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Media Optimization for Penicillin Production in *Penicillium chrysogenum* PTCC5031 via Flux Balance Analysis

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Abstract

Penicillium chrysogenum is the principal microorganism used for the industrial production of penicillin. The widespread use of this fungus has led to certain challenges regarding antibiotic production. As the global demand for antibiotics continues to increase, the importance of nutritionally balanced and cost-effective culture media becomes increasingly evident. Traditionally, different optimization methods have been used for culture media, many of which have been useful and have improved our current knowledge of the matter. These methods have mostly been based on experimentation and refinement. However, large-scale manufacturing requires higher efficiency in terms of time and resources, as well as higher precision. In recent years, the emergence of modern computational tools such as genome-scale metabolic models (GSMMs) has greatly improved our ability to understand and predict fungal and microbial metabolic behavior, which in turn has led to improvements in the optimization of manufacturing processes. In this study, a GSMM of *P. chrysogenum* PTCC5031 was used to identify optimal nutrient settings that theoretically enhance penicillin production under defined uptake constraints. The model combined metabolic pathway analysis with flux balance analysis to evaluate different culture conditions. Among the tested conditions, maltotriose and urea were identified as the most effective carbon and nitrogen sources and showed better cellular growth kinetics and higher antibiotic production rates. Further laboratory-scale validation confirmed the predicted qualitative trends, with penicillin titers increasing by up to 11% under optimized sucrose–urea conditions compared to the baseline medium. The findings highlight the utility of GSMM-driven approaches for medium design and optimization. This predictive framework can help reduce costs and accelerate the development process in biomanufacturing and biotechnology.

1. Introduction

Penicillin is one of the most remarkable discoveries in the history of modern medicine, transforming the treatment of bacterial infections and establishing the basis of the antibiotic era. After being identified in *Penicillium notatum* and massively produced on an industrial scale, penicillin rescued millions of lives globally and continues to serve as a cornerstone of antimicrobial therapy (Ozcengiz & Demain, 2013).

Classical strain improvement, metabolic engineering, and process optimization have been used in the past for industrial production of penicillin, which has been found to yield higher than natural production (Agren *et al.*, 2013; Sawant & Vamkudoth, 2022).

Progress in fungal genomics has further confirmed the biosynthesis of β-lactam as a process governed by molecular control and the intricate interplay between enzymes, genes, and regulatory factors that determine the production of antibiotics (Zhgun, 2025).

Notwithstanding these achievements, the production of penicillin in industry is still marked by a number of difficulties, especially in the areas of resource sustainability, regulatory bottlenecks, and metabolic efficiency.

The connection between central metabolism, amino acid biosynthesis, and penicillin output is also a subject of much research, highlighting the importance of metabolic balance for stable production (Maia *et al.*, 2016). Likewise, it is essential to eliminate barriers to supply precursors, cellular robustness, and energy metabolism to develop improved industrial strains (Sawant & Vamkudoth, 2022).

The most recent approaches to the improvement of strains have paid more attention to integrating metabolic engineering with modern genomic technologies, providing a new opportunity to enhance productivity without increasing process costs (Hassane *et al.*, 2025).

An important determinant of penicillin production is the selection of carbon and nitrogen sources, which have a direct effect on metabolic fluxes and biosynthetic production. The use of traditional substrates has proven to be effective but may not be sustainable, which requires investigating alternative feedstocks in accordance with the

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principles of the circular bioeconomy (Yan *et al.*, 2024). The development of constraint-based modeling and in silico optimization has emphasized the importance of approaches in the re-routing of fluxes in response to various nutrient regimes. It has also been argued that the optimized use of substrates has the potential to enhance productivity and minimize environmental impact (Volkova *et al.*, 2020). Such strategies, combined with sustainable feedstocks, should harmonize with global priorities in green biomanufacturing (Wikandari *et al.*, 2022).

