Unique Metabolic Reprogramming in *Pseudomonas Aeruginosa* P4 Facilitates the Unusual Concurrence of P-solubilizing and Biocontrol Traits Under P-limitation

Vaishnawi Gupta
Charotar University of Science and Technology PD Patel Institute of Applied Sciences
Aditi Buch (aditibuch.biochem@charusat.ac.in)
Charotar University of Science and Technology PD Patel Institute of Applied Sciences
https://orcid.org/0000-0003-2048-464X

Research Article

Keywords: Plant-beneficial P. aeruginosa, P-limitation, P-solubilization, Biocontrol, Metabolic Reprogramming, Multipotential PGPR

Posted Date: August 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-749441/v1

Abstract

Plant-beneficial fluorescent *Pseudomonas* species with concurrent P–solubilizing and biocontrol traits could have improved rhizospheric survival and efficacy; although this rare ability is subject to diverse environmental and endogenous regulations. This study investigates the P-limitation-induced regulations enabling co-production of metabolites implied in P-solubilization and biocontrol by *P. aeruginosa* P4 (P4). P-limitation reduced the growth rate of P4 while metabolic distribution of total glucose depleted shifted to increased periplasmic gluconic acid production. Remarkably, despite a parallel reduction in glucose consumption, the production of biocontrol metabolites like pyocyanin, total siderophores and IAA enhanced under P-solubilizing conditions. The study indicates a metabolic rearrangement in P4 under P-limitation such that biomass yield is maintained while adjusting the chorismate-mediated secondary metabolism. A time course analysis on glucose and artificial root exudates, unusually reduced *aroC* expression (encoding the rate-limiting enzyme chorismate synthase) and growth phase-dependent changes in the expression of key biosynthetic genes *pchA, pchE*, *pchG, pvdQ* and *phzM* suggested that although the enhanced production of chorismate-derived secondary metabolites could be attributed to P-limitation, the underlying gene expression could be governed by multiple factors. While such metabolic flexibility could impart physiological advantage in sustaining P-starvation stress, it manifests as unique coexistence of P-solubilizing and biocontrol abilities.

Introduction

Metabolically versatile fluorescent *Pseudomonas* species, known to be aggressive root colonizers, dominate as plant growth promoting rhizobacteria (PGPR) with multiple biocontrol abilities including suppression of soil-borne diseases, nutrient competition and induction of the plant defenses (Lugtenberg et al. 2009; Dutta and Podile 2010; Kupferschmied et al. 2013). Conversely, several *Pseudomonas* spp. also improve plant growth in the absence of pathogens by direct mechanisms often associated with mineral phosphate (P) solubilization, N_2- fixation and regulation of the levels of phytohormones. However, fewer *Pseudomonas* strains demonstrate co-expression of biocontrol and P-solubilizing abilities under a given rhizospheric condition. Since, most agricultural soils are insufficient with respect to P nutrient (Gyaneshwar et al. 2002); multifunctional fluorescent *Pseudomonas* sp. exhibiting efficient biocontrol ability concurrent with P-solubilizing ability could prove advantageous for efficient establishment in the rhizosphere.

Major biocontrol abilities of fluorescent *Pseudomonas* species are commonly attributed to the production of a wide spectrum of secondary metabolites including siderophores, pyoluteorin, pyrrolnitrin, phenazines like phenazine-1-carboxamide, phenazine-1-carboxylic acid and pyocyanin, HCN, 2,4-Diacetylpholorglucinol (2,4-DAPG) and rhamnolipids to name some (Kupferschmied et al. 2013). Secondary metabolites like indole acetic acid (IAA), cytokinins and gibberellins act as phytohormones and also benefit the plant by eliciting defense responses. On the other hand, primary metabolites like gluconic acid and 2-ketogluconic acid are implicated in the direct plant growth promotion *via* inorganic P-solubilization. Production of these metabolites by fluorescent *Pseudomonas* species is differentially

influenced by various abiotic environmental factors like the nature of carbon source, pH, oxygen as well as nutrient (especially Fe and P) availability (Duffy and Defago, 1999). Of these, P-availability and nature of carbon source are most crucial since they could directly influence the functioning of the metabolic pathways responsible for the biosynthesis of the implicated metabolites. The expression of genes involved in the biosynthesis of these metabolites could also be regulated, negatively or positively, by components of plant root exudates and extracellular metabolites secreted by other beneficial and pathogenic rhizospheric microbes (Dutta and Podile 2010; Avis et al. 2008). Besides, the inherent global regulatory systems and quorum sensing mechanisms also regulate the production of biocontrol metabolites in *Pseudomonas* species (Heeb and Haas 2001). Considering such complex regulatory mechanisms influencing the production of the plant growth promoting metabolites, it can be reasonably argued that the overall beneficial impact of multipotential fluorescent *Pseudomonas* sp. on the plants would often be a net result of only those growth promoting mechanisms that may be activated simultaneously under the given rhizospheric conditions.

Eventually, for a multipotential fluorescent *Pseudomonas* strain to demonstrate concurrent P-solubilization and biocontrol abilities, ideally gluconic acid and/or 2-keto gluconic acid should be simultaneously produced with the other biocontrol metabolites, under P-limiting conditions even in the presence of other influencing edaphic factors. Parenthetically, even for the most explored *P. protegens* CHA0, negative regulation of the production of major biocontrol metabolites 2,4-DAPG and pyoluteorin by gluconic acid, makes it difficult for P-solubilizing ability to co-exist with biocontrol traits (De Werra et al 2009). However, such studies exploring the regulatory mechanisms governing the prevalence of plant growth promoting traits are relatively fewer. Most studies on multipotential fluorescent *Pseudomonas* sp. have focused on monitoring the multiple plant growth promoting traits independently *in vitro* under respective ideal growth conditions, followed by demonstrating their effects on plant growth and health.

The present work demonstrates the unusual co-existence of P-solubilization and multiple biocontrol abilities under P-limitation in a root-associated *Pseudomonas aeruginosa* P4 (P4). We demonstrate the co-production of biocontrol and P-solubilizing metabolites like gluconic acid, IAA, siderophores and pyocyanin in a P-limited minimal medium mimicking soil buffering capacity without supplementation of any precursor substrate. Subsequently, this organism has been explored as a model to understand the genetic and metabolic alterations that facilitate simultaneous enhancement in the production of these metabolites in response to P-limitation.

Materials And Methods

Bacterial strain, culture conditions and reagents

Isolate *P. aeruginosa* P4 [MCC No. 2365] used as the model strain in all the experiments, was kindly gifted by Prof. G. Naresh Kumar, Department of Biochemistry, The M S University of Baroda, India. The strain was grown and maintained on Pseudomonas agar (Hi-Media Laboratories, Mumbai, India) at 30°C, as required. The culture media, dextrose, Tris base and other routine analytical-grade salts and reagents were

procured from SD Fine Chemicals Ltd., Hi-media Laboratories and Merck Life Science Pvt. Ltd. Specific molecular biology reagents like RNase, Taq DNA polymerase with its buffer and dNTPs, lysozyme, diethyl pyrocarbonate (DEPC), RNAse Zap solution, Trizol etc. were procured from Sigma Aldrich.

Physiological experiments

A single colony of P4 was aseptically inoculated in sterile 3 ml of Luria broth and bacterial growth was facilitated by overnight incubation in a rotary shaker maintained at 30 ˚C and 150 rpm (Scigenics Biotech Pvt Ltd). The freshly grown culture was harvested by centrifugation (8000 rpm for 5 minutes at room temperature), washed twice with sterile normal saline and finally resuspended in 1 ml of the same under aseptic condition. This cell suspension was used as inoculum for further experiments.

An aliquot of fresh inoculum was used to inoculate 100 ml of Tris-buffered minimal medium supplemented with 100 mM glucose and micronutrient cocktail (Buch et al. 2008) to give an initial cell density of OD_{600nm} 0.01–0.03. Tris-minimal medium supplemented with 10 mM Pi was used as a P-sufficient condition while the same with 0.066 mM Pi was used to represent P-limited condition. Batch culture studies were performed by shaking 250 ml conical flasks containing 100 ml of the inoculated media on a rotary shaker (30 ˚C, 150 rpm). Two-milliliter culture samples were withdrawn aseptically at regular intervals to measure bacterial growth as a function of OD_{600nm} and media pH. The observations were continued until the pH of the medium decreased to less than 5, under both P-sufficient and P-deficient conditions. At the point of termination, cell-free culture supernatants were obtained by centrifuging the harvested cultures (10,000 rpm, 5 minutes, room temperature) and stored at -20 ˚C until further analysis of metabolites.

The glucose concentration in the growth medium was monitored using the GOD-POD kit (ARKRAY Healthcare Pvt. Ltd., India). The difference between the glucose concentrations measured in the initial and terminal samples were used to calculate the total glucose depleted during the bacterial growth; while the difference between the total glucose depleted and gluconic acid produced was used to measure the amount of glucose consumed. Biomass yield was calculated as dry cell mass produced per unit of glucose consumed. The dry cell mass was determined by centrifuging a fixed volume of the terminal bacterial culture, discarding the supernatant, washing the cells using distilled water and weighing the cell pellet dried at 60 ˚C for 3 days. Growth rate (μ) and specific glucose depletion rate (Q_{Glc}) were calculated using log phase culture growth recorded as OD_{600nm}; for which the cell number was derived using the proportionality constant of 1 OD_{600nm} = 1.5 10^9 cells ml^{-1} (Buch et al., 2008).

