Thi-Qar Medical Journal (TQMJ):Vol.(26),No.(2),2023

Web Site: https://jmed.utq.edu Email: utjmed@utq.edu.iq

ISSN (Print):1992-9218

Comparison between Two Fermentation Media in Production of Antibacterial Crude Extracts from Penicillium Chrysogenum

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Abstract

Background: Filamentous fungi such as Penicillium chrysogenum is widely used as hosts for the industrial products such as proteins and secondary metabolites.

Aim of study:

Determine which two fermentation media used in this study is the best for the production of crude antimicrobials from Penicillium chrysogenum. A strain of Penicillum chrysogenum UNIJAG.PL.242was isolated from water sample (0.5 ml) of the Hammar marsh area in Thi-Qar Province, south of Iraq. The isolation was carried out using potato dextrose agar (PDA) at 25°C for 5 days and sub-culture was conducted on Malt extract agar (MEA) and Czapez dox agar (CDA) besides PDA at the same conditions of the isolation. P. chrysogenum was identified depending on the morphological and molecular characteristics in which ß-tubulin genes within the fungal genome were detected and then sequenced. This fungus was preliminarily tested against Staphylococcus aureas, Streptococcus pyogens, Bacillus cerues, Escherichia coli and Psuedomonus aeroginosa by which P. chrysogenum inhibited growth of Staphylococcus aureas. The fungus fermented using penicillin production medium (PPM) and corn steep liquor medium (CSLM) and the crude extracts of the fungal filtrate were harvested and tested against these bacteria that the extract of PPM inhibited S.aureus, S.pyogenes, B.cerues. And E.coli but P.aeroginosa was resistant to this extract. Relatedly, the extract of CSLM did not produce inhibitory values against B.cerues and P.aeroginosa which the remaining tested bacteria were affected. Both crude extracts were analyzed using HPLC which showed the numbers of peaks which some of them were similar to peak of pure penicillin G which was tested a control in this process.

Conclusion:

The present study concluded this fungus produced antimicrobial compounds and PPM is better fermentation medium than CSLM.

Key worlds: Penicillium chrysogenum, antimicrobial compounds, PPM, CSLM.

Introduction

Penicillium.chrysogenum is a one of the filamentous fungi that are frequently found in nature. In addition, this fungus is prevalent etiology of the fungal food contamination [1&2]. The manufacturing of antibiotics is linked to Penicillium chrysogenum, making it one of the most crucial microorganisms in the industrial world. P. chrysogenum is a mesophilic microbe that can thrive at temperatures as low as 4ºC, as high as 37ºC, and anywhere in between. Penicillium chrysogenum grows best in an acidic environment with a PH of 4 to 6. The ideal growing environment, which includes temperature, medium or nutrient content, aeration, and agitation, determines the development of antimicrobial agents as secondary metabolites [3]. Many biologically active substances can be found in abundance in Penicillium. Which also contains immunosuppressants, drugs to lower cholesterol, and antibiotics. Additionally, more anti-HIV and anti-tumor shops [1]. The majority of these are filamentous fungi, exhibiting remarkable metabolic complexity. Particularly, they are renowned for their synthesis of enzymes and secondary compounds, many of which have been utilized. Elicitors are substances that, when added to cultures as non-nutrient additives, raise the concentration of metabolites. This phenomenon is referred to as "elicitation." Elicitors are categorized based on their origin and molecular structure as physical or chemical, biotic or abiotic, and complex or defined. Filamentous microorganisms like fungi and Streptomyces are extensively employed for industrial-scale production of secondary compounds. The impact of oligosaccharide elicitors as enhancers of secondary metabolite synthesis has been examined in both plant cell systems and fungal cultures recently [2]. P. chrysogenum has long served as a model to enhance our understanding of secondary metabolite production and its correlation with the microorganism's physiological traits. However, only a few studies have investigated this relationship in the presence of oligosaccharide elicitors, with the most potent being mannan oligosaccharides derived from locust bean gum. Elicitation studies on P. chrysogenum to enhance penicillin G, a representative secondary metabolite, have demonstrated significant increases in antibiotic concentration when mannan oligosaccharides are present. Our recent investigation focused on the correlation between penicillin G production and fungal morphology. The addition of oligosaccharide elicitors to P. chrysogenum cultures resulted in a maximum increase of 47% in hyphal tip number, accompanied by a maximum increase of 120% in penicillin G levels. Intracellularly, in P. chrysogenum, elicitors not only impact secondary metabolite production but also affect the production of other metabolites such as ROS (reactive oxygen species). In the latter case, the presence of oligosaccharide elicitors led to a decrease of up to 54% in cellular ROS levels. The influence of elicitors on pigment production has also been observed in P. chrysogenum [4].