The development of genome-scale metabolic models (GSMMs) has profoundly changed the approach to microbial metabolic research and secondary metabolite engineering (Wang *et al.*, 2024). These models provide a systematic framework to map metabolic networks, predict flux distributions, and design rational engineering strategies. Platforms like RAVEN, BiGG, KEGG, and ChEBI have provided an invaluable resource for constructing models, standardization, and annotation, providing cross-comparisons and data integration (Agren *et al.*, 2013; Hastings *et al.*, 2013; Kanehisahi *et al.*, 2016; King *et al.*, 2016). Recent studies have applied GSMMs to filamentous fungi and antibiotic-producing organisms and have discovered the main metabolic bottlenecks in specialized metabolism and proposed new engineering opportunities (Qiu *et al.*, 2023; Sawant & Vamkudoth, 2022). Hybrid modeling methods have also been suggested to address the gap between in silico prediction and real-world industrial performance by supplementing GSMMs with real-time process data (Albino *et al.*, 2024).

Advances in metabolic modeling and genome editing align with new ideas of microbial bioeconomy cell factories (Yan *et al.*, 2024). This tendency underscores the strategic role of fungal platforms for known antibiotics, e.g., penicillin, as well as for the discovery and synthesis of new bioactive compounds (Hassane *et al.*, 2025). Comparative studies, such as the genomic analysis of *Acremonium chrysogenum*, further display how the future of engineering can be guided by classical strain improvements (Zhgun, 2025). Recent studies on pathway re-engineering in other microorganisms, including improved production of Iturin A in *Bacillus amyloliquefaciens*, indicate the capacity of integrating metabolic rewiring, protease modulation, and energy optimization to improve the production of secondary metabolites (Xu *et al.*, 2020).

Although many GSMMs exist for bacteria, fungal models are limited, even for important producers like *P. chrysogenum*. Agren *et al.* (2013) used RAVEN to build a GSMM for *P. chrysogenum* and applied flux balance analysis (FBA) to study limits on penicillin production. Their predictions were not tested experimentally, but suggested that increasing the availability of precursors could improve strain engineering. Our work builds on this by validating GSMM predictions using cost-effective media, connecting computational results with practical use. While traditional methods and metabolic engineering have improved penicillin yields, challenges remain in applying model predictions on an industrial scale. This study tests GSMM-based strategies for *P. chrysogenum*, with a focus on using sustainable feedstocks and improving the process.

2. Materials and Methods

2.1. Model Curation and Analysis

The GSMM initially designed by Agren *et al.* (2013) was adopted as the platform framework for this study and was rationalized and thoroughly refined before analysis. The most important parameters—such as reaction stoichiometry, elemental and charge balance, and reaction reversibility—were assessed and revised. The refinement of the models was completed using the publicly available biochemical and metabolic pathway repositories, especially KEGG, BiGG, and ChEBI (Hastings *et al.*, 2013; Kanehisa *et al.*, 2016; King *et al.*, 2016). Flux balance analysis (FBA) was used to estimate optimum flux distributions under various nutrient availability conditions. Simulations were carried out by applying the COBRA Toolbox presented in the MATLAB environment. To ensure accurate metabolic modeling, we imposed uptake restrictions on substrates such as glucose, ammonia, sulfate, phosphate, oxygen, thiamine, pimelate, phenylacetic acid, and water.

The lower limit of uptake rates was adjusted at -1000 mmol g dry cell weight $(DCW)^{-1}$ h^{-1} unless restricted by medium composition. To evaluate the effect of nutrients under direct investigation on network behavior, we set the uptake limits to -10 mmol $gDCW^{-1}$ h^{-1}.