The effect of artificial root exudates (ARE) on growth and secondary metabolite production by P4 was monitored using a similar experimental setup, where 100 mM glucose in the Tris-buffered minimal medium composition described above was replaced using a synthetic root exudate cocktail. The final concentrations of ARE components in the medium were adjusted as follows: 18.4 mM glucose, 18.4 mM fructose, 9.2 mM xylose, 9.2 mM sodium citrate, 18.4 mM sodium gluconate, 13.8 mM sodium succinate, 9.2 mM alanine, 9.2 serine, 9.2 glycine. The choice and the level of the carbon source ingredients in ARE

was based on their known occurrence in peanut root exudates and the other ARE compositions used for similar study (Baudoin et al., 2003; Dutta et al., 2013)

Metabolite analysis

Cell-free culture supernatants derived as above, from cultures at the point of experimental termination were subjected to various analytical techniques to estimate the levels of selected metabolites. A portion of the stored culture supernatants was filtered through 0.2 µm nylon membranes (MDI Advanced Microdevices, India) before subjecting to HPLC analysis (equipment from Waters India Pvt. Ltd.).

Gluconic acid levels were quantified using an RP-18 column operated at room temperature using a mobile phase of 20 mM NaH_2PO_4 at a flow rate of 1.0 ml min^{-1}. The column eluates were monitored using a UV detector at 210 nm and gluconic acid was detected and quantified by comparing the retention time and peak areas obtained for pure gluconic acid standard (10 mM) subjected to similar analytical conditions.

Total siderophores were quantitated using Chromo-azurol S solution assay of P4 culture supernatants and expressed as EDTA equivalents (Leclère et al. 2009). Siderophore pyochelin was detected and quantified by HPLC using an isocratic gradient comprising of 70 % Solvent A (H_2O, 0.1 % trifluoroacetic acid) and 30 % Solvent B (95 % acetonitrile, 0.1 % trifluoroacetic acid) and a flow rate of 1 ml/min at 254 nm (Youard et al. 2007). Pyochelin standard was prepared using layer chromatography (TLC) of the supernatant of 48 h old P4 culture grown on King's B medium. A 50 µl aliquot of the culture supernatant was spotted on pre-coated Silica Gel G sheets (0.2 mm; Xtra SILG/UV254, Macherey Nagel GmbH & Co. KG), air-dried and chromatographed with the solvent system, chloroform:acetic acid:ethanol (50:5:2.5 vol/vol) (Farmer and Thomas 2004). The appearance of characteristic yellow fluorescence upon visualization under UV light at R_f ~0.35 cm; turning typical red-brown when sprayed with 0.1 M $FeCl_3$ in 0.1 M HCl indicated the presence of pyochelin. The silica portion at around the appropriate spot was scraped and used to extract pyochelin in 100 µl of methanol, for further use as the reference standard in HPLC. The area under the appropriate peak, as compared with the standard, was used as a measure to quantify pyochelin. An untargeted metabolite detected in HPLC chromatograms was further identified using LC-MS and HR-LC-MS, respectively obtained as an outsourced service from SAIF, IIT, Bombay and Sophisticated Instrumentation Centre for Applied Research and Testing (SICART), Vallabh Vidyanagar, Gujarat, India. LC-MS was performed using LCQ Fleet and TSQ Quantum Access with Surveyor Plus HPLC System, Thermo Scientific, USA while HR-LC-MS was performed using 1290 Infinity UHPLC System, Agilent Technologies, USA by employing the aforementioned chromatography conditions with minor modifications. Pyoverdine production was determined by measuring characteristic fluorescence at 400 ± 10/460 ± 10 nm excitation/emission of 0.1 ml culture supernatants and expressed as relative fluorescence units (RFU) (Dumas et al. 2013). Pyocyanin levels were measured spectrophotometrically at 690 nm using the culture supernatants extracted with chloroform (1:2 v/v) and were calculated using the extinction coefficient of ε = 4,130 M^{-1} cm^{-1} at pH 7 (Dietrich et al. 2006). IAA levels were determined spectrophotometrically at 535 nm using Salkowski reagent (Ahmad et al. 2008), against a standard curve

generated using pure IAA. Each metabolite level was normalized to the amount of glucose consumed, to calculate their production yields.

Effect of nature and concentration of carbon source on production of secondary metabolites was determined in a time course analysis. Supernatants derived from culture aliquots harvested at regular time intervals during growth on 100 mM Tris-buffered medium with (i) 100 mM glucose and (ii) ARE, as sole carbon source. were used to measure the production of total siderophores, pyocyanin and IAA.

RT-qPCR based expression analysis of selected genes

P4 culture was allowed to grow under shaking conditions on P-sufficient and P-limited media and the cells were harvested across two growth stages (i) the experimental endpoint and (ii) mid-log phases. The cells were immediately used for total RNA isolation using Qieasy RNA isolation kit as per manufacturer's recommendations with minor variations. RNA yield and purity were determined as A260/A280 and A260/A230 ratios using QIAxpert nano-spectrophotometer. Subsequently, 1 µg of RNA was reverse transcribed to yield cDNA using oligo$(dT)_{18}$ primers at 42°C using Maxima H Minus reverse transcriptase (ThermoFisher Scientific) under following conditions: 25 ºC for 10 minutes, 42 ºC for 50 minutes and 85 ºC for 5 minutes. The cDNA synthesized was stored at − 80°C until further use. Subsequently, the real-time PCRs were conducted in Qiagen Rotor-Gene Q3000 5Plex HRM platform using Kapa SYBR Fast Universal qPCR kit as per manufacturer's instructions. The thermal cycle program used was as follows: initial denaturation at 95 ºC for 10 min followed by 35 cycles of denaturation at 95 ºC for 10 s, primer annealing 60 ºC for 1 min and extension at 72 ºC for 1 min, with a final extension at 72°C for 10 min. Specific gene fragments were amplified using primer sets either designed using Primer3 tool or adopted from published literature (Huang et al. 2009; Lopez-Medina et al. 2015; Supplementary material-I]. Specific amplification was verified using melt curve analyses. For relative quantification, the expression of each target gene was normalized to *rpoD* used as an internal control. Relative fold change in expression of the target gene under P-limitation as compared to P-sufficiency was determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Before subjected to real-time PCR, the specificity of the selected primer pairs was validated by using them for standard PCR amplification using P4 genomic DNA and confirming the presence of the desired amplicon as a single band with predicted size on ethidium bromide-stained 2 % agarose gel (Supplementary material-I).

Statistical analysis

Experimental data was derived from substantial number of replicate observations and expressed as standard statistical measures as indicated in the table footnote and the figure legends. For comparing the parameters across the two experimental conditions the statistical significance of differences was determined by two-tailed student's t test computed using Microsoft Excel (Microsoft office version 10).

Results

Growth, media acidification and gluconic acid production under P-sufficient and P-limited conditions

As a prerequisite to monitoring the production of plant beneficial metabolites, it was necessary to demonstrate the impact of P-sufficient and P-limited conditions on the growth of P4. When allowed to grow on glucose as sole carbon source, acidification of extracellular medium to pH < 5 was achieved irrespective of the Pi status; however, the time required for acidification increased from 48 h on P-sufficient condition to 72 h under P-limitation. At these respective time points, the maximum cell density achieved for P4 on the P-sufficient medium (1.71 ± 0.29 OD_{600nm}) was about ~ 2.5 fold higher as compared to that on P-limited medium (0.71 ± 0.11 OD_{600nm}); with a corresponding difference in the growth rate apparent (Fig. 1). An equivalent amount of glucose was depleted by P4 at the end of 48 and 72 h of growth under P-sufficient and P-limited conditions, respectively. However, of the total glucose depleted, about 78 % was metabolized intracellularly *via* phosphorylative oxidation (as indicated by the amount of glucose consumed) under P-sufficiency, which reduced to around 52 % under P-limitation (Table 1). The remaining of the total depleted glucose was utilized to produce gluconic acid through the periplasmic direct oxidation pathway. Accordingly, the total glucose consumed by P4 under P-limitation reduced by ~ 1.5 fold while the gluconic acid yield under P-limitation increased by ~ 2 folds as compared to that under P-sufficiency (Table 1). Gluconic acid production is chiefly responsible for the media acidification by P4 and thereby for its excellent P-solubilizing ability (Buch et al. 2008). Remarkably, the P-status did not alter the biomass yield of P4 (Table 1).

Influence of P-status on co-existence of P-solubilization and biocontrol traits

The concurrence of biocontrol traits with P-solubilization in P4 was monitored as a function of the production of selected metabolites viz. siderophores, IAA and pyocyanin, in the same 48 and 72 h old spent media (pH < 5) that was used to determine the gluconic acid levels. The influence of P-status on the production of these metabolites was determined by comparing their yields. The production of the antifungal metabolite pyocyanin and the phytohormone IAA under P-limitation increased by ~ 16 and ~ 3 folds, respectively as compared to that under P-sufficiency (Table 1). Total siderophore production under P-limitation also increased significantly by ~ 3 folds as compared to the P-sufficient condition (Table 1). Usually, *P. aeruginosa* species is known to produce two major types of siderophore *viz* pyoverdine and pyochelin (Dumas et al. 2013; Lopez-Medina et al. 2015). The fluorimetric detection of pyoverdine in the culture supernatants as a peak at ~ 467 nm and its quantitation revealed that its production under P-limitation increased by ~ 2-fold (Fig. 2, Table 1). Pyochelin was detected in both P-sufficiency- and P-limitation-derived culture supernatants, as a characteristic peak retained at ~ 15 minutes as per the HPLC chromatograms (Fig. 3). The relative pyochelin yield under P-limited condition was enhanced by ~ 6.5 folds as compared to that on P-sufficient condition (Table 1). The significant presence of an additional peak at R_t ~30.5 minutes in the chromatograms and its comparative analysis indicated ~ 1.5-fold decrease in the yield of the corresponding untargeted metabolite under P-limitation as compared to P-sufficiency. The LC-MS and HR-LC-MS analysis identified this metabolite as 5-hydroxytryptophan with an R_t ~31.99 and m/z ratio of 225.08 (Fig. 4).