Materials and Methods

Isolation and identification of Penicillium chrysogenum

Penicillium chrysogenum was isolated from the waters of the Hammar marsh area, south of Thi-Qar, by collecting a 20 ml water sample using a sterile cup container and 0.5 ml of the sample was 0.5 spread aseptically on a plates of the PDA and the incubation was at 25 °C for 5 days. The sub-culture was done using MEA and CDA as well as PDA at the same condition of the incubation. P.chrysogenum was identified depending on the morphological and molecular properties. Microscopic identification was performed using slide culture and based on the presence, shape, and arrangement of the conidia, phialides, and conidiophores. Lactopheol cotton blue stain was used in this identification under the magnification (10x, 40x) of a light microscope. While the macroscopic characterization was given according to morphological properties of the fungal colonies on these media [5]. The identification of P.chrysogenum was confirmed using DNA primers were forward: GGTAACCAAATCGGTGCTTTC. Reverse: ACCCTCAGTGACCCTTGGC were used to detect beta tubulin genes in a genome of this fungus. PCR process was performed as in (Table, 1). Concerning fungal DNA was extracted according to the instruction of Bio basic/Canada DNA kit. The PCR products were sequenced using MEGA program and then submitted into bioinformatics in the BLAST of NCBI [6].

Table, (1): PCR program

	Temperature D		
Initial Denaturation	**95°C**	**5 Min**	
Denaturation	**95°C**	**30 Sec**	
Annealing	**60°C**	**30 Sec**	
Extension	**72°C**	**30 Sec**	
Final Extension	**72°C**	**7 Min**	
	10˚C	**10 Min**	

Preliminary examination of P. chrysogenum antibacterial activity

This test was done according to [7]. Discs (6 mm in diameter) were aseptically done in a PDA Petri dish of 7 days aged Penicillium chrysogenum. In addition, five isolates of 24 days old pathogenic bacteria were also prepared in tubes contained nutrient broth (NB). These bacteria were S.aureus, S .pyogenes, E.coli, B.cerus,and P. aerogenosa. Also, dishes of Muller Hinton agar (MHA) were prepared and inoculated using a sterile cotton swab with 100 µL (1.5×10^8) cell per ml according to McFarland standard solution of each isolate and a disc of the P. chrysogenum colony was placed on each bacterial MHA dish incubated at 37 °C for 24 hrs. Then inhibitory zones (IZs) were measured by mm.

Fermentation process

In this process, penicillin production medium (PPM) and corn steep liquor medium (CSLM) were used in this process. The PPM and CSLM were prepared according to [8&9]. The PPM consisted of 5g , 75 g , 4g , 4gm , 5 g , K_2HPO_4 2.12 g, KH_2PO_4 5.1 g, of the glucose, lactose, urea, Na_2SO_4 , CH_3COONH_4 , K_2HPO_4 , respectively. All components were dissolved in 1 liter of the distilled water. While components of the CSLM were 20g, 20 g, 0.5g, 3g, 0.125g, 5g consisted of the corn steep liquor, lactose, KH_2PO_4, $NaNO_3$, $MgSO4.H_2O$, and chalk, respectively and dissolved in 1 liter of the distilled water. The pH values of both media were adjusted at 6±2 and then autoclaved. Five tubes contained PPM in which each tube had 10 ml of this medium. Four tubes were inoculated with 100 µL of the P. chrysogenum inoculum that the fungal volume contained 112.5×10^5 spores per ml based on the following equation: Number of spores / ml $=\frac{\text{number of spores}}{5} \times 25 \times$ dilution factor $\times 10^4$ [10] and fifth tube was left a negative control. All tubes were incubated at 25 °C using rotary shaking incubator (150 rpm) for 8 days. Also, this fungus was fermented in CSLM as same process of the PPM. In addition, P. chrysogenum grew in autoclaved two flasks of both these media that each flask contained 400 ml of each medium and at the same conditions of the fermentation tubes. In this process, two flasks of the PPM and CSLM were used negative control.

Examination of pH, growth curves, and antibacterial secondary screening

The tubes which were used in the above fermentation process, they were tested after completing 2 days of the incubation period in which one tube was tested after 48 ago, second tube was examined after 4 days and thus until getting 8 days of the period. The examination reported pH values of fungal filtrate in each tube which had growing P.chrysogenum as well as number of the growing fungal spores the filtrate to record the growth curves of this fungus in both PPM and CSLM at 25 °C using rotary shaking incubator (150 rpm) for 8 days using spectroscopic device. Also, the fungal filtrate was tested after of P. chrysogenum was grew in two fermentation media PPM and CSL to period (2, 4, 6, 8) days against S. aureus, S. pyogens, B. cerues, E. coli, and P. aeruginosa using the same method which was described in the preliminary examination of P. chrysogenum antibacterial activity but the fungal disc was replaced by 100 µL of the filtrate were loaded in a well (6 mm in diameter) located the center of a MHA plate inoculated with each one of the bacterial species. The antibacterial examination of the fermented fungal filtrate was tested to detect the secondary screening of the antibacterial production which produced from P.chrysogenum in the PPM and CSLM.

