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Original Article

Enhancing penicillin production in *Penicillium chrysogenum* through gamma radiation-induced mutagenesis and carbon source optimization

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ABSTRACT

Discovered by Alexander Fleming in 1928, penicillin has earned its reputation as a revolutionary antibiotic, profoundly altering the treatment of bacterial infections and marking a pivotal moment in medical history. *Penicillium chrysogenum*, a high-yielding fungal species, remains central to the industrial production of penicillin. Efforts to improve the yield of penicillin remain an active area of research, with different approaches, such as induced mutagenesis, applied to maximize production efficiency. This study aims to investigate the effects of induced mutations and the utilization of various carbon sources on penicillin biosynthesis in *Penicillium chrysogenum*. Mutant strains developed through gamma radiation exposure were evaluated against wild-type strains for antibiotic production in culture media containing lactose, sucrose, and various combinations of both at different concentrations. Penicillin production was quantified using high-performance liquid chromatography (HPLC), with 96 analyses conducted in triplicate. The study found a marked increase in penicillin production in mutant strains compared to wild-type strains, particularly in sucrose media. However, the relationship between antibiotic yield and carbon source concentration was nonlinear. Increasing carbon source concentration was not always associated with increased penicillin production. The research suggests optimizing carbon source concentration and mutation methods to enhance penicillin production. It has implications for the schematic development of bioprocesses for the production of industrial antibiotics.

KEYWORDS

Antibiotics, HPLC, Mutation, Wild-type.

INTRODUCTION

Penicillium chrysogenum, a filamentous ascomycete, exhibits the exceptional ability to produce penicillin, other β-lactam antibiotics as well as various secondary metabolites (Frisvad et al. 2004, Guzmán-Chávez et al. 2018, Kumar et al. 2018, Wu et al. 2020). *P. chrysogenum* is the most extensively studied fungus with regard to penicillin production, owing to its multiple copies of the penicillin biosynthesis gene (Cuero et al. 1986, Fierro et al. 2022). These genes carry out the biosynthesis of various compounds with complex and highly regulated enzymatic pathways (Li et al. 2018, Fatima et al. 2019). Understanding the metabolic pathways of *P. chrysogenum* is crucial for optimizing antibiotic biosynthesis at an industrial scale and discovering new biologically active compounds (Abraham et al. 1941, Nair 2007). *Penicillium chrysogenum* cultures generally produce limited quantities of penicillin, even under optimal cultivation conditions (Fernandez-Canon et al. 1989, El-Sayed 2021). However, there are methods to increase penicillin production by changing the composition of growth media combined with the appropriate use of

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physical factors (Dharmarha et al. 2019). It has been shown that low doses of ionizing radiation (200-400 Gy) may enhance spore germination and mycelial growth, leading to increased penicillin production (Aljeldah et al. 2019). Gamma irradiation is another treatment that influences the penicillin production in *P. chrysogenum* and warrants further research for its potential in pharmaceutical manufacturing (Veerapagu et al. 2008, Ibrahim et al. 2023).

Gamma irradiation, a form of ionizing radiation, has been extensively studied for its potential to create mutant strains of microorganisms for use in industrial penicillin production processes (Havn Eriksen et al. 1994, Onyegeme-Okerenta et al. 2009). There is evidence that gamma irradiation induces mutations in *P. chrysogenum,* altering its genetic makeup and enhancing its ability to produce penicillin (Mesquita et al. 2013, Hardianto et al. 2015).

Researchers have investigated various parameters such as radiation dosage, exposure duration, and their impact on penicillin production. Some studies have demonstrated that certain mutant strains show significant potential, producing higher quantities of antibiotics compared to the wild-type strain (Davey and Johnson, 1953, Aljeldah et al. 2019). The increase in penicillin production following gamma irradiation is influenced by several factors, including the irradiation conditions, the microorganism's genetic characteristics, and the composition of the growth medium (Douma et al. 2011, Amadi 2020). Enhanced penicillin production may also be linked to increased sporulation levels and morphological changes in the mutant strains of *P. chrysogenum* (Pieniążek et al. 1973, Grijseels et al. 2017).