RT-qPCR-based expression analysis of selected genes involved in the biosynthesis of biocontrol metabolites

A genetic basis for the enhanced production of the biocontrol metabolites by P4 in response to P-limitation was investigated by monitoring the relative expression of crucial biosynthetic genes. The selection of the genes was based on the existing knowledge about the biosynthetic pathways as well as organization, specific roles and regulation of the biosynthetic genes in *P. aeruginosa* species. Accordingly, *pchA*, *pchE* and *pchG* were selected to represent pyochelin biosynthesis, where *pchA* is the last gene in *pchDCBA* operon and encodes the rate-limiting enzyme in the biosynthesis of an essential precursor salicylic acid while *pchE* and *pchG* encode non-ribosomal peptide synthetase and a reductase, crucial for converting salicylate to dihydroaeruginoate and subsequently to pyochelin, respectively (Reimmann et al. 2001; Gaille et al. 2003). Since pyoverdine biosynthesis is a complex cellular process involving a large number of genes, for the present study the representative genes were selected based on their critical involvement in various phases of pyoverdine biosynthesis and its regulation. The gene *pvdH* encodes an enzyme essential for the synthesis of an unusual amino acid L-2,4-diaminobutyrate involved in the formation of core chromophore, further leading to the biosynthesis of the precursor ferribactin. This ferribactin undergoes maturation in the periplasm to form pyoverdine by the action of *pvdQ* encoded hydrolase; while *pvdS* functions as an alternative sigma factor regulating pyoverdine biosynthesis (Ringel and Bruser 2018). The *phzM* gene was selected considering its important role in converting phenazine-1-carboxylic acid to pyocyanin (Sterritt et al. 2018). The gene *aroC* was selected to represent the entry point of overall secondary metabolism while the *rpoD* gene was included in the study as the internal control.

PCR amplification of these selected genes with specific primers was optimized using P4 genomic DNA as a template and was validated by ensuring the presence of amplicons as a single band of the desired size, through agarose gel electrophoresis (Supplementary material-I). The RT-qPCR-based expression pattern for these genes was analyzed using P-sufficiency- and P-limitation-grown endpoint cultures of P4 harvested at the time of media acidification (pH < 5), to correlate the biocontrol gene expression under P-solubilizing conditions. Total RNA isolated from appropriately grown P4 culture replicates was quantified to be in the range of 400–900 ng/µL with 260/280 and 260/230 ratio ranging from 2.07–2.16 and 1.8–2.3, respectively. Subsequently synthesized cDNA could yield specific real-time PCR amplification as confirmed based on the melt curve analysis. Relative gene expression analysis revealed that in response to P-limitation the expression of the genes *pvdQ*, *pchA*, *pchE*, *pchG* and *phzM* significantly increased by ~ 1.3, 1.2, 1.3, 1.4 and 1.3 folds, respectively while the expression of *pvdS* and *pvdH* genes remained unaltered (Fig. 5). Remarkably, the expression of *aroC* significantly reduced by ~ 1.5 fold under P-limitation (Fig. 5). The expression profile of five out of six genes showing altered expression in the endpoint cultures, was again monitored using similarly grown P4 cultures harvested in mid-log phase (24 and 36 h of growth under P-sufficiency and P-limitation; media pH ~ 7), to determine the true impact of P-limitation and rule out plausible impact of pH and growth phase. In the mid-log phase cultures of P4, the expression of *pchA* and *pchE* increased by ~ 1.6 and 2-fold, respectively while that of *aroC* reduced by ~ 1.5 fold; however, the expression of *pvdQ* and *pchG* reduced by ~ 1.5 and 2-fold, respectively unlike that in the endpoint cultures. The expression of *phzM* was not monitored in mid-log phase cultures of P4 since induction of its expression under P-limitation in *P. aeruginosa* has been clearly established (Bains et al. 2012).

Combined effect of P-status and carbon source on secondary metabolite production-A time course analysis

The purpose of the *in vitro* culture analysis so far was to study P-status driven modulations in the biosynthesis of the secondary metabolites mediating plant-beneficial interactions, using a defined minimal medium containing excess of glucose as a standard carbon source. This is unlike in rhizospheric scenario the plant root exudates, usually comprising variety of sugars, organic acids amino acids and a range of aromatic compounds, serve as the major carbon source. Nature and quantity of carbon source is yet another determinant of secondary metabolite production by PGPR (REF). Since demonstrating such plant-beneficial phenotypes *in planta* would be difficult, a closely mimicking system was constituted to by replacing excess glucose in the defined minimal medium by an artificial root exudates (ARE) cocktail. Furthermore, cell density and growth phase are also known to influence the production of selected secondary metabolites. Hence, to examine the effects of these multiple factors, a time course analysis was performed to measure the production of selected secondary metabolites.

Even when grown in presence of ARE as carbon source, growth rate of P4 under P-sufficient conditions was ~ 2 fold higher than that observed under P-limitation (Fig. 1 C; Table 1). At the point of experimental termination, the maximum cell density attained for ARE-grown P4 on the P-sufficient medium after 48 h (1.28 ± 0.12 OD_{600nm}) was only ~ 1.2 fold higher as compared to that on P-limited medium after 78 h (1.00 ± 0.02 OD_{600nm}). No media acidification was observed in any case (Fig. 1 D). Production of total siderophores, IAA and pyocyanin at the experimental endpoint under P-limitation increased by ~ 2.5, 1.2 and 13 folds respectively, as compared to that under P-sufficiency (Table 1). While the levels of IAA and total siderophores produced upon growth on ARE remained similar to those produced in presence of 100 mM glucose, the pyocyanin production on ARE increased by ~ 3 fold as compared to that using glucose as sole carbon source (Table 1).

Combined effect of carbon source and growth stages was determined by monitoring the production of selected secondary metabolites at regular time intervals. When grown on 100 mM glucose, irrespective of the P-levels, the production of total siderophores, IAA and pyocyanin was apparent through mid-log phase of growth. Subsequently, under P-limitation, IAA and pyocyanin production increased and peak during late log phase, while siderophore production apparently remained consistent through the growth phases (Fig. 6 A, C, E). Under P-sufficiency the metabolite levels were more or less maintained through the growth phases. P-limitation driven peaking in IAA and pyocyanin production towards the late-log phase when grown on glucose as sole carbon source was further evident when the measurements were normalized to cell densities (Fig. 6 D, F). However, siderophore production showed a relative decrease with increasing cell mass (Fig. 6 B). Metabolites levels in the earlier time points were either undetected or not measured due to poor cell growth.

On the contrary, upon growth using ARE as carbon source, production of total siderophores and pyocyanin marginally peaked towards late-log phase and then stabilized (Fig. 7A, E) while IAA production apparently decreased towards the late log and stationary phase (Fig. 7C). Under P-limitation, IAA and

total siderophore production increased from that observed in mid-log phase, peaked towards the late log phase and then stabilized through the stationary phase (Fig. 7A, C) but pyocyanin production showed an interesting diauxic pattern of production near the late-log phase (Fig. 7E). Again, the P-status of dependent increase in the production of the selected metabolites was clearer upon normalizing the measurements with cell densities (Fig. 7B, D, F).

Discussion

Multipotential fluorescent *Pseudomonas* species are promising bio-inoculants owing to their ability to secrete a wide range of secondary metabolites; however, their impact on plant growth and health may not be necessarily cumulative owing to their inability to coproduce these metabolites under natural conditions where the soil is mostly nutrient-limited. Especially with respect to commonly prevalent P-limitation in agricultural soils, *Pseudomonas* with concurrent P-solubilizing and diverse biocontrol abilities could have clear advantage in terms of successful establishment and performance in P-starved rhizosphere. Irrespective of the rhizosphere, *P. aeruginosa* species predominate as the multipotential PGPR demonstrating both P–solubilizing and biocontrol abilities (Gupta and Buch 2019). P4 is an excellent P-solubilizer owing to high gluconic acid production (Buch et al. 2008) and is also capable of producing biocontrol metabolites like siderophores, IAA, pyocyanin and HCN under ideal experimental conditions (Gupta et al. 2020). This work aimed to investigate co-production of the aforementioned metabolites by P4 under P-limitation and understand the genetic and metabolic modulations that could facilitate this unusual co-existence of P-solubilization and multiple biocontrol abilities.