Extraction of the crude filtrate extract and examining their antibacterial activity

After completing fermentation process of two flasks in which P.chrysogenum grew, the flasks of the PPM and CSLM were used to separate the fungal filtrate from mycelia using a filter paper and

3 g of an activated charcoal were added into the filtrate and left at a fridge for 24 hours. Then this filtrate was filtrated using 8 layers of a gauze for getting charcoal free – fluid product which was mixed with absolute ethyl acetate using a separator funnel. The upper layer of the mixture was selected and left at 40°C until evaporation of the ethyl acetate for obtaining crude solid extract which was dissolved in the distilled water and sterilized by filtration (0.2 µm sized Millipore filter paper). The sterile extract was kept at the freeze. The MHA plates were prepared and inoculated with 100 µL (1.5×10^8) cell per ml of S. aureus, S. pyogens, B. cerues, E. coli, and P. aeruginosa separately. Two wells (6 mm in diameter of each well) were done in each plate. 100 µL of PPM extract was placed in one well and same volume of the CSLM extract was loaded in the second well. All plates were incubated at 37 C for 24-48 hour until IZs formed and measured by mm.

TLC chromatography and HPLC analysis of the fungal filtrate extracts

The fungal filtrate extracts were dissolved in the distilled water and they were used in the TLC chromatography and HPLC analysis. A plate (15 × 50) cm were used and very small amount was placed on the one end of the plate. As well as, dissolving pure penicillin G was tested a positive control and the solvent system was shown in (Table, 2). The Refection factor (Rf) was examined according to the below equation. Related to the HPLC process, the extract was tested according to the parameters which were shown in (Table, 3).

$$\text{Rf} = \frac{\text{The distance traveled by the material from the starting region}}{\text{The distance traveled by the solvent from the starting region}}$$ [11].

Statistical analysis

This data was statistically analysis by using SPSS (Statistical Package of Social Science version 26), based in using one way ANOVA and LSD at p. value < 0.05.

Results

Identification of Penicillium chrysogenum

Morphologically, P. chrysogenum appeared colonies on the PDA were greenish blue contained grooves and no aerial growth of the colonies. Also, the exudates were noticed on the colonies as well as the colonies had white edges, and reversible was yellow. This fungus gave growth on the MEA and its colonies were slightly greenish blue without exudates, grooves, and aerial growth besides brownish reverse. Concerning CDA, P. chrysogenum produced poor growth on this medium and its colonies were yellowish white and no exudates, grooves, and aerial growth. In addition, colony reverse was yellow when the fungus grew on CDA. Microscopically, slide culture revealed ovoid conidia and conidiophores had phialides (Fig. 1). This identification was confirmed by molecular technique which PCR resulted in tubulin genes in the fungal genome located at a base pairs (550bp) and the results of sequencing showed this identification belonged to the P. chrysogenum (Fig.2).

