our knowledge) no commercial antibody is available for *B. subtilis* house-keeping protein, there are no control bands of a house-keeping protein in this figure. The results were normalized to cell amounts.

Figure 4 Media optimization for amorphadiene production and studies on its underlying mechanisms

A: combinations of various concentrations of pyruvate (final concentration: 0 8, 16, 24 and 32 g/L) and dipotassium phosphate (final concentration: 0, 8, 16, 24, 32, 40 and 48 g/L) were examined for optimizing amorphadiene production in the engineered *B. subtilis*. The amorphadiene titer was measured at 48h after induction. B: gene transcription of dxs-idi and 6xR.ads was not altered during addition of the two chemicals. C: most DXP metabolites (DXP, MEP, CDP-ME, CDP-MEP and MEC) were also over-produced by addition of the two chemicals, indicating that the flux through the DXP pathway was enhanced. D: addition of pyruvate and dipotassium phosphate significantly enriched co-factors (ATP and CTP) and precursor (phosphoenolpyruvate (PEP)) of the DXP pathway. DAHP (dehydro-deoxy-arabino-heptonate phosphate) is the first intermediate in *E. coli* aromatic synthetic pathway, which also derives from PEP (Supplementary file S2). Addition of dipotassium phosphate also buffered the medium pH during cell growth, and this is discussed in Supplementary file S6. The cells were induced by 0.1mM IPTG and 0.1% xylose. The presented data were average of three replicates, and standard errors were plotted on the graph. Three representative conditions were examined in the transcription and metabolite analysis (N.A.: No chemical addition; +P: addition of 8g/L pyruvate; +P+K: addition of 8g/L pyruvate and 32g/L dipotassium phosphate).

Supplementary files

Supplementary file S1 Screening of chemical additives

Supplementary file S2 Illustration of metabolic pathway related to this study

Supplementary file S3 Nucleotide sequence of codon optimized ads

Supplementary file S4 Primers used in this study

Supplementary file S5 Control experiment (RT minus) for the transcription quantification

Supplementary file S6 Effects of dipotassium phosphate on medium pH during cell growth