Under experimentally attained P-solubilizing conditions i.e. at the time of media acidification under P-limited growth conditions with glucose as sole carbon source, P4 simultaneously enhanced the production of the primary metabolite gluconic acid as well as that of the secondary metabolites like siderophores pyoverdine and pyochelin, pyocyanin and IAA, despite reduced growth rate. The selection of these metabolites for the study was considering that they are typically produced by *P. aeruginosa* species and are implied in major direct and indirect plant growth promoting mechanisms, through their primary or secondary actions; except IAA which it rarely produces (Hu et al. 2010). In this regard, gluconic acid primarily responsible for P-solubilization by fluorescent *Pseudomonas* species is also known to act as an antifungal agent (Kaur et al. 2006). Likewise, both pyoverdine and pyochelin, apart from improving Fe availability for plants and restricting the same for phytopathogens, can also trigger ISR in plants (Kaur et al. 2006; Aznar and Dellagi 2015). In fact, increased pyoverdine synthesis could explain improved sustained peanut root colonization by P4, as in *P. putida* S11 (Ponraj et al. 2013; Gupta et al. 2020). Pyocyanin, a phenazine with a major role in antibiosis of fungal pathogens, is demonstrated to elicit ISR against *Magnaportha grisea* (De Vleesschauwer et al. 2006). PGPR-produced IAA with a primary role in root development, can also prime ISR response in plants as well as signal for establishing beneficial plant-microbial interactions (Spaepen and Vanderleyden 2011; Mei et al. 2014). HCN production by P4 was not included in the present study since pyocyanin was already included to represent antibiosis.

Enhanced production of pyoverdine, pyochelin and pyocyanin by P4 in response to P-limitation, was in accordance with the several earlier studies which demonstrated that production of these metabolites by *P. aeruginosa* species is inversely correlated with P levels (Zaborin et al. 2012; El-Fouly et al. 2015; Ringel and Bruser 2018). Relative to pyoverdine, pyochelin production was enhanced to a greater extent, probably because the biosynthesis of the latter under P-starved conditions could be metabolically cheap (Dumas et al. 2013). However, an increase in tryptophan-independent IAA production by P4 under P-limitation was unusual. Reduced production of an untargeted metabolite 5-hydroxytryptophan (not known to directly influence plant growth promotion) may imply a redistribution of the anthranilate flux towards increased IAA biosynthesis by P4 (Supplementary material-II). Several *Pseudomonas* species including *P. aeruginosa* are known to convert L-Tryptophan into 5-hydroxytryptophan using phenylalanine hydroxylase homologs (Ma et al. 2016). Influence of P-levels on IAA production by fluorescent *Pseudomonas* species is largely unexplored except a recent study that showed a negative correlation between P-levels and IAA production by *P. putida* (Srivastava and Srivastava 2020). On the other hand, unlike in *P. protegens* CHA0 and *P. putida* S11 where gluconic acid repressed the production of 2,4-DAPG and pyoverdine, respectively (De Werra et al. 2009; Ponraj et al. 2013), it didn't influence the production of the biocontrol metabolites by P4. From an efficacy point of view as multipotential PGPR, such enhanced co-production of P-solubilizing gluconic acid and the prime biocontrol metabolites by P4, even under P-limitation is remarkable and suggestive of unique metabolic and genetic regulations.

Under P-limitation, a relatively increased gluconic acid production with concomitantly reduced glucose consumption, suggested that P4 efficiently redirected glucose towards periplasmic oxidation by a PQQ-dependent glucose dehydrogenase while reducing the cytoplasmic glucose catabolism. A similar metabolic channelling of glucose by P4 under P-deficiency has been demonstrated previously (Buch et al. 2008). Correlating the findings of the previous and the current studies, a metabolic scenario was predicted to explain the intracellular fate of the glucose consumed under P-limitation (in terms of its distribution across catabolic pathways) and its impact on the growth and metabolite yields. Accordingly, under P-limitation the cytoplasmic glucose catabolism *via* ED pathway and TCA cycle gets relatively compromised causing reduced growth rate. The PP pathway which is crucial for both anabolism and shikimate pathway-mediated secondary metabolism, probably redirects its carbon flux towards biomass production as reflected by unaltered biomass yield. This in turn implied relatively reduced availability of carbon for secondary metabolism, typically chorismate biosynthesis at the end of the shikimate pathway. Biosynthesis of chorismate could be crucial considering that it serves as the branch-point metabolite in the synthesis of aromatic amino acids as well as the secondary metabolites including pyoverdine, pyocyanin and pyochelin (Sterritt et al. 2018). Significantly reduced *aroC* expression in P4 under P-limitation irrespective of the growth phase substantiated the hypothesis that P-limitation caused reduced biosynthesis of chorismate in P4. Enhanced production of the selected chorismate-derived biocontrol metabolites, despite reduced chorismate supply under P-solubilizing conditions (P-limitation, media pH < 5) could be subsequently attributed to the upregulated expression of key biosynthetic genes *pvdQ*, *phzM*, *pchA, pchE* and *pchG*. Although the apparent alterations in the gene expressions were marginal, they

could lead to significant physiological impact given that most of the selected genes encoded the branch-point, rate-limiting or standalone enzymes crucial for respective biosynthetic pathways.

A similar P-depletion induced expression of pyoverdine, pyochelin and phenazine biosynthetic genes is also reported in *P. aeruginosa* PAO1 (Bains et al. 2012; Zaborin et al. 2012). These studies have reported the transcriptional responses to P-starvation in *P. aeruginosa* using 0.1mM or 0.2 mM Pi; thus, suggesting that use of 0.066 mM Pi as P-limiting conditions in the present study could have sufficiently induced the P-depleting conditions for P4. Bains et al. (2012) has reported only those changes in gene expression under P-starvation that were 2-fold or greater. Accordingly, *phzM* (7.2-fold) and *pvdS* (4.3-fold) expression increased under P-starvation; while in present study, although *phzM* expression increases, *pvdS* expression remained unaltered. Similarly, Zaborin et al (2012) have reported increased expression of *pvdQ* (1.7-fold), *pvdH* (2.6-fold), *pchA* (6.3-fold), *pchE* (2.5-fold) and *pchG* (2.4-fold) under P-depleting conditions. While the similar trend of change in gene expression was seen for *pvdQ, pchA* and *pchE* in mid-log phase cultures of P4, with the expression of *pchA* and *pchE* in mid-log phase being higher than that in the stationary phase. The overall differences in the fold changes in the gene expression as compared to the reported studies could be attributed to the fact that the earlier studies have used P-depleted cultures of late-log phase, unlike the present study where mid-log and stationary phase cultures have been used. Eventually, the expression of pyoverdine and pyochelin biosynthesis genes is subject to complex regulations by multiple signals ranging from iron starvation to quorum sensing (Ringel and Bruser 2018). The protein PvdQ apart from its role in maturation of pyoverdine is also implicated in regulating 3-oxo-C12-HSL mediated quorum sensing in *P. aeruginosa* under P-depletion (Meng et al. 2020). For plant-beneficial rhizobacteria, quorum sensing is a critical determinant of effective rhizospheric colonization (Zúñiga et al. 2013). This indicates that a very complicated regulation mediated by multiple factors could influence the *pvdQ* expression in the mid-log and stationary phase cultures of P4, which cannot be sufficiently explained in the present study. The possibility of cross-regulation or feedback regulation by the co-produced secondary metabolites cannot be negated completely. On the other hand, pyochelin is known to auto-induce the entire *pchE* operon encoding the enzymes converting salicylate to pyochelin (Reimmann et al. 2001; Gaille et al. 2003). However, our results could not directly explain the growth-phase dependent expression of *pchG* in P-limited P4 cultures. PchG is a standalone NADPH-dependent reductase unique to *P. aeruginosa* species and is crucial for final tailoring of pyochelin before secretion (Schalk et al. 2020). In *P. aeruginosa* PAO1 *pchE* and *pchG* are probably organized as a part of the same operon (Reimmann et al., 2001). While similar gene organization in other strains of *P. aeruginosa*, has not been explored in detail; that in other pyochelin producing *P. fluorescens* strains Pf-5 and CHA0 differs from that in *P. aeruginosa*, with no gene having obvious sequence homology with *pchG* (Schalk et al., 2020). Therefore, difference in clustering of the pyochelin biosynthetic genes across different strains of *P. aeruginosa* cannot be negated, especially considering that P4 used in the present study has rhizospheric origin with plant-beneficial traits. A possible explanation to the different expression patterns of *pchE* and *pchG* could be an unusual occurrence of a cryptic regulatory element upstream of *pchG* within the operon in P4 genome, which could influence the *pchG* expression under the experimental conditions. No study over pyochelin biosynthetic genes so far have indicated such a

possibility, but occurrence of non-contiguous operons with complex regulations are well reported in bacterial system (Price et al., 2006; Sáenz-Lahoya et al., 2019). The present study is not enough to conclude on such a regulatory mechanism directly; however, it has interesting prospects for future investigations. Furthermore, the presence of IAA induced *pvdS* expression by ~ 2 fold in *P. aeruginosa* PAO1 (Lee et al. 2009), unlike in P4 where despite the significant enhancement in the production of IAA, *pvdS* expression remained unaltered.