Furthermore, recent developments show the effectiveness of UV radiation in stimulating penicillin production in the mutant strains of *P. chrysogenum,* suggesting it as another potential mutagenic approach (Clutterbuck et al. 1932, Veerapagu et al. 2008). This research focuses on how gamma radiation increases fungal growth and penicillin production, even when cultured in various glucose-lactose media using a *P. chrysogenum* mutant.

Systematic treatment of fungi with gamma rays is expected to confer some mutations that enhance penicillin production. This work will focus on assessing antibiotic-producing fungal strains, including genetic analyses of the observed mutants. Given the changes in penicillin production levels between mutated and wild strains grown on different sources of carbohydrate, we aim to unravel the optimal raw materials required for effective antibiotic production. This research is expected to aid in identifying optimal culture conditions and developing new genetically modified *P. chrysogenum* strains that are more effective and aligned with the industrial requirements for penicillin production. The importance of this study lies in its potential to shift penicillin production toward a more cost-effective and eco-friendly direction. Our goal is to develop enhanced biosynthetic strains by harnessing the mutagenic effects of gamma rays, thereby reducing production costs and increasing the availability of this important antibiotic. In addition, the results of this work may support achieving similar outcomes in the production of other important secondary metabolites and contribute to enhancing the overall efficiency of fungal biotechnology.

MATERIALS AND METHODS

Fungal Sample Preparation

Penicillium chrysogenum (PTCC5031) was obtained from the Mycology Laboratory at the Sari Agricultural Sciences and Natural Resources University. It was cultivated on potato dextrose agar (PDA) medium and incubated at 25°C. Once the fungus had grown sufficiently, a spore suspension was created and kept at 4°C. This suspension was then sent to the Agricultural Research Institute of the Karaj Nuclear Science and Technology Research Institute for additional experiments.

Production Culture Conditions

P. chrysogenum and its mutant isolates were cultured in 96 Erlenmeyer flasks, each containing 50 mL of medium initially, with a total working volume of 100 mL per flask. The experiments were conducted using sixteen different media formulations, each replicated three times for both *P. chrysogenum* and its mutant isolates. All media had a fixed base composition, including peptone, yeast extract 3.5 g/L, and standard salts ($MgSO_4 \cdot 7H_2O$ 0.005 g/L, $KH_2PO_4$6.5 g/L, $ZnSO_4 \cdot 7H_2O$, 0.02 g/L). The variable components (the carbon sources: sucrose and lactose) were tested at three different concentrations (1 g, 3 g, and 6 g/L) individually and in combination, forming the sixteen distinct media. The initial pH of all culture media varied between 4 and 5, and fermentation was carried out at 24°C. These modifications in carbon source type and concentration allowed us to investigate their effects on penicillin production under moderate conditions (Tan and Ho 1991).

Extraction of penicillin

The liquid-liquid extraction technique was used to recover penicillin from a variety of shaking flask cultivation systems (Prasad and Prasad, 2010). It consisted of 96 extractions, each with three replicates among *P. chrysogenum* and its mutant isolates. Fifty milliliters of the Penicillium culture filtrate grown in site broth (rich in penicillin) was extracted triple times using ethyl acetate in a liquid-liquid extraction procedure. Mix them well to transfer penicillin into the organic phase-ethyl acetate. The ethyl acetate containing penicillin was separated into two phases. The ethyl acetate containing penicillin was back-extracted into an aqueous solution of 2% sodium acetate to ensure all the penicillin transferred completely into the aqueous phase. The solution left with penicillin was collected to analyze (Aljeldah et al. 2019).

HPLC analysis

A total of 96 penicillin extractions from *P. chrysogenum* and its mutants were prepared and injected into the HPLC system for analysis. The penicillin analysis was performed using High-Performance Liquid Chromatography (HPLC). The analysis was executed under specific conditions: a C-18 column and a UV detector set to 220 nm for optimal detection. The mobile phase was made up of two components: (A) 10 mM ammonium acetate (pH 4.5, adjusted with acetic acid) and (B) acetonitrile, mixed in a 75:25 (A) ratio. The flow rate was kept at 1 mL/min. For comparative purposes, the results were evaluated against commercially available penicillin injection standards (Aljeldah et al. 2019).

