

## Effect of Gamma-Rays on the Growth and Penicillin Production of *Penicillium chrysogenum*

Mohammed M. Aljeldah<sup>1</sup>, Hosam El-Sayyad<sup>2\*</sup>, Nasreldin Elhadi<sup>3</sup> and Ali A. Rabaan<sup>4</sup>

<sup>1</sup>Department of Medical Laboratory Technology, College of Applied Medical Sciences, University of Hafr Albaten, Hafr Albaten Saudi Arabia. <sup>2</sup>Middle Eastern Regional Radioisotope Centre for the Arab Countries, Dokki, Giza, Egypt. (Al-Ghad International College for Applied Medical Sciences Dammam, Kingdom of Saudi Arabia). <sup>3</sup>Department of Clinical Laboratory Science, College of Applied Medical Sciences, Imam Abdulrahman Bin Faisal University, P.O. Box 2435, 31441 Dammam, Kingdom of Saudi Arabia. <sup>4</sup>Molecular Diagnostic Laboratory, Johns Hopkins Aramco Healthcare, Dhahran, Saudi Arabia.

### Abstract

This study aimed to investigate the effects of different doses (25, 50, 75, 100, 125, 150, 200, 250, 500, 1000 and 2000 Gy) of gamma radiation on spore germination in *Penicillium chrysogenum* and on the production and efficacy of penicillin in this fungus. A dose of 200 Gy of gamma radiation increased spore germination by 70.2% after 20 hours of incubation compared to control cultures. It was also found that penicillin yield increased by 95.2% at this dose of gamma radiation. The efficacy of penicillin was remarkably enhanced, resulting in a 99.6% reduction in the total count of *Staphylococcus aureus* treated with penicillin from irradiated *P. chrysogenum* compared with that of *S. aureus* treated with commercially available and purified penicillin from nonirradiated fungi. The results obtained highlighted the potential of this technology for production of penicillin.

**Keywords:** *Penicillium chrysogenum*, penicillin, gamma radiation.

\*Correspondence: helhalby@gc.edu.sa, 0966562983169

(Received: 18 February 2019; accepted: 20 April 2019)

**Citation:** Mohammed M. Aljeldah, Hosam El-Sayyad, Nasreldin Elhadi and Ali A. Rabaan, Effect of Gamma-Rays on the Growth and Penicillin Production of *Penicillium chrysogenum*, *J Pure Appl Microbiol.*, 2019; **13**(2): 779-788. doi: 10.22207/JPAM.13.2.13

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## INTRODUCTION

Many microorganisms are used for the production of pharmaceutical agents, including antibiotics. Among them are fungi perform environmental services of human life and provide economic benefits, e.g., *Penicillium* as a basis for a large pharmaceutical industry of antibiotics<sup>1</sup>. Penicillin is a class of antibiotics that is extracted from *Penicillium chrysogenum* or *Penicillium notatum*<sup>2</sup>. However, *P. chrysogenum* gives much more penicillin than *P. notatum*<sup>3,4</sup> because *P. chrysogenum* carry multiple copies of the penicillin biosynthesis gene cluster<sup>5</sup>. Therefore, *P. chrysogenum* is still the main fungal species used for the production of penicillin. Penicillin is more effective against gram-positive bacteria than gram-negative bacteria by disrupting the synthesis of the peptidoglycan polymerase enzyme<sup>6</sup>.

The production rate of penicillin from fungal cultures is relatively low under normal cultivation conditions. Nevertheless, the fungal growth and penicillin production rates increase when certain controlled substances are used in growth media under suitable physical conditions<sup>7</sup>. Tugay *et al.*<sup>8</sup> reported that the low doses of gamma radiation (200–400 Gy) stimulated spore germination and hyphal growth. Few reports are available on mutagenesis in fungi by gamma radiation<sup>9-11</sup>. Additionally, Thykaer and Nielsen<sup>12</sup> and Tawfik, *et al.*<sup>13</sup> revealed that irradiation of the fungus may considerably improve penicillin production due to high sporulation and morphology variation of mutant strains of *Penicillium chrysogenum*<sup>14</sup>. Recently, UV irradiation was used to increase the production of penicillin in a mutant strain of *P. chrysogenum*<sup>15-16</sup>.