Thi-Qar Medical Journal (TQMJ):Vol.(26),No.(2),2023

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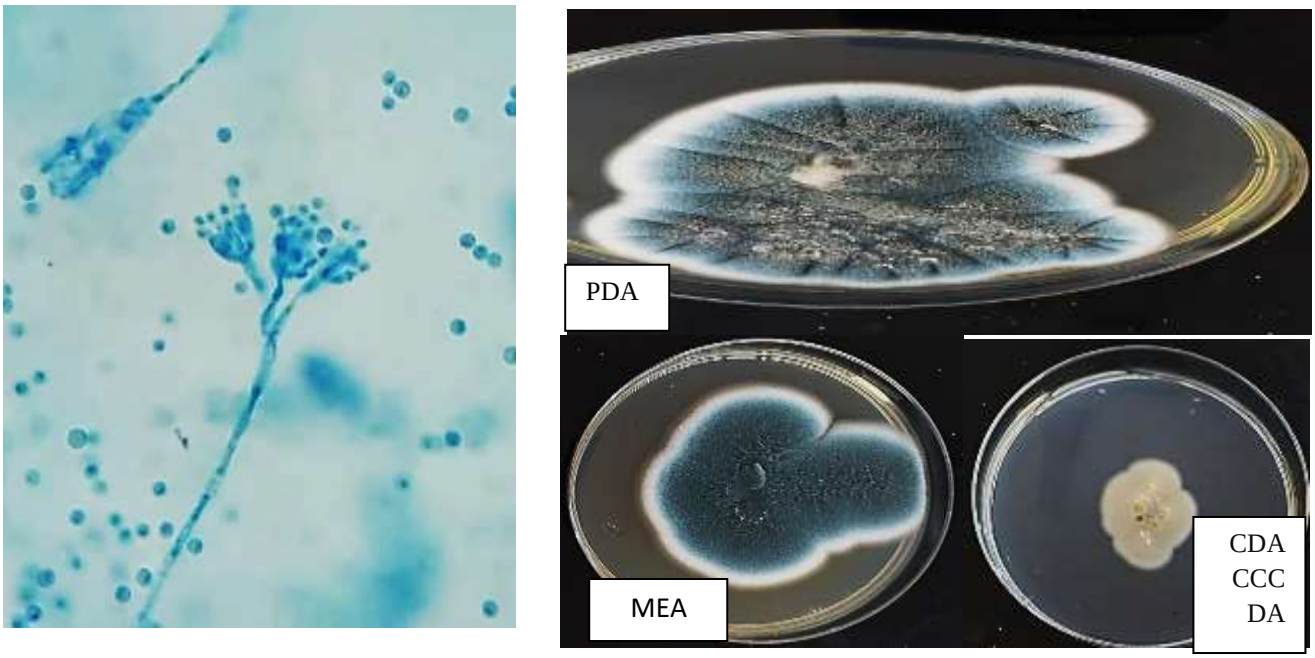
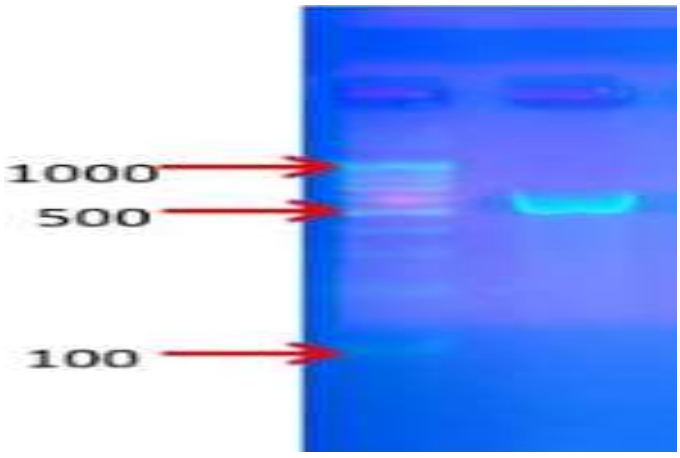
ISSN (Print):1992-9218



Figuer. (1) Morphology of P. chrysogenum



Penicillium chrysogenum strain UNIJAG.PL.242 beta-tubulin (Tub) gene, partial cds

Sequence ID: MT304424.1Length: 466Number of Matches: 1

Range 1: 1 to 431GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
712 bits(385)	0.0	416/431(97%)	1/431(0%)	Plus/Minus

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Query  22   CTGACCG-ATACGAATTTGTCGGGGCGTACAAGCTTGCCGAAGGGACCGGAGCGGACAGC  80
           ||||||| | ||||| |||||||||||  | ||||||||||||||||||||||||||||||
Sbjct  431  CTGACCGAAGACGAAGTTGTCGGGGCGGAAAAGCTTGCCGAAGGGACCGGAGCGGACAGC  372
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Figuer (2). Molecular identification of P. chrysogenum in which (ß-Tub) gene sequencing match appeared.