2.2. Strain, Medium, and Cultivation Protocol

The industrial strain *P. chrysogenum* PTCC5031 from the Persian Type Culture Collection was used to validate the experiments. Cultivation was carried out in a two-stage procedure: pre-culture and production. In the first stage, pre-culture, ~10^6 conidiospores were inoculated into Sabouraud Dextrose Broth (50 mL) using 250 mL Erlenmeyer flasks. The cultures were then incubated under constant shaking at 200 rpm at 25 °C. The initial pH was adjusted to 6.5 using 1 M NaOH or HCl prior to sterilization, and allowed to grow for 40 hours. The production medium contained 13 g/L lactose monohydrate (or other carbon sources), 5.0 g/L $(NH_4)_2SO_4$, 1.0 g/L KH_2PO_4, 0.15 g/L $Na_2SO_4 \cdot 7H_2O$, 0.4 g/L phenylacetic acid (PAA), 0.4 g/L pimelate, and 0.4 g/L thiamine. Each production flask was inoculated with 5 mL of actively growing mycelia from the pre-culture. In some experiments, the nitrogen source was changed to study its effect on production. All cultures were grown under the same conditions and collected after six days.

2.3. Analytical Procedures

The accumulation of biomass was measured as dry cell weight (DCW). To extract penicillin from culture supernatants, we carried out liquid-liquid partitioning with ethyl acetate (1:1) under acidic conditions (pH 2). Quantification of the recovered antibiotic was performed using high-performance liquid chromatography (HPLC,

Kenvar smartline system) equipped with a UV detector. Separation was achieved on an ODS C18 column (4.6 × 250 mm, 5 µm particle size; packing L1) maintained at 50 °C. The mobile phase consisted of 0.01 M potassium phosphate buffer and ethanol (65:35, v/v), delivered at a flow rate of 1.0 mL min^{-1}. We also optimized detection and quantification parameters to ensure accuracy and reproducibility across all experimental runs.

3. Results and Discussion

3.1. Model Validation and Predictions

Throughout this analysis, we also improved and updated our metabolic model. After curation, all reactions were checked for consistency using mass balance and reversibility according to biochemical resources such as KEGG, BiGG, and ChEBI (Hastings *et al.*, 2013; Karnik *et al.*, 2016; King *et al.*, 2016). A detailed comparison between the original and updated models is provided in Table S1. FBA was then applied to the model under minimal culture conditions, resulting in initial solutions for growth rate and penicillin production of 0.8937 and 0.2926 mmol $gDCW^{-1}$ h^{-1}, respectively. These baseline outputs provided a foundation for development and further optimization strategies.

3.2. Carbon Source Evaluation

FBA simulations were run for 36 distinct carbon sources, including a range of mono-, di-, and oligosaccharides, sugar alcohols, and organic acids. The predicted growth rates and penicillin production yields are presented in Figure 1, showing the metabolic flexibility of *P. chrysogenum*. The specific type and concentration of the carbon source used in the cultivation medium were shown to regulate and affect the expression patterns of the core penicillin biosynthetic enzymes—ACVS, IPNS, and IAT (Sánchez *et al.*, 2010). Among the evaluated substrates, oligosaccharides like maltotriose, raffinose, and chitobiose led to increased biomass and penicillin production, which shows that *P.* chrysogenum can use the carbon flux for both primary and secondary metabolism. The improved performance observed with oligosaccharides such as maltotriose and raffinose may be related to their relatively slower uptake compared to glucose. In filamentous fungi, rapid glucose assimilation is known to trigger carbon catabolite repression (CCR), primarily mediated by the CreA regulatory system, which can downregulate genes involved in secondary metabolism (Sánchez *et al.*, 2010). Chitin, a polysaccharide derived from fungal biomass, was also another substrate of interest. Its utilization demonstrated metabolic compatibility and a promising route for sustainable bioproduction. As previously reported, filamentous fungi have remarkable potential in the advancement of circular bioeconomy strategies by valorizing renewable feedstocks (Wikandari *et al.*, 2022). The *P. chrysogenum* capacity to metabolize chitin provides economic and environmental advantages, making this fungus a suitable candidate for sustainable industrial applications (Wikandari *et al.*, 2022). The model emphasized the carbon usage pattern as the main factor of penicillin production, though our experimental evidence supported the role of nutrient limitation on the productivity of secondary metabolites. These findings are consistent with previous reports on the interdependence of the central metabolism, amino acid biosynthesis, and antibiotic synthesis in filamentous fungi.