Basically, this study aimed to investigate the efficiency of gamma radiation in improving fungal growth and producing a natural type of cost-effective, sustainable penicillin from mutant derived strain of *P. chrysogenum*.

## MATERIALS AND METHODS

### Isolation of *Penicillium chrysogenum* from spoiled fruit

Since *P. chrysogenum* is a common fungus found in spoiled food and moist soil, a stock culture was isolated from spoiled orange fruits. The general plating method was employed to isolate the organism and plate it onto Sabouraud Dextrose

agar media (peptone, preferably mycological, 1%, dextrose 4%, agar 1.5%, pH 5.6). Several replicas of pure cultures were extracted to expose the cultures to different durations and doses of gamma radiation.

### Identification of *P. chrysogenum*

One  $\mu$ l (per spot) of fungal spore suspensions isolated from spoiled orange fruits were inoculating onto sterile Sabouraud Dextrose agar media and then incubated at the standard temperature of 25°C for 7 days.

After 7 days, The fungal cultures were identified on the basis of macroscopic (colony morphology, color, texture, shape, diameter, degree of sporulation, colour of mycelia, the presence and colours of soluble pigments and exudates, colony reverse colours, and degree of growth) characteristics<sup>17</sup> and microscopic (mycelium septations, presence of specific reproductive structures, shape and structure of conidia, and presence of sterile mycelium) characteristics<sup>18-19</sup>.

### Maintenance of the microorganism

Experimental cultures were descendants of the pure organism and were maintained on agar slants of modified Dox's medium.

### Source of gamma irradiation

The spores of *P. chrysogenum* were exposed to gamma rays from a Cobalt-60 gamma cell source located at the National Center for Radiation Research and Technology – Atomic Energy Authority, Nasr City, Cairo with a dose rate of 13.3 Gy/min for the experimental time.

### Spore preparation and irradiation

Sufficient quantities of spores were extracted by performing a spore print from *P. chrysogenum* cultures. This was accomplished by placing a spatula down on sterile petri dishes. The spores were then scraped as the colony was flooded with sterile saline (0.85% NaCl) and were diluted into a suspension of  $5 \times 10^6$  spore/ml in a sterile beaker. The spore suspension was then distributed into sterile Eppendorf tubes (1.5 ml each) and was irradiated with gamma rays separately at doses of 25, 50, 75, 100, 125, 150, 200, 250, 500, 1000 and 2000 Gy. Three Eppendorf tubes were used as controls, without being irradiated<sup>20-21</sup>. Spore germination and mycelial growth were described as the following and compared for each dose.

### Determination of spore germination

Spore germination percentages were determined by inoculating separately 200 µl of irradiated and nonirradiated (control) fungal spore suspensions on sterile agar plates containing 2% water-agar at 27°C. The resulting cultures were then examined under a microscope at different intervals (4, 8, 12, 16, 20 and 24 hours) post inoculation<sup>22</sup>. The spore germination percentage was calculated as follows:

$$\text{Spore germination percent} = \frac{\text{Total count of germinated spores}}{\text{Total count of spores}} \times 100$$

### Determination of mycelial growth

The fungal mycelial growth for each culture was determined by inoculation of 1 ml of irradiated and nonirradiated fungal spore suspensions into sterile 250 ml conical flasks, each containing 100 ml of Sabouraud dextrose liquid media, followed by incubation at 27°C for 15 days.

Following this period of incubation, flasks were filtrated, and the resulting mycelia were dried at 50°C<sup>23</sup>.

### Extraction of penicillin

The liquid-liquid method was executed for the extraction of penicillin from different shaking flask cultivation systems<sup>24</sup>.

Penicillin recovery from penicillin-rich aqueous broth (100 ml) was performed by liquid-liquid extraction (solvent extraction). Penicillin was extracted from an aqueous phase using acetyl acetate as the solvent. Finally, the extraction was transferred from acetyl acetate into a 2% sodium acetate solution.

### Using TLC for the detection of penicillin G.

Thin layer chromatography (TLC) was used to detect the presence of penicillin isolated from the first and second generations of irradiated and non-irradiated *P. chrysogenum* cultures by acetyl acetate. The obtained samples and standards of penicillin were then developed with benzene: ethylacetate: acetic acid (40:40:20) as the solvent<sup>25</sup> on a TLC plate. The plate was sprayed with ninhydrin reagent<sup>26</sup> and heated at 110°C for 5 min.