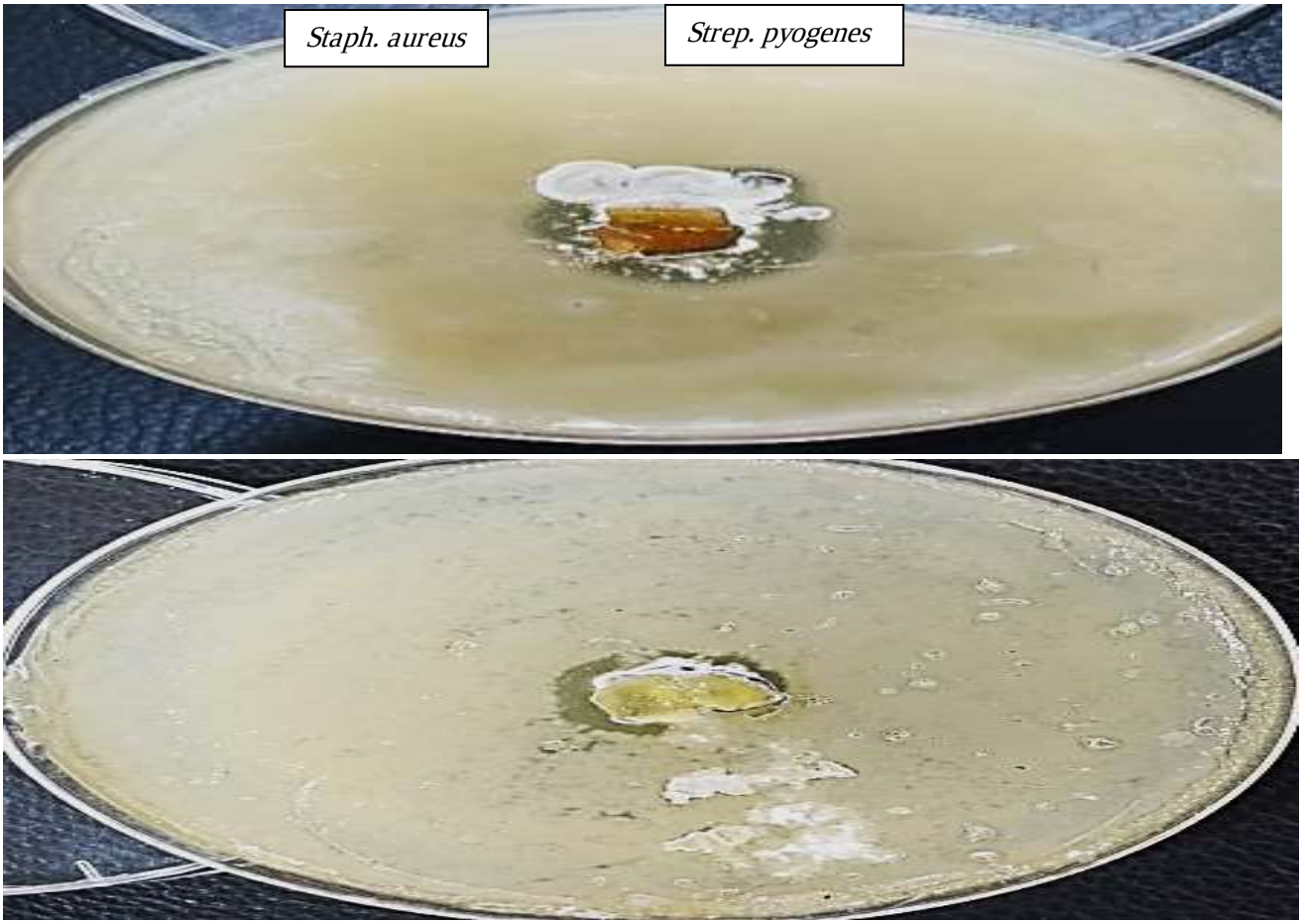
Preliminary and secondary examinations of P. chrysogenum antibacterial activity

P. chrysogenum produced IZs against a growth of the S. aureus, and S. pyogenes only and B. cereus, E. coli, and P. aeruginosa were resistant (Fig.3). Fungal filtrates of PPM and CSLM resulted in different values of the IZs against these tested bacteria. Filtrate of the PPM revealed good antibacterial results compared with CSLM filtrate (Table, 2).

Table (2). Antibacterial activities of the fungal filtrates against five species of bacteria

Bacterial Type	Day	Inhibition Zone In Media/ Mm	
		CSL	PPM
Staph. Aureus	2	0	0
	4	0	8
	6	0	10
	8	8	15
Strep. Pyogenes	2	0	0
	4	0	0
	6	0	0
	8	0	12
E.Coli	2	0	0
	4	0	10
	6	0	18
	8	9	25