3.3. Nitrogen and Sulfur Sources

Comparison simulations using alternative sources of nitrogen indicated that urea was more effective in supporting the production of penicillin when compared to ammonium salts. While ammonia supported sufficient growth, ammonium ions are also recognized to have an inhibitory impact on penicillin gene clusters, suppressing the secondary metabolism (Ozcengiz & Demain, 2013). Urea, on the other hand, offered growth stimulation, in addition to positive biosynthetic results, which underscores its industrial value.

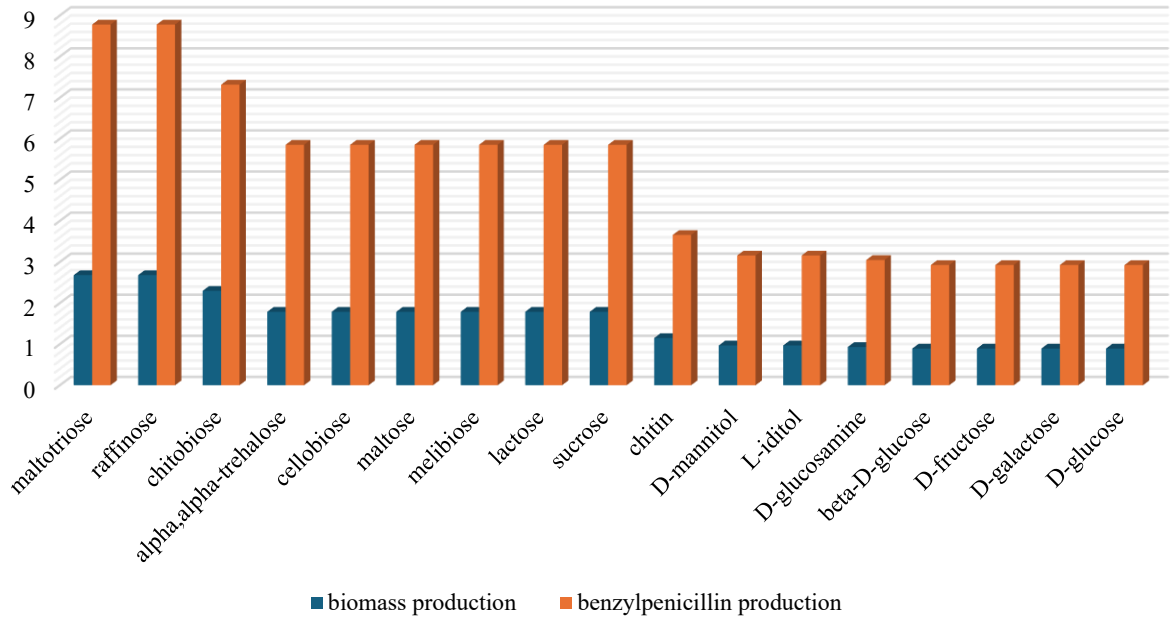

Figure 1. Predicted biomass formation and penicillin production across alternative carbon sources. Flux values are expressed as mmol $gDCW^{-1}$ h^{-1}.

A systematic assessment of sulfur metabolism was also conducted. Among the sulfur donors tested, sulfate was efficient in glucose-sufficient conditions that were capable of promoting high yields of penicillin.

These results are in agreement with the necessity of sulfur in penicillin biosynthesis in *Penicillium chrysogenum*, since sulfur is necessary for cysteine formation and forms part of the thiazolidine ring of the β-lactam structure (Nasution *et al.*, 2008). Sulfide, however, did not support sufficient growth or production, probably because of toxicity and poor metabolic incorporation. In addition, supplementation with methionine increases penicillin titers, which corroborates previous reports that certain amino acids act as metabolism-specific boosters, but not as nutrients (Nasution *et al.*, 2008; Ozcengiz & Demain, 2013).