### HPLC analysis of penicillin

HPLC analysis of penicillin was conducted in the Institute for Research and Medical Consultations (IRMAC)-Cell Signaling

Lab, Department of Genetic Research – Imam Abdulrahman Bin Faisal University, Saudi Arabia, with a UV detector set at 220 nm and using a C-18 column. The mobile phase consisted of [A] 10 mM ammonium acetate (pH 4.5 acetic acid): [B] acetonitrile, (75:25, A:B) at a flow rate of 1 mL/min<sup>27</sup>. The standards used for comparison were from the commercially available penicillin injection.

### Characterization of purified penicillin

#### Microorganism

*Staphylococcus aureus* (*S. aureus*, obtained from King Fahad Hospital, Imam Abdulrahman Bin Faisal University, Saudi Arabia) was used for testing the antibacterial activity of extracted penicillin.

#### Inoculation and growth conditions

Two milliliters of penicillin extracted from each fungal culture was placed in sterile test tubes with fresh cultures of *S. aureus* at an initial concentration of 1-3 x10<sup>5</sup> CFU/ml, and the tubes were then incubated at 37°C. Viable counts were performed on overnight incubated bacterial cultures. Viable populations were recorded as colony-forming units per milliliter (CFU/ ml) after incubation at 37°C overnight on blood agar plates.

#### Statistical analysis

All data represented herein are the average of triplicates for each experimental procedure and were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 20 software<sup>28</sup> using one-way analysis of variance (ANOVA). Significant differences were calculated ( $P < 0.05$ ) using least significant differences (LSD).

## RESULTS

### Isolation and Identification of *P. chrysogenum*

The data obtained from examinations confirmed that *P. chrysogenum* was the isolated fungus (Fig. 1).

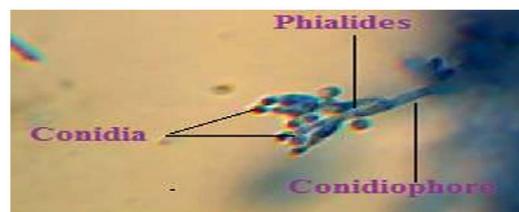


Fig. 1. A microscopic picture of *Penicillium chrysogenum*

### Influence of different doses of gamma rays on the germination of *P. chrysogenum* spores

The effect of gamma irradiation on spore germination in *P. chrysogenum* was investigated. Table (1) indicates that during the first 4 hours of incubation, there was no sign of germination of irradiated and nonirradiated (control) spores in *P. chrysogenum*. After a period of 4 hours, the table shows that the spore germination rate increased with time in all cultures of both irradiated and control spores. Following twenty-four hours of incubation, high germination percentages of

nonirradiated spores (56.8%) were observed in *P. chrysogenum*. At low experimental doses of gamma rays (25, 50, 75, 100, 125, 150 and 200 Gy), a stimulatory effect of spore germination was noticed, and the highest percentage of spore germination (70.2%) was recorded at 200 Gy after 24 hours of incubation (Fig. 2). At radiation doses higher than 200 Gy, the percentage of spore germination significantly decreased, reaching 39.1% at a dose of 2000 Gy compared to the controls (Table 1).

**Table 1.** Spore germination percentages of *Penicillium chrysogenum* after irradiation with gamma rays

Dose(Gy)	Spore germination %					
	4 h	8 h	12 h	16 h	20 h	24 h
Control**	0	29.1±1.9	37.9±2.1	43.2±1.7	49.5±1.5	56.8±1.1
25	0	35±3.1	43±2.5	48±2.6	51±1.2	57.7±2.0
50	0	33±1.5	40±1.0	47±1.7	50±0.9	58.2±1.0
75	0	34±2.5	45±2.0*	48±2.7	51±1.3	59.0±2.0
100	0	32±1.0	40±1.0	45±0.8	52±1.8	58.0±1.1
125	0	39±1.5*	46±1.9*	47±1.8	53.2±2.0	57.9±1.0
150	0	38±2.0*	45±1.3*	46±1.1	52.6±1.9	57.1±0.9
200	0	41±1.0*	49±2.0*	54±2.0*	61±1.9*	70.2±1.0*
250	0	31±1.5	39±1.1	44±1.1	49±0.3	53.5±2.0
500	0	28±2.2	30±2.8*	37±1.0*	42±1.0*	49.5±1.3*
1000	0	22±3.0*	27±2.0*	28±1.3*	34±2.1*	40.3±1.9*
2000	0	20±2.0*	25±1.0*	27±1.4*	33±2.2*	39.1±2.0*

\*Significant differences from the control (P<0.05).; \*\* Control (nonirradiated spores).