B.Cerus	2	0	0
	4	0	0
	6	0	7
	8	0	10
P.Aurogenusa	2	0	0
	4	0	0
	6	0	0
	8	0	0



Figuer. (3). Preliminary screening of P. chrysogenum's antibacterial production

Thi-Qar Medical Journal (TQMJ):Vol.(26),No.(2),2023

Web Site: https://jmed.utq.edu Email: utjmed@utq.edu.iq

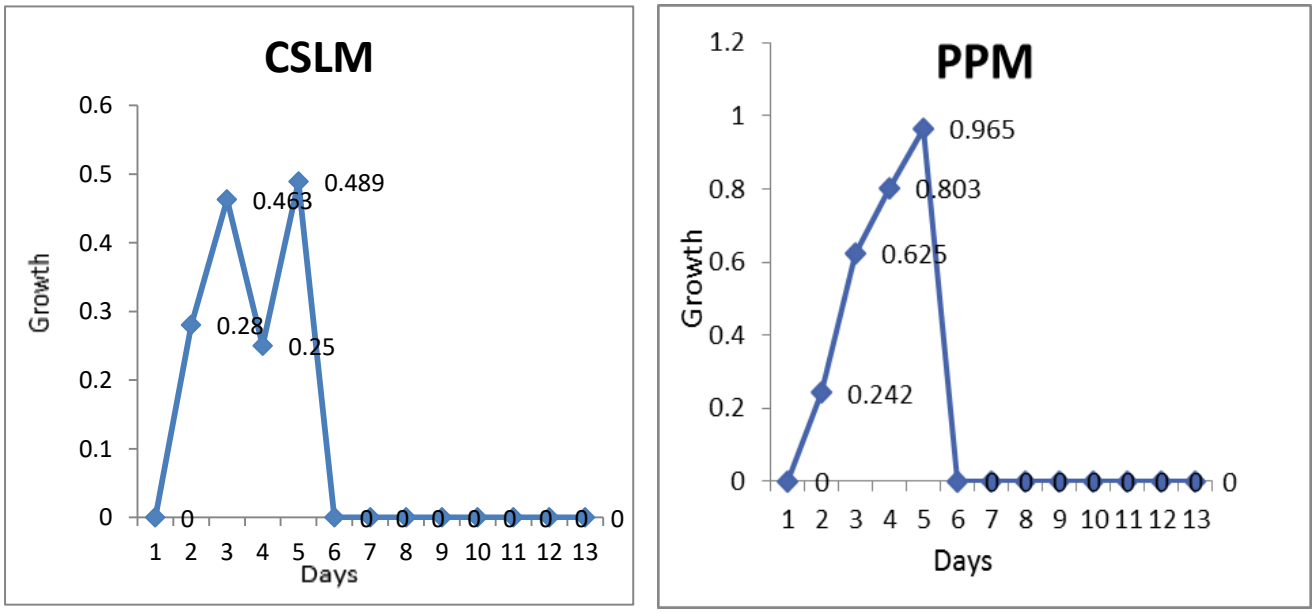
ISSN (Print):1992-9218

PH values of the fermentation media and growth curves of Penicillium chrysogenum

The pH values of both PPM and CSLM decreases from 6 into 4.5 (Table, 3) and curves of the fungal growth revealed PPM appeared better growth than CSLM (Fig. 4).

Table (3). PH value in fermentation media.

Ph	Day	Media
6±2	0	Ppm
6	2	
5.5	4	
5	6	
4.8	8	
6±2	0	Csl
6	2	
5.8	4	
5	6	
4.5	8	



Figuer. (4): curves of the fungal growth revealed PPM appeared better growth than CSLM.

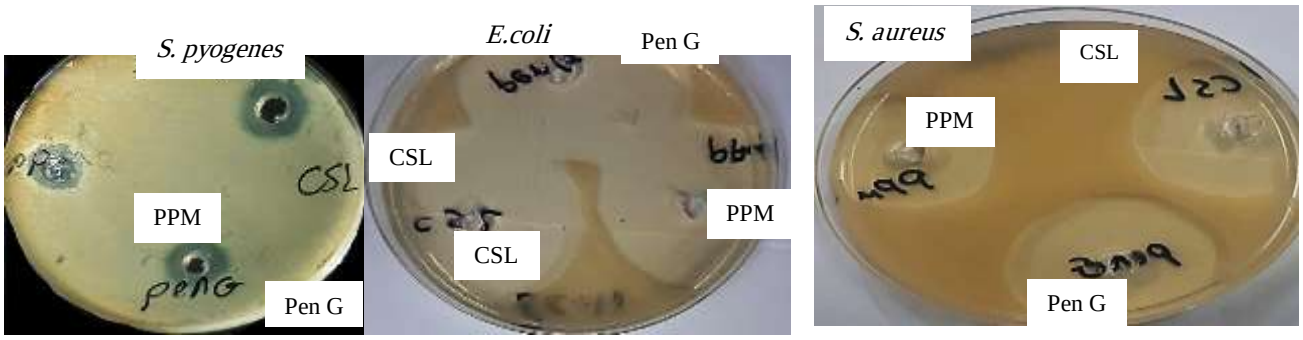
Antibacterial activities of the crude fungal filtrate extracts

Both crude fungal filtrated extracts of the PPM and CSLM produced inhibition against S. aureus, S. pyogenes, and E.coli. About B. cereus, it was inhibited by extract of the PPM only while P. aeruginosa was resistant into both extract. Regarding pure

penicillin G, it produced IZs against all tested bacteria except for P. aeruginosa which was resistant (Table, 4) and (Fig. 5).

