ISSN: 0067-2904
GIF: 0.851

The Optimum Conditions for Pleurotin Production by *Pleurotus* spp. Local Isolates

Israa A. Al-Temimay[1*], Muna H. AL-Jibouri[1], Abdulla A. Hassan[2], Farooq I. Mohammad[3]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
[2] Department of Plant Protection, College of Agriculture, Tikrit University, Tikrit, Iraq.
[3] Biotechnology Research Center, Al Nahrain University, Baghdad, Iraq.

Abstract

The study includ selection of six species of the fungi related to genus *Pleurotus* were evaluated for their ability to produce of Pleurotin, one of them, *Pleurotus ostreatus* (P.11) was isolated and identified in the present study. Pleurotin was detected by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). The maximum absorption of Pleurotin was 1.6 nm at 250 nm. Pleurotin was purified with two methods using chloroform and ethyl acetate, the results showed the ethyl acetate was more efficient in pleurotin production resulting in 14.6 µg/ml compared to 9.8 µg/ml with chloroform. The local isolate, *P. osteratus* (P.11) showed significant high Pleurotin production (14.6 µg/ml) when was grown on the modified Czapek-Dox liquid media supplemented with 5% sawdust compared to (0-7.8 µg/ml) the rest fungi in the same medium. By screening various growth conditions, production of Pleurotin by *P. ostreatus* (P.11) increased to 38.5 µg/ml using modified Czapek-Dox liquid medium supplemented with 10% Rice husks, pH 7, incubation for 5 weeks at 30 °C.

Keywords: Pleurotin, optimum conditions to pleurotin production.

الظروف المثلى لانتاج البليروتين المنقى من الفطر الغذائي *Pleurotus* spp. محلياً

اسراء احمد التميمي[1*]، منى حمودي الجبوري[1]، عبد الله عبد الكريم حسن[2]، فاروق ابراهيم محمد[3]

[1]قسم علوم الحياة ، كلية العلوم ، جامعة بغداد ، بغداد ، العراق.
[2]قسم حماية النبات ، كلية الزراعة ، جامعة تكريت ، تكريت ، العراق.
[3]مركز بحوث التقنيات الاحيائية ، جامعة النهرين ، بغداد ، العراق.

الخلاصة

شملت الدراسة اختيار ستة انواع من الفطريات العائدة للجنس *Pleurotus* وتقييم قدرتها على انتاج البليروتين، وأحد هذه الفطريات *P. ostreatus* (P.11) والذي تم عزله وتشخيصه في الدراسة الحالية. حيث تم الكشف والتحري عن البليروتين بواسطة TLC و HPLC . وكانت اعلى امتصاصية للبليروتين 1.6 نانوميتر عند 250 نانوميتر. وتم استخلاص وتنقية البليروتين بطريقتين الاولى باستخدام خلات الايثيل و الثانية باستخدام الكلوروفورم، واظهرت النتائج ان خلات الايثيل اكثر كفاءة في عيار البليروتين بنتيجة 14.6 ميكروغرام/مل مقارنةً بالكلوروفورم 9.8 ميكروغرام/مل. العزلة المحلية *P. ostreatus* (P.11) اظهرت ارتفاعاً معنوياً لانتاج البليروتين 14.6 ميكروغرام/مل عندما نُمّيَ على الوسط المحور السائل Czapek–Dox مُدعّم ب 5% نشارة الخشب مقارنةً ببقية الفطريات التي اعطت 0–7.8 ميكروغرام/مل في وسط التنمية

نفسهُ. ومن خلال غربلة ظروف النمو المختلفة فان انتاج البليروتين من قبل P. ostreatus (P.11) ارتفع الى 38.5 ميكروغرام /مل باستخدام الوسط المحورالسائل Czapek-Dox مُدعّماً ب 10% قشور الرز، الرقم الهيدوجيني 7، وبمدة حضن بلغت خمسة اسابيع في درجة حرارة 30 °م.

Introduction

Edible mushrooms have been part of human diet for centuries. The nutritional value of mushrooms is twice that of any vegetable or fruit [1,2]. At least 270 species of mushrooms are considered to possess therapeutic properties [3], such as antitumor, antibacterial, antiviral [2]. Medicinal mushrooms belonging to Basidiomycetes are estimated that about 650 mushrooms possess medicinal properties, but only several edible, such as *Pleurotus* spp. [4]. *Pleurotus* mushrooms, commonly known as oyster mushrooms, grow in the wild and are easily artificially cultivated. They are healthy foods, low in calories and in fat, rich in protein [5,6], chitin, vitamins and minerals [7]. Important characteristics are observed concerning other chemical compounds, for example polysaccharides, mannans and ß-glucans [8]. Pleurotin is a naphthoquinone antibiotic, is a fungal metabolite found in extracts of some mushrooms such as *Pleurotus griseus*, as well as *Hohenbruehelia geogenius* and *Hohenbruhelia atrocaerulea*. Extraction and Purification of Pleurotin was whole fermentation broths were processed with Homogenizer then extracted by partitioning twice against an equal volume chloroform according to [9] and ethyl acetate (EtOAc) according to [10]. The aims of this study was: Isolation and purification of Pleurotin from the local isolate *Pleurotus* spp., Evaluation of cultural conditions including media, temperature, pH, supplementation and inoculation rate for maximum Pleurotin production**.**