The *in vitro* culture based analysis of plant-beneficial phenotypes as a function of coexisting production of secondary metabolites, although reflects unique metabolic flexibility of P4, it certainly cannot explain how these will translate into in planta phenotype, considering that the plant root exudates are going to be major carbon sources in the rhizosphere. Besides, the production of the secondary metabolites considered here is largely subject to multiple factors including growth phases and cell quorum as discussed earlier. Time course analysis of secondary metabolite production provided some clearer insights on these lines. With respect to carbon source, gluconic acid production in presence of ARE as carbon source becomes redundant since organic acids in ARE could be the preferred carbon sources for P4; thus, explaining the lack of media acidification even after 78 h of cultivation. Irrespective of the carbon source, the secondary metabolite production observed through the mid log phase, through the late log phase until the stationary phase, was significantly higher under P-limitation as compared to P-sufficiency. These observations suggest that enhanced levels of secondary metabolite production could be largely considered as a metabolic response to P-limitation.

Collectively, in response to P-limitation, P4 drastically redistributes the intracellular carbon such that production of chorismate-mediated secondary metabolites is augmented while retaining the biomass yield; without any adverse influence from the concurrently enhanced periplasmic production of gluconic acid. Such metabolic and genetic adaptations, while offering physiological advantage to the bacteria, gets manifested as unusual concurrence of P-solubilizing and multiple biocontrol traits; plausibly imparting an obvious advantage to P4 and other similar rhizospheric *Pseudomonas* species in terms of survival and efficient execution of plant-beneficial impact even under P-starvation stress. Such studies may prove useful in predicting the actual efficacies of multifaceted PGPR under given soil nutrient conditions; thereby helping in identifying efficient bioinoculants for sustainable agriculture.

Declarations

Funding:

This research was supported by Charotar University of Science and Technology (CHARUSAT)

Conflicts of interest:

The authors have no conflict of interest to disclose

Ethics approval:

Not applicable

Consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and material:

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability:

Not applicable

Authors' contributions:

Aditi Buch: Conceived the study, analyzed data, wrote the paper

Vaishnawi Gupta: Performed research, Analyzed data

Acknowledgements:

The authors thank Dr Jisha Elias and Dr Hina Mir for helping with the analysis of gene expression data. The authors are thankful to Dr Naina Patel's Akanksha Hospital & Research Institute, Anand, Gujarat, India for supporting the RT-qPCR related work. This research was supported by Charotar University of Science and Technology (CHARUSAT). We are grateful to Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay and Department of Science and Technology, Government of India for facilitating the HR-LC-MS analysis.

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Tables

Table 1

Influence of P-status on production of selected plant growth promoting and biocontrol metabolites by P. aeruginosa P4

Growth promoting and biocontrol metabolites		Yield of the metabolites produced			
		100 mM Glucose + P-sufficient condition	100 mM Glucose + P-limited condition	ARE + P-sufficient condition	ARE + P-limited condition
Gluconic acid	(mg/mg glc consumed)	0.23 ± 0.05 (5)	0.47 ± 0.11 (5) **	–	–
	(mg/ml/OD_{600nm})	1.72 ± 0.44 (5)	6.59 ± 2.2 (5) **	–	–
	Actual concentration in culture supernatants (mg/ml)	3.16 ± 0.81 (5)	5.99 ± 2.01 (5) *	–	–
IAA	(µg/mg glc consumed)	0.32 ± 0.07 (4)	1.01 ± 0.17 (4) ***	–	–
	(µg/ml/OD_{600nm})	1.79 ± 0.35 (4)	7.62 ± 1.59 (4)	5.71 ± 0.38 (3)	7.48 ± 0.25 (3)m**
	Actual concentration in culture supernatants (µg/ml)	4.14 ± 1.15 (7)	6.65 ± 1.03 (7) ***	6.28 ± 0.55 (3)	7.71 ± 0.26 *
Pyocyanin	(µg/mg glc consumed)	1.09 ± 0.65 (4)	15.90 ± 2.79 (6) ***	–	–
	(µg/ml/OD_{600nm})	8.49 ± 2.72 (10)	99.21 ± 17.88 (9) ***	21.91 ± 12.19 (3)	310.77 ± 27.63 (3) ***
	Actual concentration in culture supernatants (µg/ml)	13.66 ± 5.77 (7)	101.80 ± 15. 55 (9) ***	24.35 ± 13.87 (3)	312.73 ± 34.85 (3) ***
Total siderophores	(EDTA equivalent/mg glc consumed)	0.042 ± 0.025 (9)	0.091 ± 0.011 (9) **	–	–
	(EDTA equivalent/OD_{600nm})	0.19 ± 0.13 (12)	0.65 ± 0.21 (12) ***	0.19 ± 0.03 (3)	0.51 ± 0.02 (3) ***
	Actual concentration in culture supernatants (EDTA equivalent/ml)	0.28 ± 0.16 (12)	0.55 ± 0.16 (12) *	0.21 ± 0.03 (3)	0.53 ± 0.03 (3) ***

Growth promoting and biocontrol metabolites		Yield of the metabolites produced			
		100 mM Glucose + P-sufficient condition	100 mM Glucose + P-limited condition	ARE + P-sufficient condition	ARE + P-limited condition
Pyoverdine	(RFU/0.1 ml/mg glc consumed)	36.79 ± 2.71 (4)	61.95 ± 7.94 (4) **	–	–
	(RFU/0.1 ml/ OD_{600nm})	256.00 ± 76.29 (4)	513.89 ± 68.37 (4) **	–	–
	Actual concentration in culture supernatants (RFU/0.1ml)	380.3 ± 36.41 (4)	362.25 ± 95.06 (4) ^{ns}	–	–
Pyochelin	(Area under the peak/mg glc consumed)	21024.34 ± 10837.53 (4)	136875.58 ± 23022.12 (6) ***	–	–
	(Area under the peak/OD_{600nm})	129348.62 ± 67797.80 (4)	1024729.29 ± 48072.01 (6) ***	–	–
	Actual concentration in culture supernatants (Area under peak/0.03 ml)	221693.25 ± 128211.17 (4)	868808 ± 115140.87 (6) ***	–	–
Untargeted metabolite at R_t~30.5 minutes	(Area under the peak/mg glc consumed)	748888.11 ± 147211.35 (4)	513823.17 ± 50779.85 (4) *	–	–
	(Area under the peak/OD_{600nm})	4663394.99 ± 1361359.55 (4)	4759607.61 ± 1685869.03 ^{ns}	–	–
	Actual concentration in culture supernatants (Area under peak/0.03 ml)	7802129.5 ± 2009218.97 (4)	3997292.33 ± 1471959.75 (4) *	–	–
Growth parameters					
OD_{600nm} at the experimental endpoint	–	1.82 ± 0.18 (9)	1.07 ± 0.15 (9)	1.28 ± 0.12 (3)	1.00 ± 0.02 (3)
pH at the experimental end point	–	4.56 ± 0.28 (9)	4.64 ± 0.33 (9)	8.09 ± 0.04 (3)	8.36 ± 0.01 (3)

Growth promoting and biocontrol metabolites		Yield of the metabolites produced			
		100 mM Glucose + P-sufficient condition	100 mM Glucose + P-limited condition	ARE + P-sufficient condition	ARE + P-limited condition
Time of experimental end point	h	48 h	72 h	48 h	78 h
Specific growth rate	μ (h^{-1})	0.33 ± 0.05	0.11 ± 0.06 (5) ***	0.32 ± 0.04	0.16 ± 0.02 **
Specific rate of glucose depletion	Qglc (g/g/h)	0.18 ± 0.02	1.15 ± 0.32 (5) ***	–	–
Total glucose depleted	(mg/ml)	13.38 ± 0.41 (5)	12.52 ± 1.16 (5) ^{ns}	–	–
Glucose consumed	(mg/ml)	10.22 ± 0.65 (5)	6.63 ± 0.97 (5) ***	–	–
Biomass yield	(mg dcw/glc utilized)	0.24 ± 0.07 (5)	0.18 ± 0.07 (5) ^{ns}	–	–

Results are represented as Mean ± S.D. of independent observations as indicated in the parenthesis for each parameter

* P<0.05; ** P<0.01, ***P<0.001, ^{ns} non-significant when compared between P-sufficient and P-deficient conditions for a particular carbon source