Statistical analysis

Before conducting statistical analysis, the data were assessed for normality and homogeneity of variances using the Jarque-Bera test. (Jarque and Bera 1987) and Levene's test (Levene 1960), respectively. If required, the data was transformed to (x+1). Analysis of variance (ANOVA) was then applied to assess all biological and damage indicators using a factorial randomized complete block design, followed by the mean comparisons using Fisher's Protected Least Significant Difference (LSD) method.

RESULTS AND DISCUSSION

Comparison of Antibiotic Production

This study examined the impact of mutations and varying carbon sources on the antibiotic production capacity of *P. chrysogenum*, with a particular focus on penicillin synthesis. Comparative experiments assessed the antibiotic yields of mutant and wild-type strains when cultured on lactose, sucrose, and their combination.

The results revealed that mutant fungal isolates consistently exhibited significantly higher penicillin production rates compared to the wild-type strains. Our findings highlight the complex relationship between carbon source concentration and antibiotic production efficiency. Interestingly, increasing the concentration of carbon sources in the growth medium did not universally enhance antibiotic synthesis. In certain cases, increased concentrations of carbon sources led to a reduction in penicillin production, indicating the existence of an optimal concentration threshold for each carbon source, beyond which production efficiency diminishes.

Furthermore, we explored the effects of using single versus combined carbon sources. The data indicated that combining carbon sources does not always enhance antibiotic biosynthesis. In some instances, the use of multiple carbon sources had a detrimental effect, significantly reducing penicillin production. Overall, as shown in (Fig. 1), our findings emphasize the complex interactions between gamma radiation-induced mutations, the type and concentration of carbon sources, and their combined influence on penicillin biosynthesis in *P. chrysogenum*.

These results demonstrate a positive correlation between sucrose concentration and penicillin production for both strains, with production increasing up to a certain threshold (Fig.1). The mutant strain consistently outperformed the wild type under all conditions. For the wild-type strain, penicillin production peaked at both 1 g/L sucrose and 6 g/L sucrose, with a decrease at 3 g/L sucrose. In contrast, the mutant strain exhibited maximum production at 3 g/L sucrose, with a decline at 6 g/L sucrose. These results highlight the superior biosynthetic capacity of the mutant strain, particularly in the S3 (3 g/L sucrose) culture medium.

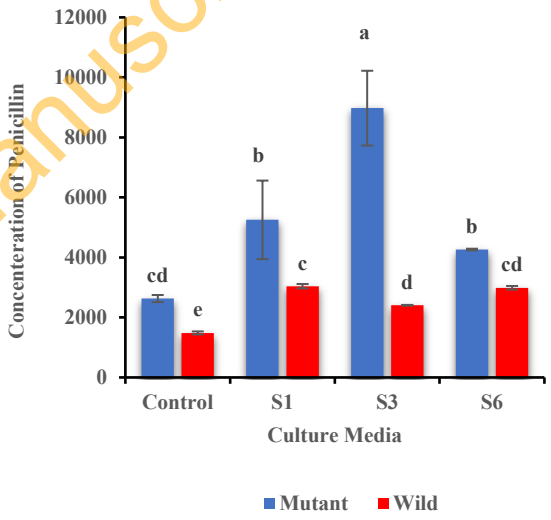

Fig.1. Comparison of the mean production of penicillin antibiotic by wild strains of *Penicillium chrysogenum* and a mutant isolate in sucrose culture medium with varying concentrations. (S1 = sucrose1g/L, S3 = sucrose3g/L, S6 = sucrose 6g/L)

The Fig. 2 illustrates the effect of different lactose concentrations on penicillin production in both mutant and wild strains. The mutant strain consistently produced significantly higher levels of penicillin compared to the wild type under all conditions. Notably, the L1 medium (containing 1 g/L lactose) yielded the highest penicillin production in the mutant strain, significantly surpassing all other tested media. While the L3 (3 g/L lactose) and L6 (6 g/L lactose) media also enhanced penicillin production in the mutant strain, the levels achieved were lower than those in L1, suggesting a decline in production as lactose concentration increased. In contrast, the wild-type strain exhibited lower and

relatively consistent levels of penicillin production across all media, with the highest yield observed in L3; however, this difference was not statistically significant compared to the other conditions. These results underscore the enhanced biosynthetic capacity of the mutant strain, particularly in the L1 medium, whereas the wild-type strain demonstrated limited and uniform production across the tested conditions.