### Mycelium growth of *P. chrysogenum* affected by irradiation with gamma rays

The effect of gamma ray exposure doses on mycelial growth was investigated. Table (2) represents the average mycelial dry weights of *P. chrysogenum* after irradiation by gamma rays. The results in this table reveal that the doses of 100, 125, 150 and 200 Gy significantly increased the mycelial dry weight.

The highest yields were observed at a dose of 200 Gy (1.2 g/100 ml) compared to those in the controls (0.57 g/100 ml); however, with higher gamma radiation doses, the growth significantly decreased, reaching a minimum value of 0.02 g/100 ml dry weight at a dose of 2000 Gy.

### TLC analysis of purified penicillin

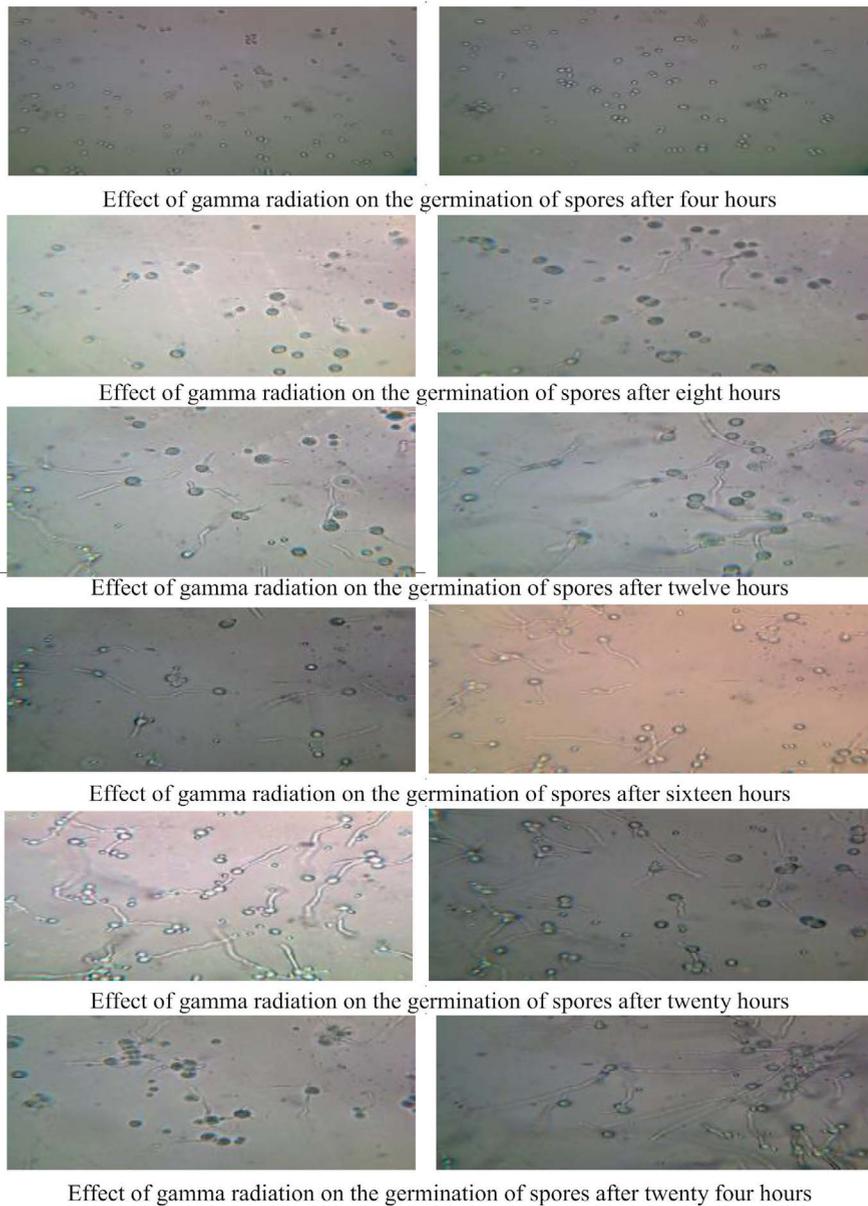
In this study, the irradiated and nonirradiated *P. chrysogenum* grew well in