– is Not Determined

Figures

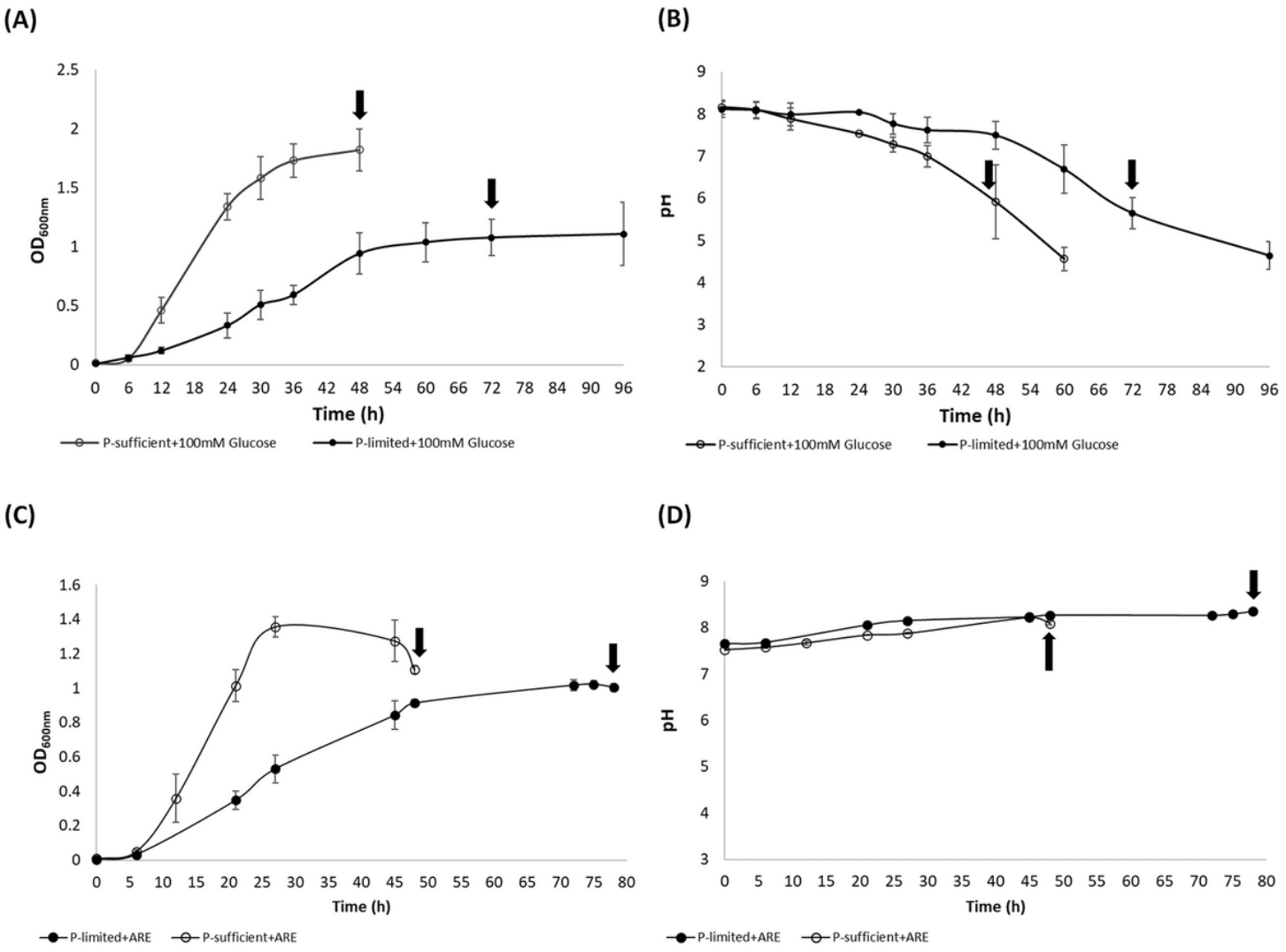

Figure 1

Effect of P-status on growth and media acidification profiles of P4 (A) Growth and (B) pH profile of P4 culture grown in presence of 100 mM glucose, under P-sufficient and P-limited conditions; (C) Growth and (D) pH profile of P4 culture grown in presence of ARE, under P-sufficient and P-limited conditions, respectively. Results are expressed as Mean ± SD of 3-5 independent observations. The arrows indicate the experimental end points for sampling.

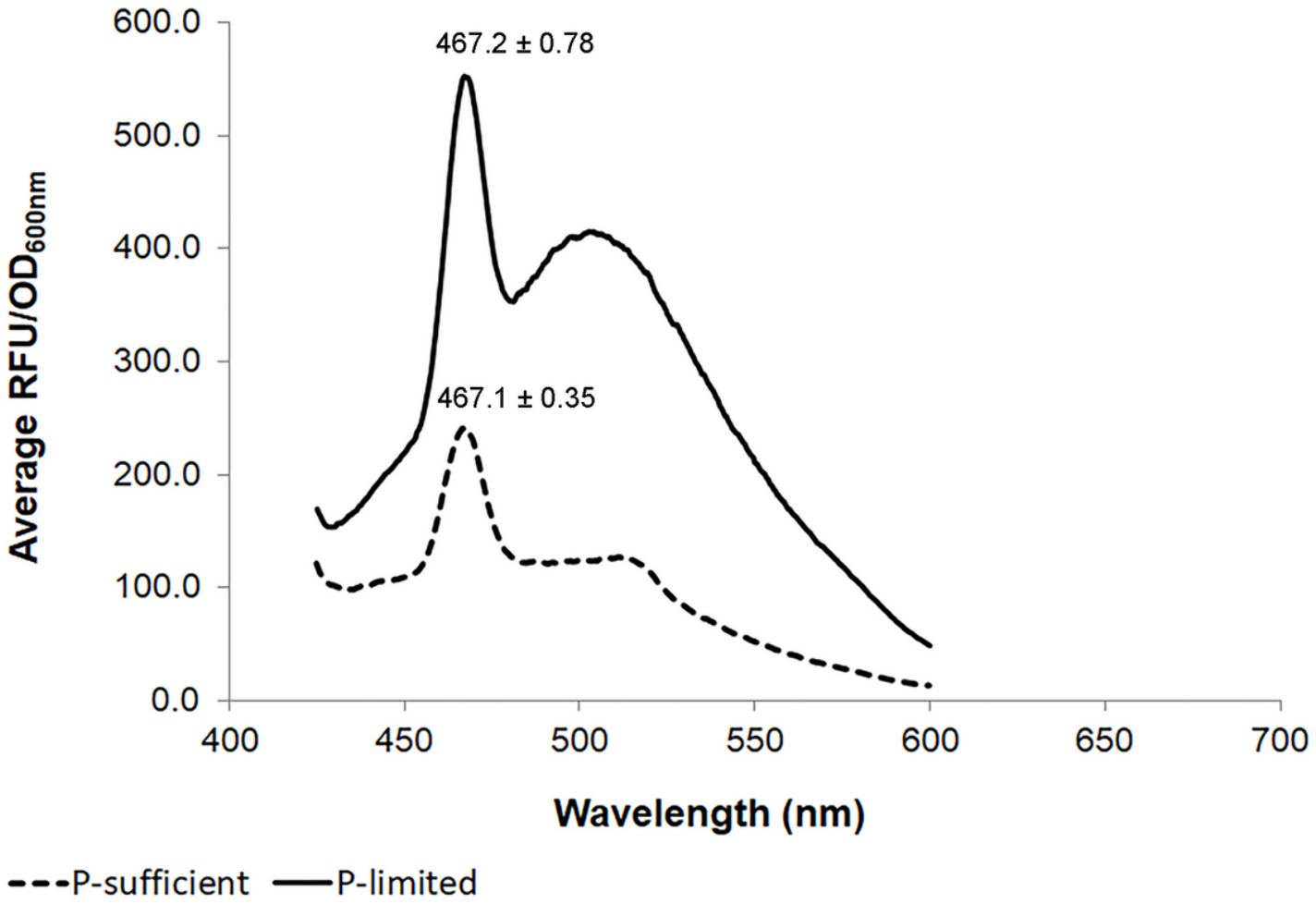

Figure 2

Fluorescence-based quantitation of pyoverdine produced by P4 under P-sufficient and P-limited conditions Pyoverdine production was measured using supernatants of 48 and 72 h old P4 cultures grown under P-sufficiency and P-limitation, respectively. Fluorescence properties of pyoverdine were determined as an emission peak generated at ~467 nm after excitation at 400 nm. Results are expressed as Mean ± SD of 4 independent observation