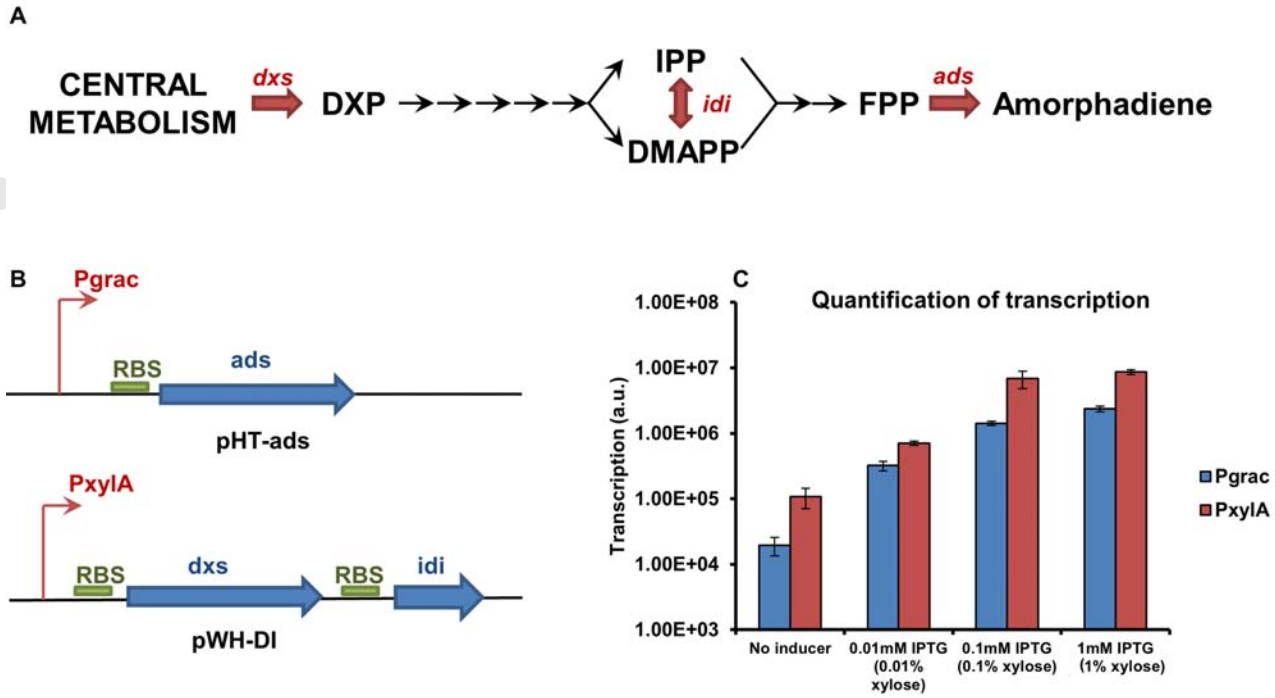

Figure 1

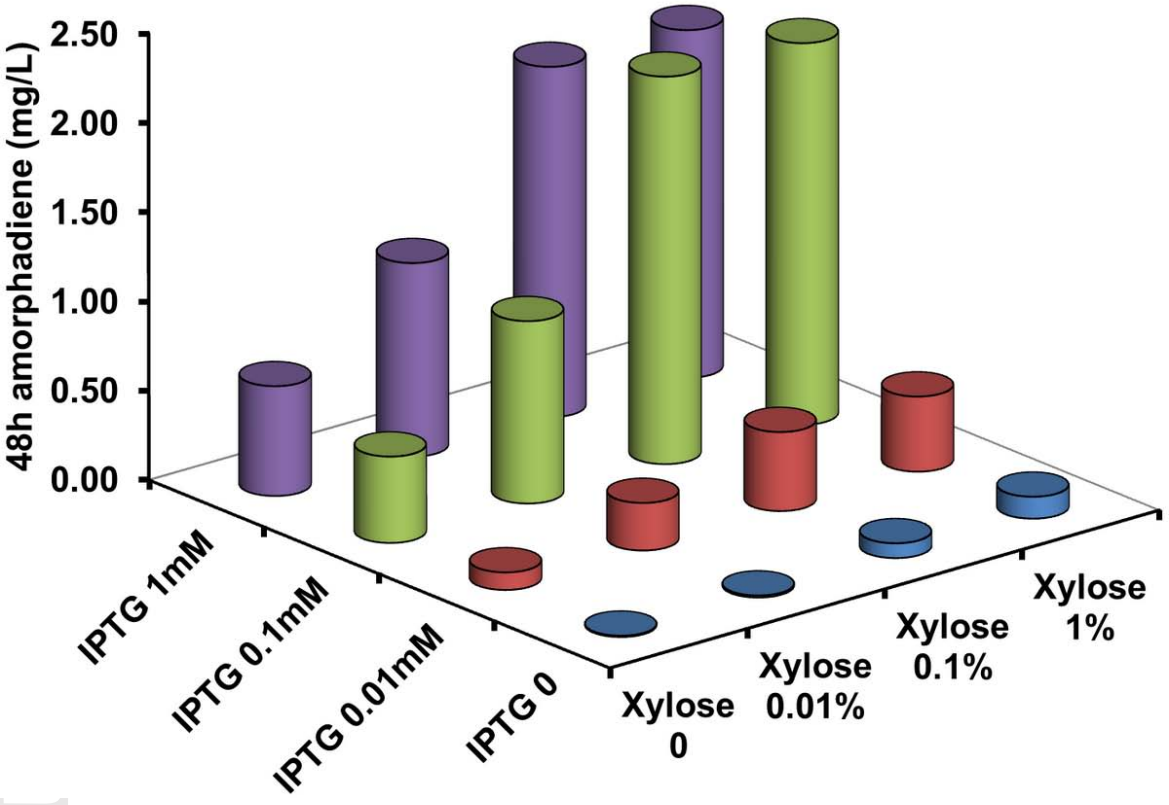

Figure 2