**Table 2.** Influence of gamma rays on dry weight (g/100 ml) of *P. chrysogenum*.

Doses (Gy)	Mean Dry weight (g/100 ml)
Control**	0.57±0.02
25	0.52±0.02
50	0.54±0.03
75	0.53±0.03
100	0.61±0.05*
125	0.85±0.04*
150	0.88±0.02*
200	1.20±0.05*
250	0.43±0.07
500	0.03±0.02*
1000	0.03±0.01*
2000	0.02±0.02*

\*Significant differences from the control (P<0.05).

\*\* Control (nonirradiated spores).



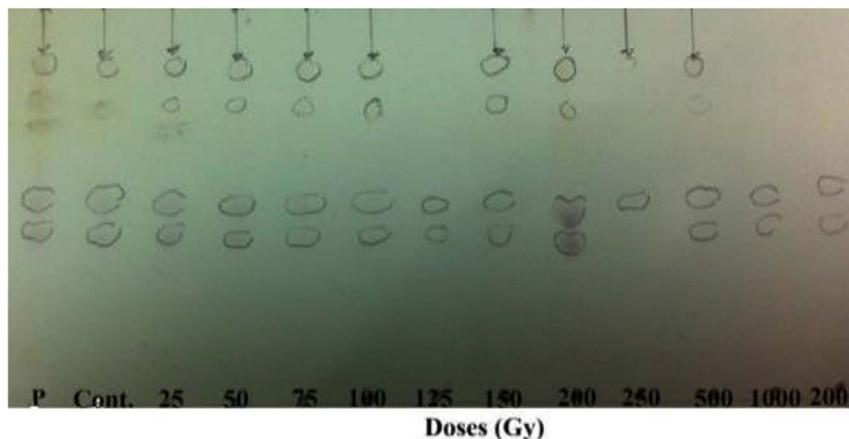
**Fig. 2.** Germination of *Penicillium chrysogenum* spores either gamma radiated at a dose of 200 Gy or nonirradiated

Sabouraud Dextrose liquid media and produced substances inhibiting the growth of bacteria. The anti-critical composition of penicillin in this medium was determined using the TLC method and was compared with that of standard penicillin G (Fig. 1). The purified penicillin from irradiated and nonirradiated samples was confirmed using analytical TLC. The samples showed pale spots at all gamma irradiation doses except at 200 Gy, which showed clear colored bands. However, there

were no bands detected at doses of 1000 and 2000 Gy after spraying with ninhydrin (Fig. 3).

#### HPLC analysis of purified penicillin

Comparisons of spore germination, mycelial growth, and antibacterial activity of penicillin were carried out to evaluate the most effective dose of gamma irradiation. According to the data presented, the dose of 200 Gy was found to be the most effective and thus was selected for the HPLC analysis. Table (3) shows penicillin



**Fig. 3.** Analytical TLC image of various doses of gamma ray samples of penicillin extracted from *P.chrysogenum*

**Table 3.** Production of penicillin by wild-type and mutant strains of *P. chrysogenum*

Doses (Gy)	Concentration (mg/ml)	Percentage
Control**	2.54 ± 0.61	0.0
200 Gy	4.96 ± 0.75*	95.2

\*Significant differences from the control (P<0.05).

\*\* Control (nonirradiated spores).

production by wild-type and selective mutant strains of *P. chrysogenum*. The mutant strain of *P. chrysogenum* irradiated with 200 Gy showed the highest yield of penicillin (4.96 mg/ml) compared with the wild-type strain (2.54 mg/ml).