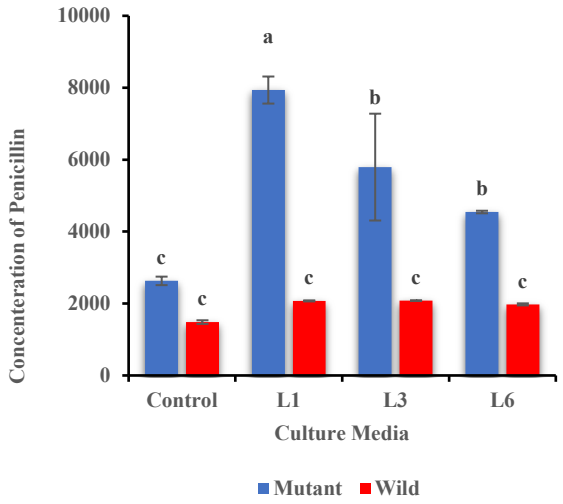

Fig. 2. Comparison of the mean production of penicillin antibiotic by wild strains of *P. chrysogenum* and a mutant isolate in lactose culture medium with varying concentrations. (L1 = lactose 1 g/L, L3 = lactose 3 g/L, L6 = lactose 6 g/L)

In (Fig. 3), penicillin production was compared in culture media with different combinations of sucrose and lactose for both wild-type and mutant isolates of *Penicillium chrysogenum*. Across all mixed cultures, the mutant isolate consistently outperformed the wild type in penicillin production. When sucrose was set at 1 g/L, the highest penicillin production by the mutant isolate occurred with 3 g of lactose (Fig. 3A). With 3 g sucrose, the peak yield was observed with 1 g lactose (Fig. 3B). At 6 g sucrose, maximum production was achieved with 6 g lactose (Fig. 3C). Overall, the mutant strain showed the highest penicillin production in media containing 3 g sucrose with 1 g lactose and 6 g sucrose with 6 g lactose.

In contrast, the wild strain produced the most penicillin in media with 1 g of sucrose and varying lactose concentrations. However, increasing sucrose and lactose concentrations had a counterproductive effect, reducing penicillin production in the wild strain. These findings underscore the mutant strain's superior ability to synthesize penicillin under specific media conditions, whereas the wild type exhibited limited responsiveness to changes in sucrose and lactose concentrations.