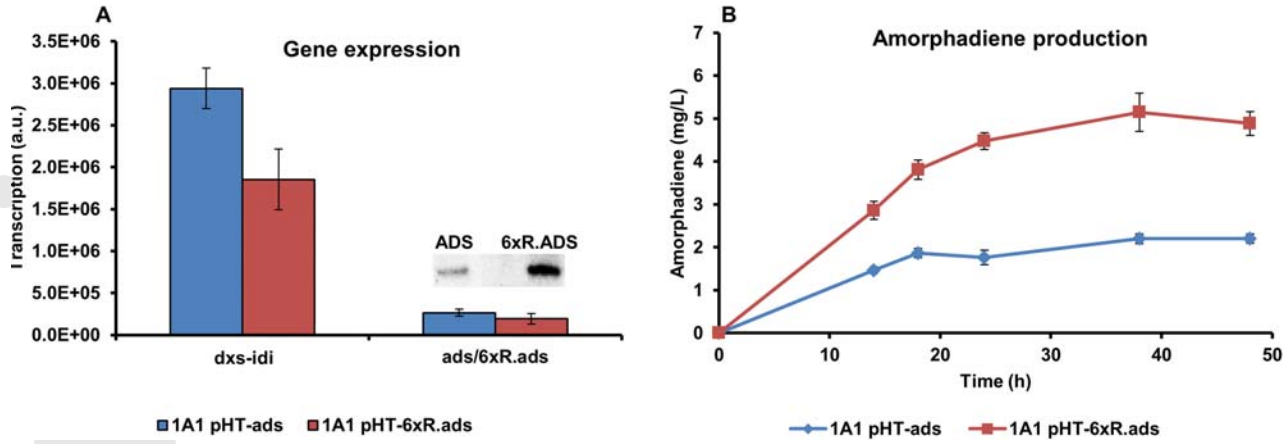

Figure 3

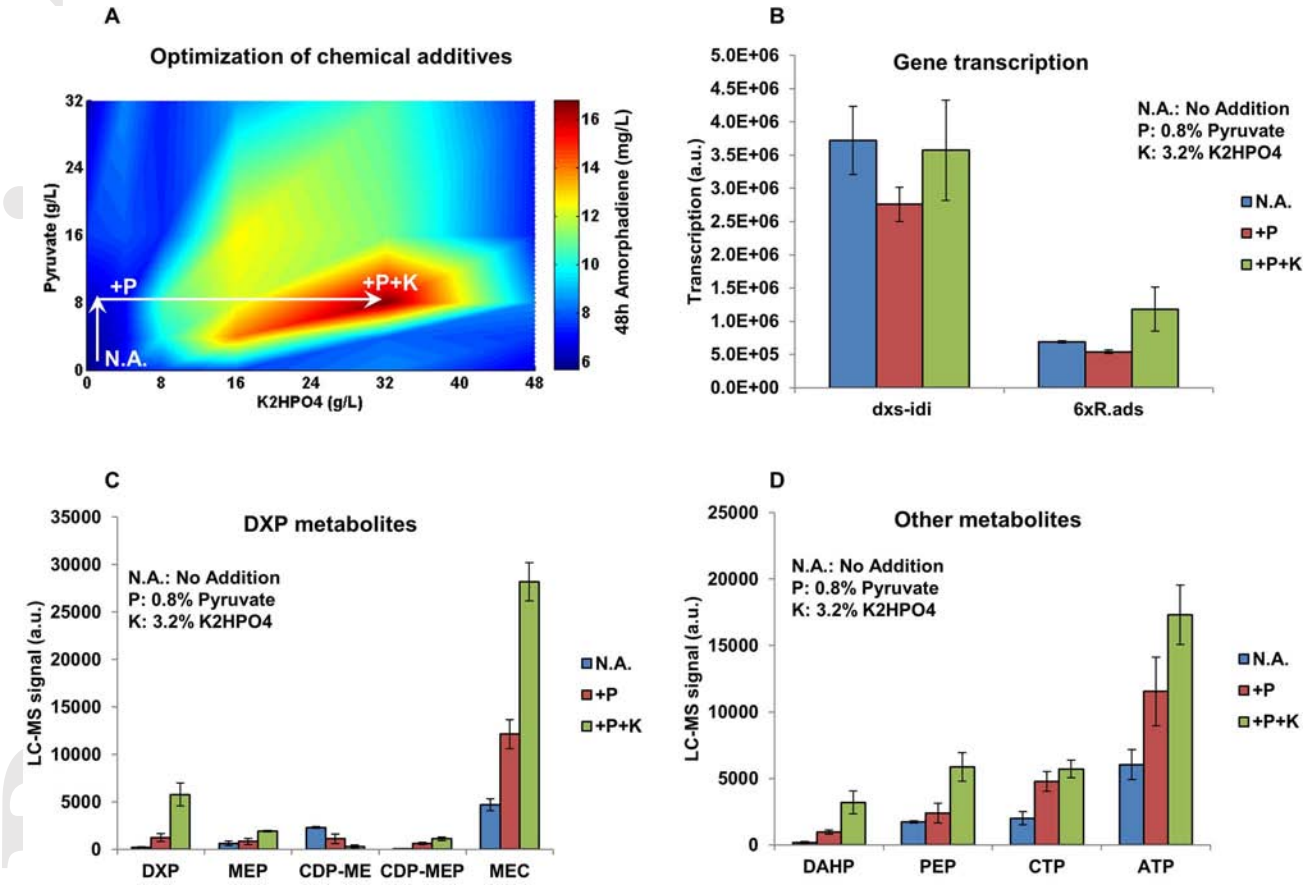

Figure 4