Figure 2 depicts the results of analyses on the phenotype phase plane, and shows that combined increases in lactose and sucrose uptake have positive effects on penicillin biosynthesis. Of note, glucose stimulated biomass accumulation but inhibited penicillin production because of carbon catabolite repression. This finding justifies dual-sugar feeding strategies, in which glucose is employed to support rapid initial growth, followed by lactose to induce secondary metabolism. These metabolic changes are in line with the classical studies indicating the interrelation between amino acid metabolism and penicillin biosynthesis (Nasution *et al.*, 2008). However, unlike earlier static approaches, the incorporation of dynamic flux analysis in this study offers a more predictive perspective on metabolic trade-offs (Wikandari *et al.*, 2022). Penicillin production yields increased as sucrose and urea concentrations were raised, up to a specific threshold. Increasing the concentration beyond that threshold showed no further increase in production yields, which demonstrated the existence of a saturation point in metabolic capacity. In our model, using sucrose as the sole carbon source resulted in a twofold increase in penicillin yield. However, when both sucrose and urea were increased simultaneously, no significant change in penicillin production was observed. These findings highlight the critical role of the carbon-to-nitrogen (C:N) ratio in determining medium composition. Similar integrative approaches have been recently conducted for other antibiotic-producing microorganisms, including *Saccharopolyspora spinosa* (Wang *et al.*, 2014) and *Acremonium chrysogenum* (Zhgun, 2025), where genomic and metabolic engineering were used to enhance antibiotic production. Comparative genomic studies could validate this method, as classical strain improvement often causes extensive rewiring of metabolic and regulatory networks (Zhgun, 2024). Looking ahead, breakthroughs in extremophilic fungi highlight untapped opportunities for the biosynthesis of novel antimicrobials, suggesting that strategies developed for *P. chrysogenum* can be generalized to other fungal species (Dishliyska *et al.*, 2025).

3.4. Oxygen and Redox Balance

The presence of oxygen proved to be an important factor in the biosynthesis of penicillin. The results of the simulation and literature indicate that oxygen limitation places the antibiotic productivity under significant constraints. Past research on metabolism has shown that penicillin production is favored when dissolved oxygen is maintained at moderate to high levels, typically above 40–60% air saturation. In contrast, pronounced oxygen limitation—particularly below approximately 10%—has been associated with a marked decline in penicillin titers

and the accumulation of intermediates such as 6-aminopenicillanic acid and isopenicillin N (Nasution *et al.*, 2008).

This alteration was explained by the increased flux via the glyoxylate shunt, a redox equilibrium process that was activated under oxygen deficiency (Qiu *et al.*, 2023). Even though biomass accumulation was not seriously influenced during low oxygen, the sharp decrease in secondary metabolism indicates a redox-based trade-off between growth and antibiotic biosynthesis. The ability to incorporate oxygen transfer limitations into future model versions could improve predictions for large-scale bioreactor conditions, where oxygen limitation is a common phenomenon.