**Characterization of purified penicillin**

Table (4 and 5) represents the antimicrobial activity against *S. aureus* by commercial and purified penicillin from *P. chrysogenum* after being irradiated by gamma rays. The purified penicillin

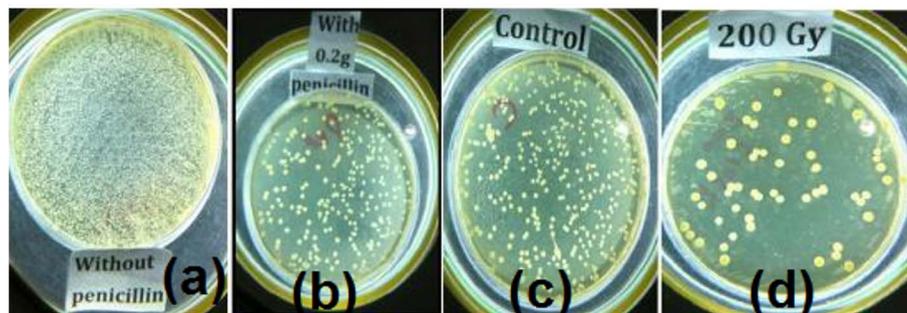
from irradiated and nonirradiated samples gave better results than commercial penicillin. The purified penicillin from *P. chrysogenum* irradiated with 200 Gy was more effective than the other samples tested (table 5). The total count of *S. aureus* treated with penicillin from the samples irradiated with 200 Gy was markedly reduced to 0.2 x 10<sup>5</sup>CFUs/ml, which is a reduction rate of 99.6% (Fig. 4), compared to that of the samples

**Table 4.** Effect of commercial penicillin on colony counts (CFUs/ml) of *S. aureus*

Type	Colony Count x 10 <sup>5</sup> CFUs/ml	Reduction %
Blank**	100	0
Commercial Penicillin***	4.0±0.6	96

\*\*Media without penicillin.

\*\*\* Media with commercial penicillin.



**Fig. 4.** Characterization of commercial and purified penicillin from *P. chrysogenum* on *S. aureus*.(a) Colony count without penicillin. (b) Colony count with commercial penicillin. (c) Colony count with purified penicillin from nonirradiated *P. chrysogenum*. (d) Colony count with purified penicillin from irradiated *P. chrysogenum*.

treated with commercial penicillin (Table 4) and purified penicillin from nonirradiated fungi (Table 5).

**Table 5.** Effect of purified penicillin on colony counts (CFUs/ml) of *S. aureus*

Doses (Gy)	Colony Count x 10 <sup>5</sup> CFUs/ml	Reduction %
Blank**	100	0
Control****	5.5±0.5	94.5
25	14.0±2.0*	86
50	24.0±2.1*	76
75	11.0±3.0*	89
100	10.0±1.0*	90
125	26.0±5.0*	74
150	17.0±1.9*	83
200	0.20±0.1*	99.8
250	31.0±4.0*	69
500	21.0±3.0*	79
1000	34.0±4.9*	66
2000	35.0±3.8*	65

\*Significant differences from the control (P<0.05).

\*\*Media without penicillin.

\*\*\* Media with commercial penicillin.

\*\*\*\* Media with extracted penicillin from nonirradiated fungi.

## DISCUSSION

One of the major problems that researchers face is low penicillin titers produced by the original *P. notatum* strain and the increasing demand of antibiotics<sup>29</sup>.

In this context, the objectives of our study were to investigate the effects of gamma irradiation on the production of penicillin by *P. chrysogenum*.

In the current study, *P. chrysogenum* spores were irradiated with different doses of gamma radiation, and the potential of *P. chrysogenum* to grow and produce penicillin was analyzed. In general, at lower radiation doses, there was an increase in the spore germination and mycelial growth of *P. chrysogenum* but a gradual decrease at higher radiation doses.

Our results revealed that there was no effect on the germination of irradiated and nonirradiated spores during the first four hours of incubation. This finding is probably attributed to the swelling of spores, which occurs before effective germination<sup>30</sup>.

The viability of fungi after irradiation also revealed that low doses of gamma irradiation significantly increased the viability of fungal spores. These results were in agreement with those of El-Abyad and Ismail<sup>31</sup>, which illustrated that the spore germination and mycelial growth of some fungi were stimulated by low dose of gamma irradiation (50 Gy). These results are also in agreement with those of Adams *et al.*<sup>32</sup>, who observed that the mycelial growth of *Penicillium expansum* and *Penicillium patulum* were stimulated by low doses of gamma rays (1.5 KGy). Some other studies<sup>33-34</sup> support the findings of this study, who reported that the simulative biological effects produced by low-dose irradiation of organisms have been studied extensively because low-dose irradiation brings about effects that differ from those produced by high-dose irradiation.