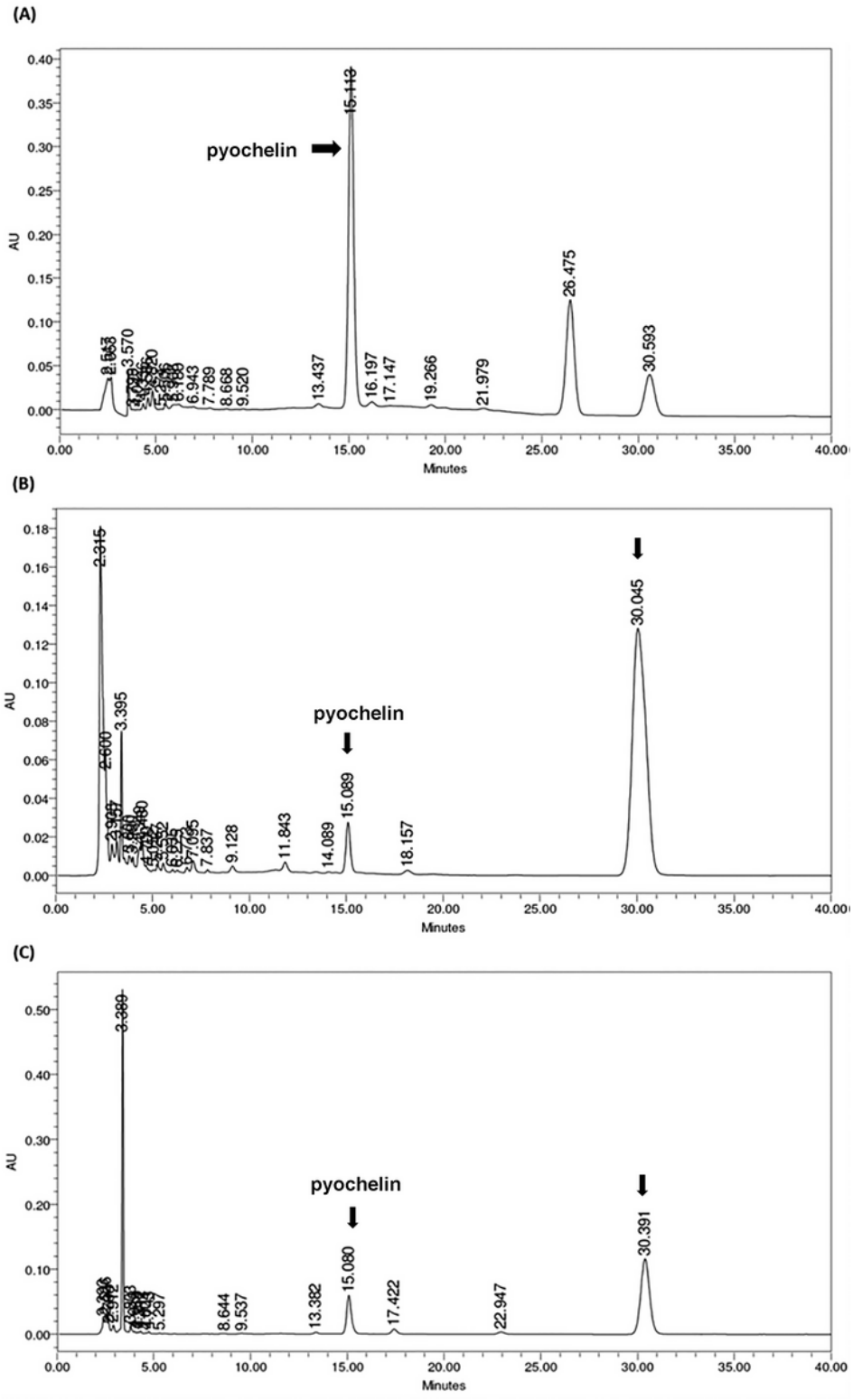

Figure 3

Pyochelin production by P4 under P-sufficient and P-limited conditions HPLC chromatograms were generated using culture supernatants of P4 grown on (A) King's B medium for 48 h (B) P-sufficient medium for 48 h and (C) P-limited medium for 72 h. Representative chromatograms in each category are depicted here.

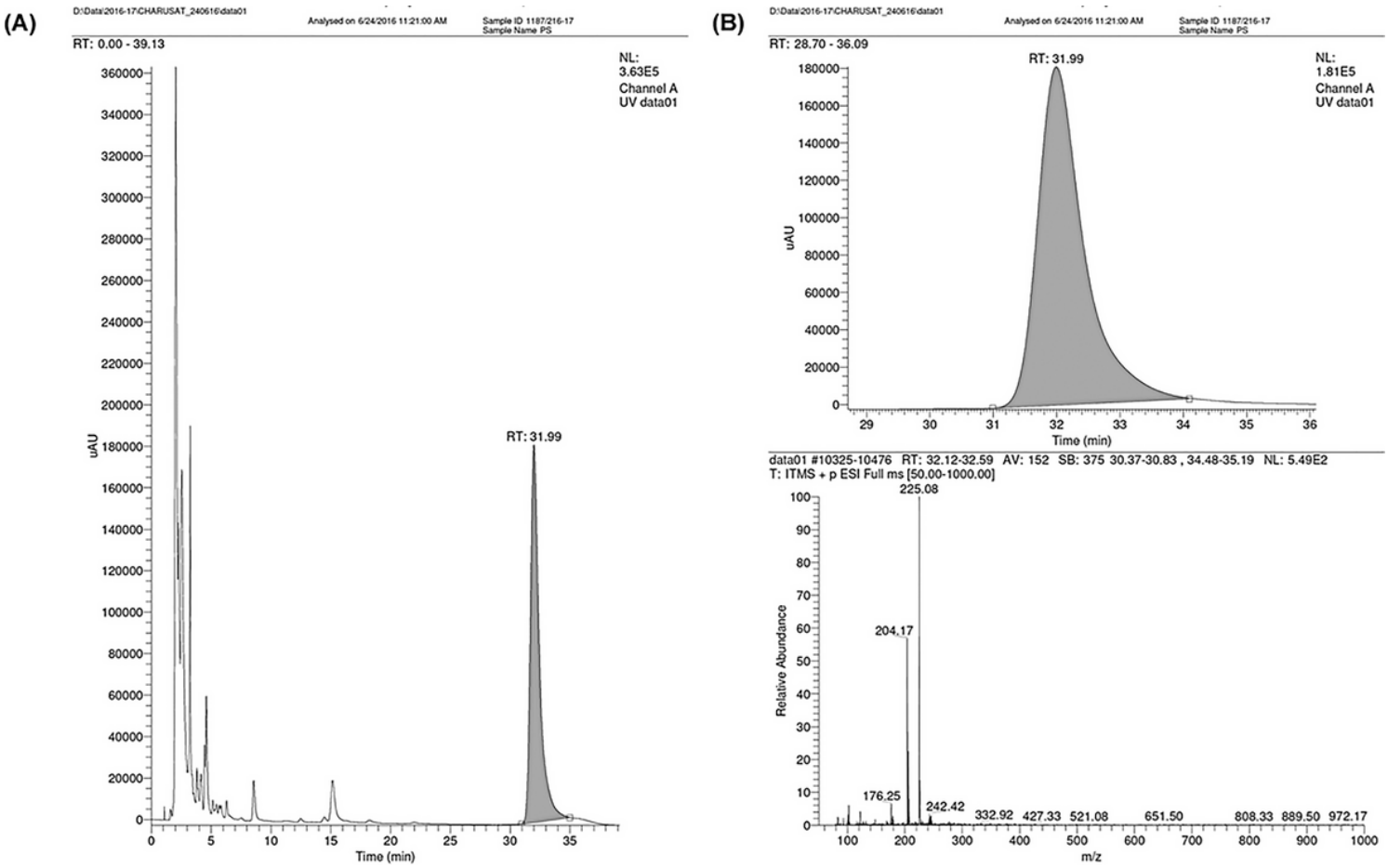

Figure 4

LC-MS-based identification of the untargeted metabolite produced by P4 (A) Unidentified metabolite retained at Rt ~31.99 (B) m/z ratio of the specific metabolite. This metabolite after HR-LC-MS analysis was identified as 5-hydroxytryptophan. P-sufficiency-derived culture supernatant was used for the analysis.

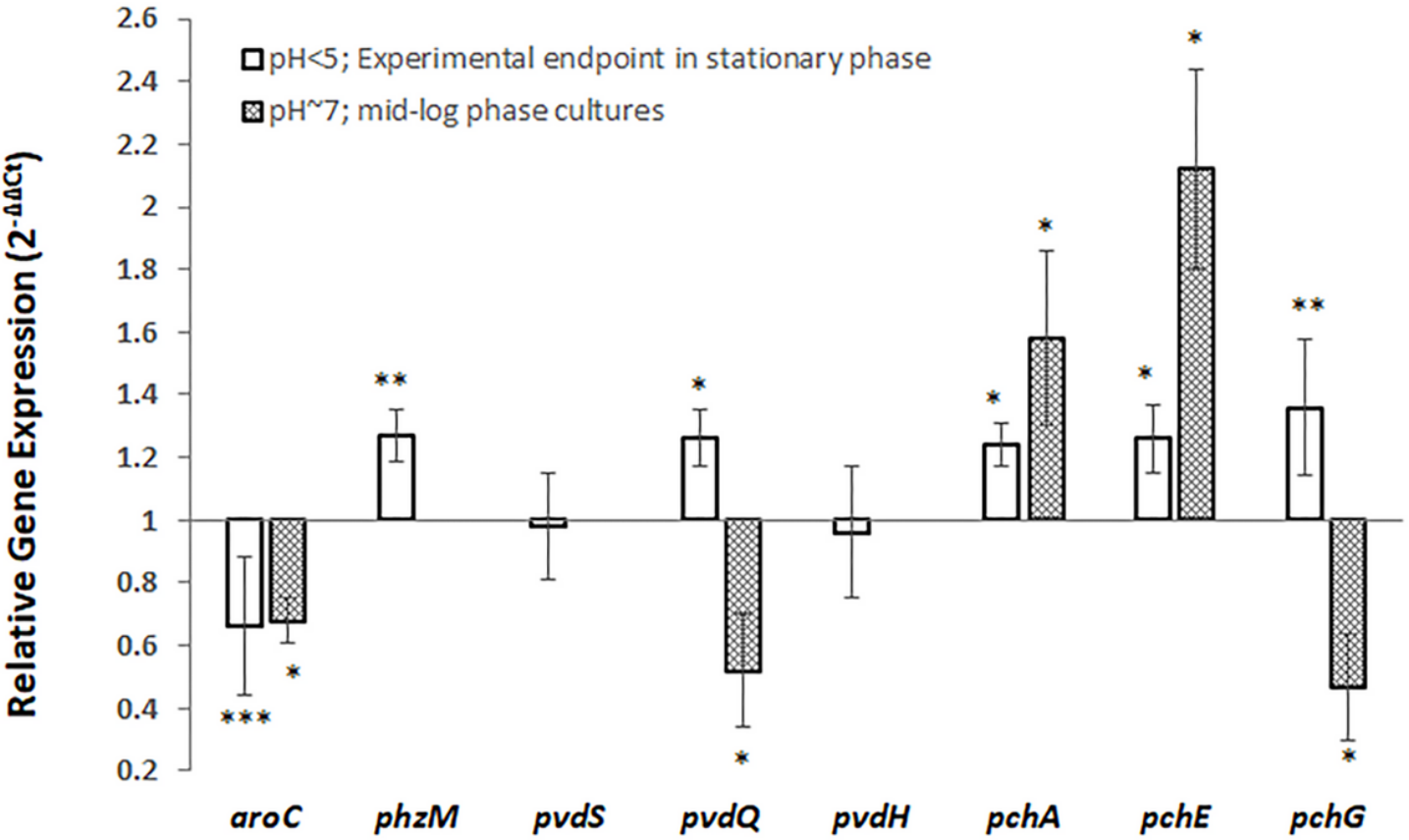

Figure 5

P-status dependent alterations in relative expression of key genes involved in the biosynthesis of biocontrol metabolites by P4 Gene expression levels were normalized to that of rpoD gene. The data is represented as Mean ± SD of observations from three independent replicates assayed in triplicates; *** $p < 0.001$, ** $p < 0.01$, *$p < 0.05$. For each gene, ΔCt values for P-limitation and P-sufficiency derived cell cultures were compared to obtain statistical significance