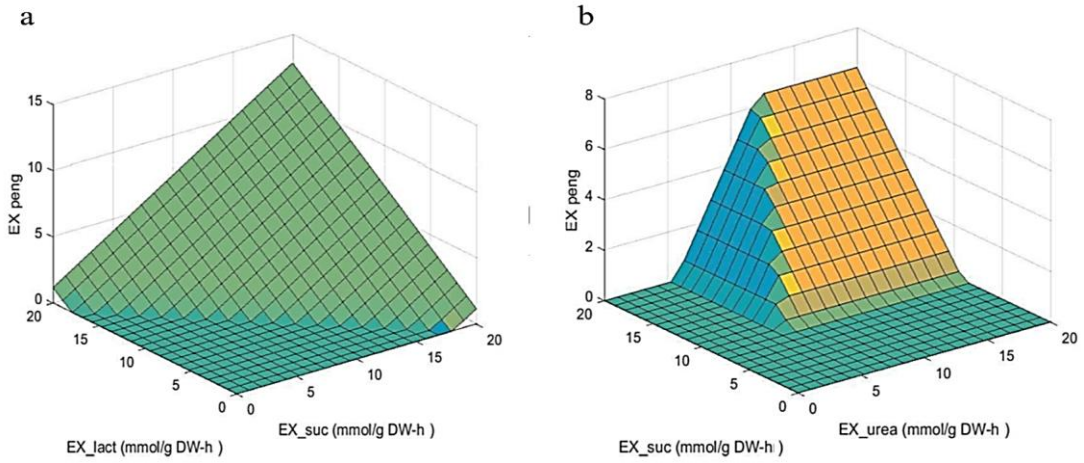

Figure 2. Phenotype phase plane analysis showing the effect of sucrose with lactose (a) and sucrose with urea (b) on penicillin production flux (EX_peng). EX_sucr, EX_lact, and EX_urea represent exchange reactions describing the uptake fluxes of sucrose, lactose, and urea, respectively. Uptake fluxes are expressed in mmol $gDCW^{-1}$ h^{-1}.

3.5. Metabolic Map-Guided Interpretation of Flux Redistribution

To improve clarity and provide a clearer overview of the GSMM-based optimization strategy, a genome-scale metabolic map was constructed to visualize the main metabolic routes, including central carbon metabolism, nitrogen and sulfur assimilation pathways, precursor biosynthesis, and penicillin production routes; see Figure 3. This map helps to summarize how metabolic fluxes are redistributed under optimized nutrient conditions and supports the interpretation of both modeling and experimental results. Flux balance analysis showed clear changes in carbon flux distribution under optimized conditions, with more carbon directed toward the pentose phosphate pathway. This shift increases intracellular NADPH availability, which is needed for secondary metabolite biosynthesis.

The use of oligosaccharides, such as maltotriose, maintained carbon flow toward acetyl-CoA and oxaloacetate pools and improved precursor availability for α-aminoadipate and cysteine synthesis, two key building blocks of penicillin. Analysis of nitrogen assimilation pathways indicated that urea provides a more balanced intracellular nitrogen supply through urease-mediated conversion to ammonium. Unlike ammonium salts, which are known to repress penicillin biosynthesis gene clusters, urea prevents excessive NH_4^+ accumulation and supports continued secondary metabolism. This behavior explains the higher penicillin yields observed under urea-supplemented conditions in both simulations and experiments. Sulfur metabolism analysis showed that sulfate is the most effective sulfur source under glucose-sufficient conditions, as it supports cysteine biosynthesis and subsequent penicillin assembly. In contrast, sulfide sources led to poor growth and very low antibiotic production, likely due to metabolic toxicity and limited bioavailability. These results highlight the importance of proper sulfur assimilation for maintaining metabolic balance during penicillin biosynthesis. Precursor supply analysis showed increased flux toward α-aminoadipate, valine, and cysteine biosynthesis under optimized media conditions. Comparison of glucose-ammonia and maltotriose-urea conditions using flux variability analysis (FVA) showed a coordinated redistribution of metabolic fluxes at the genome scale. Under maltotriose-urea conditions, flux was redirected away from growth-associated pathways and toward the biosynthesis of key penicillin precursors, including α-aminoadipate, cysteine, and valine. This shift was accompanied by reduced activity in certain growth-related pathways and enhanced precursor supply, providing a metabolic explanation for the increased penicillin production observed experimentally. Higher availability of these precursors directly contributed to increased penicillin production, which is consistent with the known relationship between amino acid metabolism and secondary metabolite formation in filamentous fungi. Oxygen uptake was strongly linked to penicillin biosynthesis. When oxygen availability was reduced, metabolic flux was redirected toward the glyoxylate shunt, which helps balance redox status but limits NADH oxidation capacity. Although biomass formation was not strongly affected under oxygen-limited conditions, penicillin production decreased noticeably, emphasizing the role of redox balance in regulating secondary metabolism. Relative to the baseline minimal medium condition, flux balance analysis predicted up to a 2-fold theoretical increase in

penicillin production when sucrose was used as the sole carbon source.