Table (4). Antibacterial activities of the crude fungal filtrates extracts against five species of pathogenic bacteria in comparison with pure penicillin G.

Diameter Of Inhibition Zone/Mm			
Bacterial Species	**Csl**	**Ppm**	**Pen G**
E.Coli	**45**	**48**	**25**
S. Aureus	**40**	**38**	**30**
S. Pyogenes	**18**	**23**	**28**
B.Cerus	**10**	**8**	**8**
P.Aurogenosa	**0**	**0**	**0**

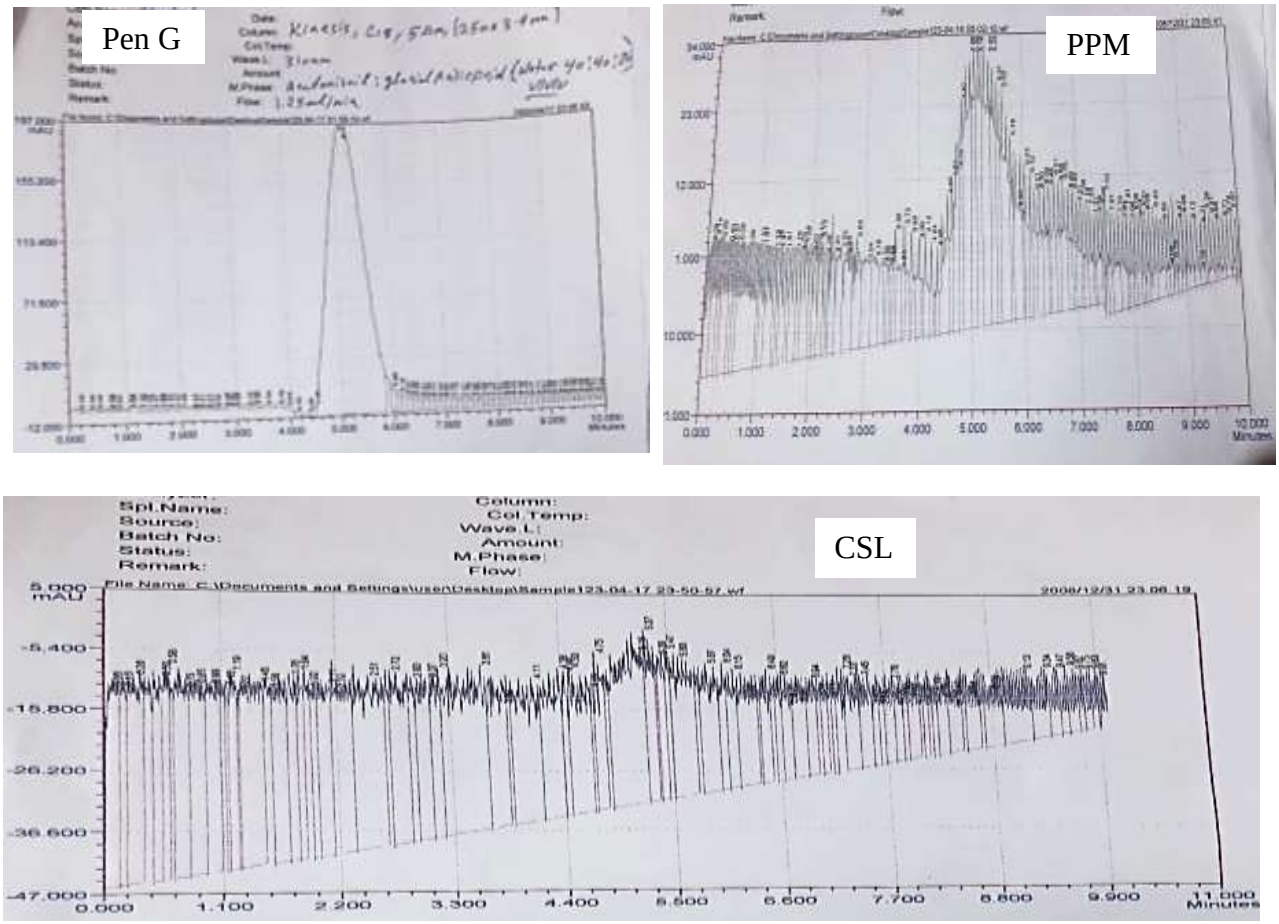


Figuer. (5): Antibacterial activities of the crude fungal filtrate extracts against five species of the pathogenic bacteria in comparison with pure penicillin G.