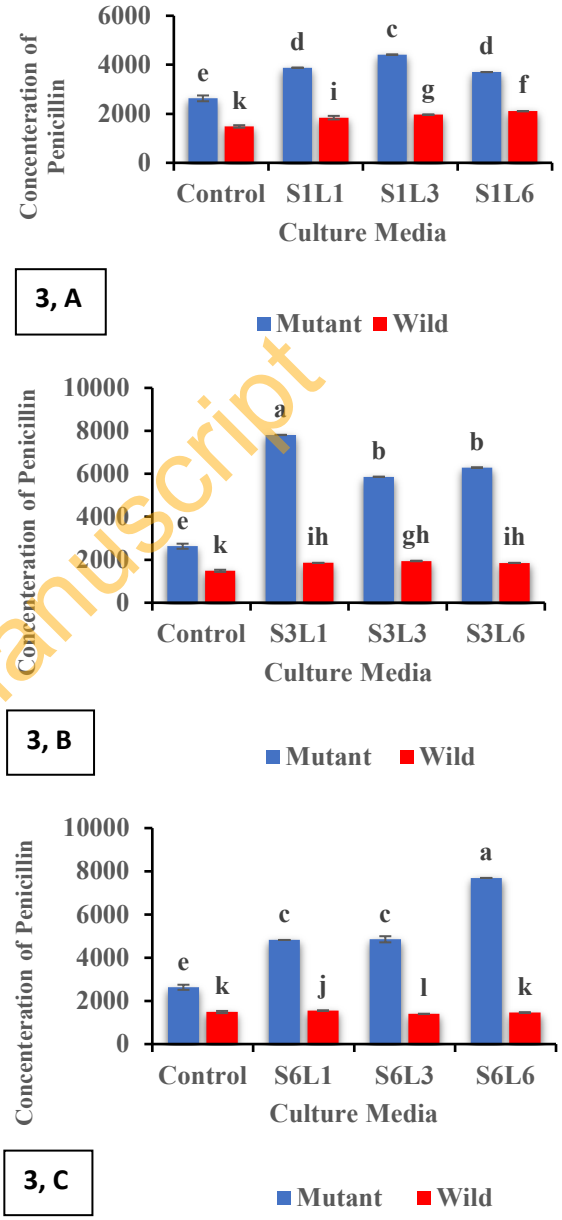

Fig. 3. Comparison of the mean production of penicillin antibiotic by wild strains of *Penicillium chrysogenum* and a mutant isolate in combined sucrose and lactose culture media. A. Penicillin production in media with 1 g/L of sucrose and three concentrations of lactose: 1 g/L (S1L1), 3 g/L (S1L3) and 6 g/L (S1L6); B. Penicillin production in media with 3 g/L of sucrose and three concentrations of lactose: 1 g/L (S3L1), 3 g/L (S3L3) and 6 g/L (S3L6); C. Penicillin production in media with 6 g/L of sucrose and three concentrations of lactose: 1 g/L (S6L1), 3 g/L (S6L3) and 6 g/L (S6L6).

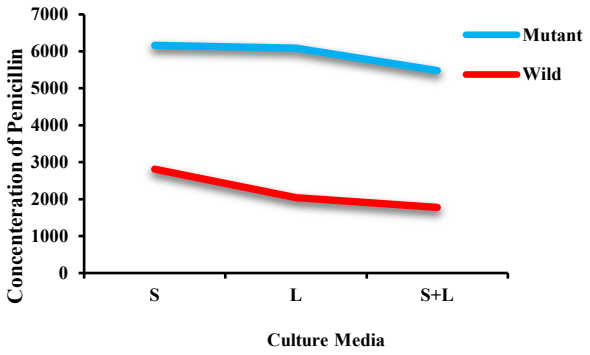

Fig. 4. Overall comparison of the mean production of penicillin antibiotic by wild strains of *P. chrysogenum* and a mutant isolate in varying sucrose culture medium, including sucrose, lactose, and the combination of sucrose and lactose.

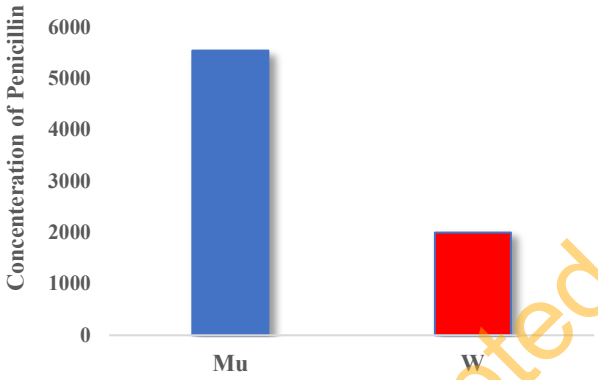

Fig. 5. The Mutant (Mu) isolate produces the greatest amount of penicillin, yielding nearly three times more antibiotics compared to the wild strain (W).