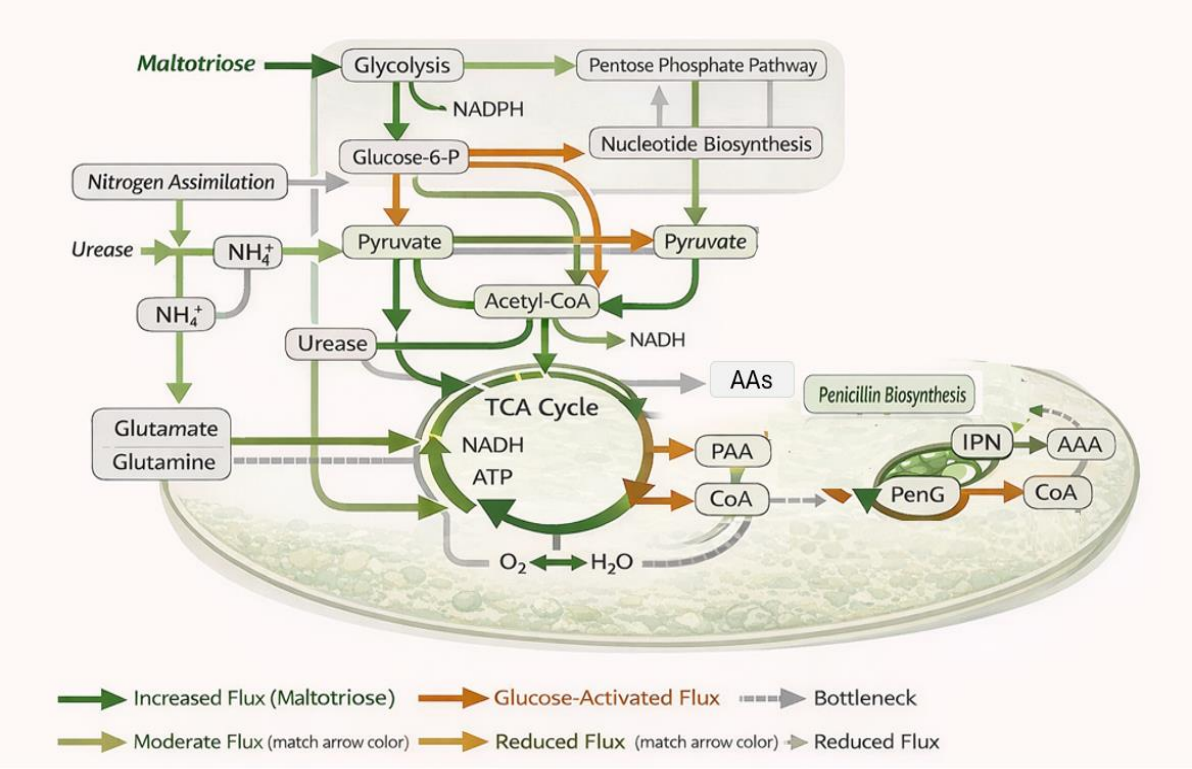

Figure 3. Schematic representation of the metabolic pathways associated with penicillin biosynthesis, created by the authors based on the GSMM predictions obtained in this study.

The combined application of sucrose and urea had approximately the same effect on the predicted penicillin flu. Experimental results followed the same trend, with penicillin titers increasing by 6% under sucrose supplementation and by 11% under combined sucrose-urea conditions, while biomass formation increased only moderately (5%). The difference between the theoretical increase predicted by FBA and the enhancement observed experimentally reflects intrinsic limitations of stoichiometric models. Constraint-based approaches estimate the maximal redistribution of metabolic fluxes under optimal assumptions and steady-state conditions (Orth *et al.*, 2010). However, penicillin biosynthesis in *P. chrysogenum* is subject to multiple layers of regulation, including carbon catabolite repression, oxygen-dependent redox balance, enzyme capacity, and precursor transport limitations. Similar discrepancies between model-derived potentials and laboratory-scale titers have been reported in other GSMM-based studies (Machado & Herrgård, 2014; Qiu *et al.*, 2023). Therefore, theoretical capacities should be interpreted as an upper bound rather than an expected experimental outcome. These findings indicate that metabolic flux was mainly redirected toward penicillin production rather than growth. Collectively, the metabolic map-guided flux analysis helps identify key bottlenecks and regulatory points controlling penicillin biosynthesis. By integrating carbon distribution, nitrogen and sulfur assimilation, precursor availability, and oxygen/redox balance, this approach provides a clear explanation for the improved performance observed under optimized culture conditions.