TLC chromatography and HPLC analysis of the fungal filtrate extracts

The TLC technique showed that the crude fungal filtrate extracts appeared spots for PPM and CSL differed from Rfs of the spots which produced from pure penicillin G (Fig. 6). About HPLC, it showed a number of the peaks which were some of them had similar retention times of the pure penicillin G which was used a positive control in the HPLC process (Fig. 7).

Figuer. (6): TLC technique showed that the crude fungal filtrate extracts appeared spots differed from Rfs of the spots which produced from pure penicillin G.



Figuer. (7): The peaks which were some of them had similar retention times of the pure penicillin G which was used a positive control in the HPLC technique.

Discussion

Penicillium chrysogenum is considered a one of the fungi which are more distributed in the environment that inhabit water, soil, air, and plants [12]. Also, Penicilllium species have difficult identification depending on the morphological characteristics, however; they are identified if the expertise is obtained. The genetic analysis helps this identification to be accurate [13]. This study, therefore, used the morphological methods, PCR, and sequencing for getting a confirming the P. chrysogenum which was isolated from a water sample of the Hammar marsh areas in Thi-Qar province is located in the south of Iraq. The current study showed a sequencing the tubulin genes of this fungus appeared the fungus is P. chrysogenum (Fig. 1 and 2).

Penicillium chrysogenum is able to inhibit bacteria due to its production of the secondary metabolites such as antibacterial products [14]. This fungal antagonism is examined in the laboratory to appear ability of this fungus to produce the antibacterial compounds, however; some bacteria are resistant into fungi [15]. Therefore, the results of the present study showed P.

chrysogenum inhibited S. aureus, and S.pyogenes but other tested bacteria were noticed resistant in (Fig. 5) and Table, 4 [16, 17&18]. Physiologically, fungi including P. chrysogenum is affected by a number of the environmental factors such as the composition of medium, temperature degrees, pH, and inoculum [19]. Fermentation media is most important factor which has effects on a growth of P. chrysogenum and producing its metabolites such as antimicrobial agents [20&21]. For examples, iron participates in a synthesis of the cytochrome P450 oxidases which are important in the production of many metabolites from fungi. In addition, magnesium phosphate is remarkable metal in the fungal metabolism that many enzymes contain the magnesium ion where a process of the metabolism carries. As well as, a stability of the proteins is performed if a medium has zinc. Also in this context, the media improve the fungal growth if they contain vitamins, sugars, nitrogen sources etc. [22]. For these reasons, this study used two fermentation media, PPM and CSLM, to evaluate growth of P.chrysogenum and its ability to give antibacterial agents that the fungus showed good growth in the PPM compared with CSLM (Fig. 4). Antibacterial compounds of P.chrysogenum including penicillin are extracellular products that their harvesting is in the fungal filtrate [23]. The present study examined the filtrate of P.chrysogenum growing in PPM and CSLM, therefore, the filtrates appeared different IZ values against bacteria by which the results of this study agreed with [24].

The antibacterial compounds of fungi are produced as crude extracts. These products are associated with other substances and need to suitable solvent for their extraction. Scientists used a number of the solvents such as ethyl acetate, chloroform, petroleum ether for the extraction [15] & [22]. In addition, activated charcoal has been used to remove the impurities, e.g., proteins, from the antibacterial extracts [22]. Based on the mentioned words, the current study used the ethyl acetate and activated charcoal in a process of the extraction. The results of this study showed agreement with results of [25]. For general screening and characterization of the crude P. chrysogenum extracts, TLC and HPLC have been used to know the components of these extracts [21] & [26]. Therefore, results of the current study appeared spots (Fig. 6) of the crude P. chrysogenum extracts as well as the HPLC appeared various peaks and different retention times (Fig. 7) within each crude fungal extract in comparison with pure penicillin G which was used in this technique. The results showed agreement with [21].

The bio-statistical analysis showed significant levels in the fermentation medium, where it was found that the highest significant difference was for PPM medium, followed by CSLM The results of the statistical analysis confirmed that the P value of the two fermentation media was less than 0.05. Through the results of biostatistical analysis to compare these media, it was confirmed that the best medium for the growth of P. chrysogenum was obtained in the fermentation process of this study.

Conclusions

A fungus P. chrysogenum present in the Hamamr marsh areas in Thi-Qar Province, south of Iraq. As well as, this fungus produced antimicrobial compounds and PPM is better fermentation

medium than CSLM. This study recommends further study to detect genes control these antimicrobial compounds which are produced from that fungus. Also, the current study recommends to use the fermentation process of P. chrysogenum using these media in the fungal taxonomy of Penicillium species.

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