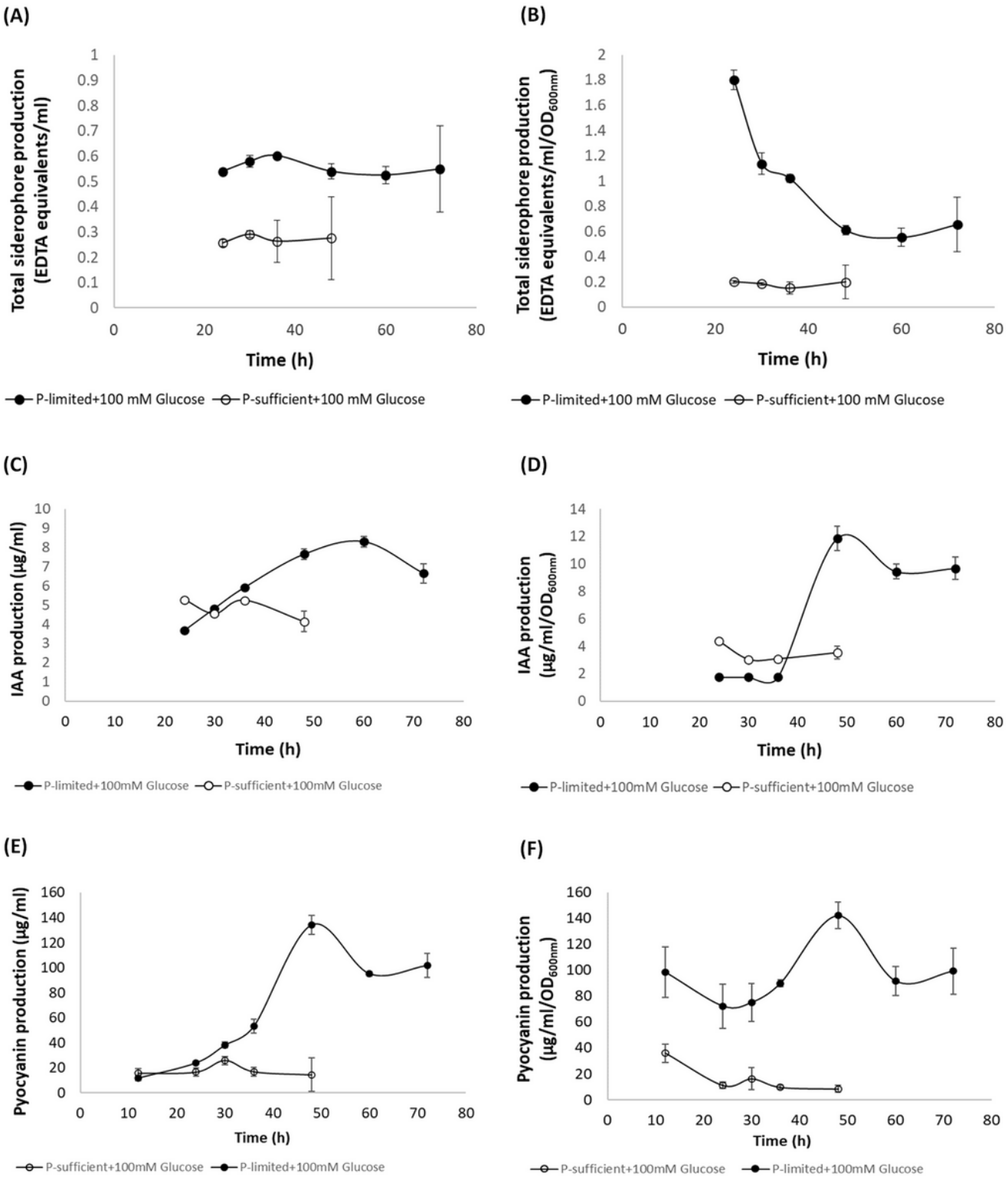

Figure 6

Time course analysis of secondary metabolite production in presence of excess glucose as sole carbon source. Actual and cell density-normalized levels of (A), (B) Total siderophores, (C), (D) IAA and (E), (F) Pyocyanin. The results are represented as Mean ± SD of observations from three independent observations. The actual metabolite levels are normalized to cell density measured as a function of OD600nm

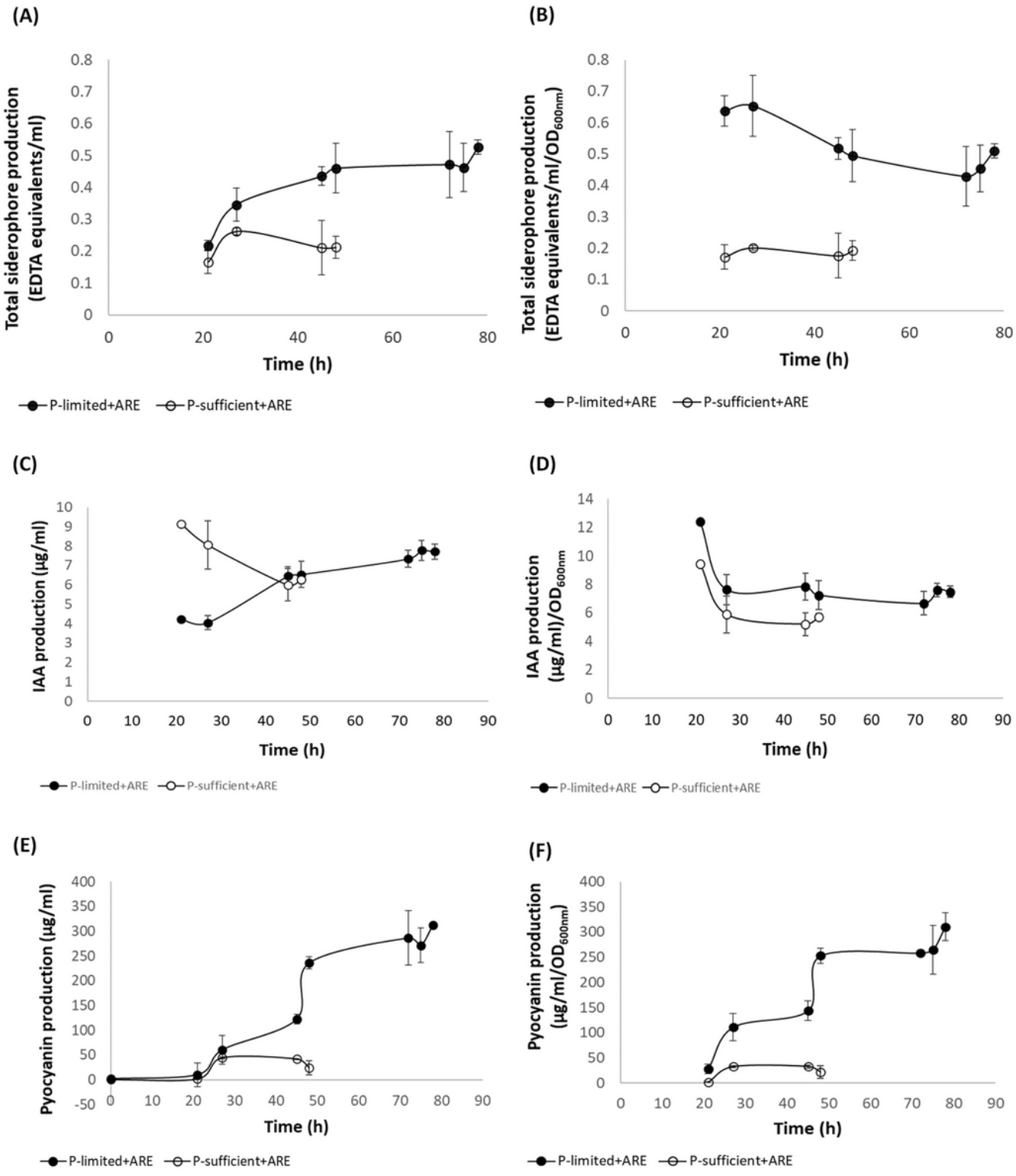

Figure 7

Time course analysis of secondary metabolite production using artificial root exudates as carbon source. Actual and cell density-normalized levels of (A), (B) Total siderophores, (C), (D) IAA and (E), (F) Pyocyanin. The results are represented as Mean ± SD of observations from three independent observations. The actual metabolite levels are normalized to cell density measured as a function of OD600nm

Supplementary Files

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- Supplementarymaterialll.docx
- SupplementarymaterialIII.tif