3.6. Model Expansion and Specialized Metabolites

Besides the biosynthesis of penicillin, the growth of the curated model offers opportunities to find out the production of special metabolites. Recent reconstructions of *P. chrysogenum* have shown that the integration of silent biosynthetic gene clusters makes it easy to anticipate cryptic secondary metabolites (G. Wang *et al.*, 2024). This method provides a roadmap to the activation of pathways in an organized manner that were not active in traditional culture conditions. It has also been mentioned that using extremophilic fungi could be highly active in the production of new antimicrobial compounds, implying the importance of incorporating such specific metabolic processes into GSMM systems (Dishliyska *et al.*, 2025). Furthermore, sustainable production of bioactive metabolites, including iturin, has already been attained through end-to-end metabolic engineering of bacterial systems (Zhang *et al.*, 2025), demonstrating the translational capabilities of these methods in fungal systems. Moreover, when integrating metabolomics data into metabolic models, the accuracy of predictions and the detection of regulatory bottlenecks have greatly increased (Volkova *et al.*, 2020). Such expansions of multi-omics promise to develop the industrial use of *P. chrysogenum* and other related fungi as general microbial cell factories.

3.7. Limitations and Future Directions

Despite its predictive power, constraint-based modeling is inherently limited by static assumptions, as it neglects regulatory dynamics and temporal flux adaptations (Wikandari *et al.*, 2022).

While curated GSMMs are highly useful for exploring metabolism, they require continuous improvement through the integration of multi-omics data, such as transcriptomics, proteomics, and metabolomics (Maia *et al.*, 2016).

The second weakness is related to the variability in the strains. The strains that are optimized in the laboratory may not fully indicate the performance of industrially optimized producers (Sawant & Vamkudoth, 2022). Future studies must prioritize the integration of GSMMs with genome editing tools, especially CRISPR-Cas systems, to validate and implement predicted metabolic interventions (Hassane *et al.*, 2025).

Moreover, comparative genomics will still play a pivotal role in the determination of genetic determinants behind higher biosynthetic capacity (Zhgun, 2025).

The study of the extremophilic strains of fungi can also open up new metabolic pathways that can find their use in the process of antimicrobial discovery (Dishliyska *et al.*, 2025). Ultimately, integrating advanced modeling, genome editing, and process engineering will presumably accelerate the shift of the penicillin production to a more efficient, sustainable, and economically feasible domain.

4. Conclusion

The predictive ability of the model was validated by experiments on secondary metabolite biosynthesis in limited nutritional conditions. Overall, the model was able to reflect the expected interplay between biomass formation and penicillin production under the tested nutrient conditions. Although some quantitative differences were observed between simulated outputs and experimental measurements, the overall trends remained consistent.

In conclusion, the integration of GSMMs, experimental validations, and genome editing technologies can lead to the identification of sustainable antibiotic production pathways. The concomitant use of computational predictions with industrial applications in our study focuses on bioeconomy and sustainability, as well as enabling innovations in fungal technologies.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Open access

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None declared.

Authors' Contributions

Fakhri Sadat Hosseini conceived and supervised the project. Masoumeh Mansoubi Hosseini performed the computational analyses and data interpretation. Fatemeh Takfallah performed the experimental validation. All authors have read and approved the final version of the manuscript.

Supplementary files

Supplementary file 1.

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