According to (Fig. 4), the relative comparison of various culture media regarding the production of penicillin by *P. chrysogenu*m and its mutant isolate exhibits that sucrose is the best medium for both fungal strains. It has also been found that the titer of penicillin decreases in media that contain only lactose and also in media containing a combination of sucrose and lactose

This study thoroughly explored the effects of gamma radiation-induced mutations and various concentrations of sucrose and lactose on penicillin production in *P. chrysogenum*. The results revealed that both genetic mutations and carbon source composition have a significant impact on penicillin biosynthesis, providing valuable insights for optimizing production processes. The findings confirmed that gamma radiation-induced genetic changes enhanced the metabolic pathways involved in penicillin synthesis, as shown by the mutant strain's ability to produce significantly higher penicillin titers under various culture conditions. These results are consistent with previous studies (Stauffer and Backus 1954, Onyegeme-Okerenta et al. 2013), which reported increased antibiotic production in *P. chrysogenum* mutant strains exposed to ultraviolet radiation (Onyegeme-Okerenta et al. 2013). This preliminary analysis highlights the need for further detailed research into genetic and cultural strategies that could further enhance antibiotic production in *Penicillium chrysogenum*.

Gamma radiation-induced mutagenesis led to the development of a mutant strain with nearly doubled penicillin production compared to the wild type. All gamma-irradiated fungal strains showed significantly altered penicillin titers compared to their wild-type counterparts. This substantial improvement underscores the potential of induced mutation to enhance penicillin production, aligning with previous research (Karunakar et al. 2012, Aljeldah et al. 2019), which reported similar results. Notably, these studies have shown that gamma radiation not only boosts spore germination but also increases penicillin production in *Penicillium chrysogenum* (Luckey 1982, Macklis and Beresford 1991).

This study highlighted the complex relationship between carbon substrate concentration and the efficiency of penicillin production. Although higher carbon levels are generally expected to enhance yield, the results indicate the existence of optimal concentration ranges beyond which production declines, as illustrated in (Figs. 3 and 4). For example, sucrose concentrations up to 6 g per culture medium were positively correlated with penicillin production, whereas higher concentrations led to reduced yields. These findings align with previous studies (Kumar et al. 2018, Amadi 2020), which reported that carbon sources such as lactose, corn starch, and corn dextrin at 3% concentrations yielded the highest penicillin production, with no further improvement at higher levels (Sánchez et al. 2010). Furthermore, this study found that combining different carbon sources does not necessarily enhance antibiotic production and may, in some cases, even suppress it, as shown in (Figs. 3A, 3B, and 3C). For instance, while the S3L1 medium resulted in elevated penicillin output (Fig. 3B), a marked decrease was observed in the S6L6 medium.

(Fig. 3C). These results highlight the importance of carefully selecting and optimizing both the type and concentration of carbon sources to maximize antibiotic yield. Studies by Hardianto et al. (2015) and Weber et al. (2012) demonstrated that ultraviolet-induced mutations can enhance penicillin production; however, the extent of this enhancement is influenced by the growth conditions and the specific composition of the carbon Our results also

show that sucrose is generally more effective than lactose in promoting penicillin synthesis (Fig. 1), likely due to sucrose's higher metabolic efficiency, which provides more energy for production. The study highlights the significant impact of mutations and carbon source ratios on penicillin production (Fig. 5). By selecting appropriate mutant strains and optimizing carbon source combinations, penicillin production can be improved, reducing costs and enhancing efficiency. These findings are consistent with previous studies (Fierro et al. 2006, Fatima et al. 2019).

Implications and Future Research

Optimizing the type and concentration of carbon sources can significantly boost yields, lower costs, and improve efficiency in penicillin production by *P. chrysogenum*. Research underscores the importance of carbon source composition, with sucrose proving more effective than lactose. Each carbon source has an optimal concentration range, and exceeding this range can lead to a decline in production efficiency. Furthermore, combining carbon sources does not always improve production and may, in some cases, decrease it, highlighting the importance of carefully selecting the right carbon sources for optimal antibiotic production.