In our study, it was found that the high dose of gamma irradiation, 2000 Gy, inhibited the growth of *P. chrysogenum*. these may be due to that the gamma rays destroy the DNA structure of cells and that cells cannot continue to function, while incomplete inhibition may result from a small injuries to cells<sup>34</sup>. These results agreed with those of previous investigations that showed that exposure to high doses of radiation resulted in the complete inhibition of spore germination<sup>35</sup>. Those previous studies also showed that *A. ochraceus* spore germination was completely inhibited at 600 Gy, while a noticeable activation was recorded at lower doses. The irradiation of fungal isolates with 400 Gy showed no observable effects on spore germination<sup>36</sup>.

This finding could be explained by proposing that higher doses of irradiation may initiate a shock to the spores, which requires more time for recovery, or it may be attributed to the possibility of toxic substance production<sup>37</sup>.

The purpose of the present investigation was to enhance the production of penicillin by subjecting the wild-type strain of *P. chrysogenum* to gamma irradiation.

Among all the mutants tested, the mutant of the *P.chrysogenum* strain treated at 200 Gy showed the highest penicillin production compared with the parent strain.

The increase in penicillin production from the irradiated strain may be a consequence

of metabolic reorganizations<sup>38</sup>. However, gamma radiation may induce the enzymes that are involved in the last step of penicillin biosynthesis, namely, acyltransferase, phenylacetyl-CoA ligase and isopenicillin N acyltransferase<sup>38-39</sup>. This may also be explained by the modifications of a microsomal cytochrome P450 monooxygenase that leads to the over production of penicillin<sup>40</sup> or the amplification of the genomic region that includes the three penicillin biosynthesis genes<sup>41-43</sup>.

These data agree with previously reported results of other studies<sup>15-16</sup> that revealed that the strain of *P. chrysogenum* irradiated with ultraviolet rays exhibited increased penicillin production over the parent strain.

In our study, the extracted penicillin from irradiated *P. chrysogenum* showed significant antimicrobial activity against the clinical isolate of *S. aureus* compared to the extracted penicillin from the parent strain and commercial penicillin. This may be due to the overproduction of penicillin and the ability of penicillin to reach intracellular spaces and denature the bacterial cell wall.

## CONCLUSION

These results demonstrate that gamma radiation is a successful method for improving the production of penicillin. The ability of gamma radiation to change the structural characteristics and bioactivities of penicillin is not only cost effective and sustainable but also an environmentally friendly approach to natural penicillin production.

## CONSENT FOR PUBLICATION

We (all co-authors, who signed this letter) certify that we have participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of the manuscript, to take public responsibility for it and have agreed to have our name listed as a contributor. We believe the manuscript represents valid work. Neither this manuscript nor one with substantially similar content under our authorship has been published or is being considered for publication elsewhere, except as described in the covering letter. We certify that all the data collected during the study is presented in this

manuscript and no data from the study has been or will be published separately. We attest that, if requested by the editors, we will provide the data/information or will cooperate fully in obtaining and providing the data/information on which the manuscript is based, for examination by the editors or their assignees.

We give the rights to the corresponding author to make necessary changes as per the request of the journal, do the rest of the correspondence on our behalf and he/she will act as the guarantor for the manuscript on our behalf.

Yours sincerely,

Author's Name

1 Mohammed M. Aljeldah: Main author

2 HosamEldin El-Sayyad: Corresponding author

3 NasreldinElhadi: Co-author

4 Ali A. Rabaan: Co-author

## ACKNOWLEDGEMENTS

The authors express their gratitude to Al-Ghad International Colleges of Applied Medical Sciences in Dammam, Saudi Arabia for providing the necessary facilities and the use of various laboratories and We are grateful also to the Deanship of Scientific Research Imam Abdulrahman bin Faisal University for supporting our research (grant No. 2014130).

## CONFLICTS OF INTEREST

All authors declares that there is no conflict of interest.

## FUNDING

This work was supported by the Deanship of Scientific Research at the Imam Abdulrahman Bin Faisal University, grant No. 2014130.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

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

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