Future studies should explore the genetic and metabolic pathways affected by induced mutations to develop strategies for improving strains through genetic engineering or metabolic optimization. Gaining a deeper understanding of the molecular mechanisms behind enhanced penicillin production could lead to more efficient strain development, resulting in higher yields and reduced production costs. Genes such as pcbAB, pcbC, and penDE are essential in the penicillin biosynthesis pathway, and mutations in these genes could boost enzyme activity, ultimately improving penicillin production (Fierro et al. 2022). Specifically, increasing the activity of ACV synthetase, encoded by pcbC, could raise the availability of ACV, a crucial precursor in the penicillin synthesis pathway. Additionally, optimizing the phenylacetic acid (PAA) pathway could further enhance penicillin yield (Zhgun 2023). Future research should aim to characterize the genetic and metabolic changes driven by mutations while examining metabolic pathways related to carbon source utilization. This approach would help identify key targets for strain optimization and lead to the development of more efficient and cost-effective production methods, ultimately benefiting the pharmaceutical industry and healthcare by making antibiotics more accessible.

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AUTHOR CONTRIBUTION

All authors contributed to the conception and design of the study. Mohammad Ali Barimani Varandi contributed to performing all tests and writing the original draft. Mohammad Ali Tajick Ghanbary contributed to the conceptualization, supervision, validation, review, and editing of the original draft. Valiollah Babaiezad contributed to software and data analysis. Zohreh Moradi contributed to the methodology, review, and editing of the original draft. William Dashtmiani contributed to optimizing tests, visualization, and investigation; All authors have read and approved the final draft of the manuscript.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

DECLARATION

The authors declare no conflicts of interest.

FUNDING

The author declares that no financial support was received during this research.

ETHICS APPROVAL

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

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بهبود تولید پنی‌سیلین در *Penicillium chrysogenum* از طریق جهش‌زایی القاشده با پرتوی گاما و بهینه‌سازی منبع کربن

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چکیده

پنی‌سیلین، که در سال ۱۹۲۸ توسط الکساندر فلمینگ کشف شد، به عنوان یک آنتی‌بیوتیک مهم شناخته می‌شود که به‌طور قابل توجهی درمان عفونت‌های باکتریایی را دگرگون کرد و نقطه عطفی در تاریخ پزشکی محسوب می‌گردد. *Penicillium chrysogenum*، به عنوان یک گونه قارچی با بازدهی بالا، همچنان نقش محوری در تولید صنعتی پنی‌سیلین ایفا می‌کند. تلاش‌ها برای افزایش تولید پنی‌سیلین تاکنون به عنوان یک حوزه پژوهشی فعال مطرح بوده و رویکردهای مختلفی از جمله جهش‌زایی القایی برای حداکثرسازی بازده تولید به کار گرفته شده‌اند. هدف این مطالعه، بررسی تأثیر جهش‌های القا شده و استفاده از منابع مختلف کربن بر تولید پنی‌سیلین در *Penicillium chrysogenum* است. سویه‌های جهش‌یافته که تحت تأثیر پرتوی گاما ایجاد شده بودند، در مقایسه با سویه‌های وحشی از نظر تولید آنتی‌بیوتیک در محیط‌های کشت حاوی لاکتوز و ساکارز و ترکیب این دو با مقادیر مختلف مورد ارزیابی قرار گرفتند. میزان تولید پنی‌سیلین با استفاده از کروماتوگرافی مایع با کارایی بالا (HPLC) اندازه‌گیری شد و در مجموع ۹۶ آنالیز در سه تکرار انجام پذیرفت. یافته‌های مطالعه نشان داد که تولید پنی‌سیلین به‌ویژه در محیط‌های حاوی ساکارز نسبت به محیط کشت های دیگر و سویه‌های جهش‌یافته در مقایسه با سویه‌های وحشی افزایش چشمگیری داشته است. با این حال، رابطه بین بازده آنتی‌بیوتیک و غلظت منبع کربن غیرخطی بود، به‌طوری که افزایش غلظت منبع کربن همیشه منجر به افزایش تولید پنی‌سیلین نشد. این پژوهش پیشنهاد می‌کند که بهینه‌سازی غلظت منبع کربن و روش‌های جهش‌زایی می‌تواند به بهبود تولید پنی‌سیلین منجر شود. این نتایج دارای پیامدهایی برای طراحی فرآیندهای زیستی در تولید آنتی‌بیوتیک‌های صنعتی است.

کلمات کلیدی: آنتی‌بیوتیک، جهش، سویه وحشی، کروماتوگرافی مایع با کارایی بالا (HPLC).

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