Materials and Methods:

Pleurotus spp. Isolates

Six species of the fungi were obtained from the Mushrooms Production Unit at the College of Agriculture, University of Tikrit. The original sources of these fungi were *Pleurotus eryngii* (King oster-KING) cod: P.w.3, *Pleurotus djamar* (Pink oster-PK) cod: P. ix, *Pleurotus ostreatus* (Bluel grey-BG) cod: P. o. G, *Pleurotus ostreatus* (White oyster-White) cod: P. white, (all four species from Mushroom Box, U.K.) *Pleurotus ostreatus,* cod: P.o. 2 (from India type culture collection/India), *Pleurotus ostreatus* (localy) cod: P. 11, Locally (isolated in the present study/Iraq). All fungal strains were routinely maintained on (PDA) slants, then kept refrigerated at 5°C and sub culturing at least monthly.

Cultivation of *P. ostreatus* in Liquid media

Czapek-Dox liquid media were added in clean Erlenmeyer flasks (500-mL capacity) each flask contain 150 ml media, then cover by cotton plug and sterilization in autoclave for 15 min. after cooling, the media were inoculated by piece (1×2 cm) of new growth colony (7 days) of *Pleurotus* spp., then incubated at 25-27 °C until the growth must be fully cover media surface (4-8 weeks).

Purification of Pleurotin

A. Extraction method by Chloroform

Pleurotin was purified as below; liquid media was filtered using gauze and cotton to remove the fungal mycelium. Then the filtrate was extracted with chloroform *via* solvent-solvent extraction in 1:1 ratio of filtrate to chloroform. The two layers were separated in separating funnel. The chloroform layer was extract by 1% sodium bicarbonate three times to acids remove. The extracting solvent was transfer to small Baker and leaves at room temperature to evaporate all chloroform. Gummy redness substance was form this substance was dissolve by added 2-5 ml hot ethanol. The Baker was cover and left in room temperature to evaporate solvents. The formed crystals were washing by 2-5 ml ether and dried by air. The products was recrystallization by added 1:1 chloroform, ether then leave to formed yellowish amber crystals this is pure pleurotin [9].

B. Extraction method by ethyl acetate (EtOAc)

Liquid media was filtered using cotton to remove the fungal mycelium. Then the filtrate was extracted with ethyl acetate (EtOAc) *via* solvent-solvent extraction in 1:1 ratio. The two layers were separated in separating funnel. The ethyl acetate layer was dried with anhydrous sodium sulphate and then filtered with Whatman filter paper. The extracting solvent was evaporated using rotary evaporator. The extract was then transferred in to a pre-weighed scintillation vial with small amount of

chloroform. The vial was left in the fume hood to allow the removal of chloroform *via* evaporation. The vial was weighed after the chloroform was completely evaporated to determine the weight of the extract obtained [10].

Detection of purified Pleurotin

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed to determine a suitable solvent system for the isolation of chemical compound (pleurotin) from the bioactive fractions of *Pleurotus* spp. Precoated silica gel 60F254 TLC plates were utilized for TLC analysis. A small drop of sample and stander were spotted about 1.0 cm from the bottom of the TLC plate by using a capillary tube. When the sample had dried, the TLC plate was placed into a TLC developing tank filled with premixed organic solvent system. The TLC plate was removed from the developing tank when it was fully developed to solvent front. Then it was dried by using hair blower and was viewed under both long and short UV Visible light. The TLC plate was then placed into an iodine vapor chamber to stain the separated chemical compounds' spots [11-13].

High Performance Liquid Chromatography (HPLC)

Column: 3µm particle (50 × 4.6 mm I.D) C-18 column. Mobile phase: solvent A: 0.1% ammonium format in ethanol, solvent ethanol, near gradients 0% B to – 100% B at 5 min. UV set at 210 nm. Flow rat 1.4 ml/min. , temp. 35°C. HPLC of the samples was performed with the HPLC system equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 AVP) and a C-R6A chromatopack data processors. Samples were centrifuged (2000 rpm for 2 min).Then filtered through a single-use 0.22 µm nylon syringe filter (Aldrich) pass through disposable filter 2.5 um prior to analysis on HPLC system under optimum separation condition, the concentration of sample were quantitatively determined by comparison the peak area of authentic standard with the peak area of samples under the same separation condition. The sequences of the eluted material of the standard wear as follow, each standard was 10 µg/ml. Retention time of pleurotin 2.45 minute with 9126 area.

Calculation:-

The concentration of the sample measuring according to the following equation:

$$\text{Concentration of sample } \mu g/ml = \frac{\text{area of sample}}{\text{area of standard}} \times \text{Conc. of standard} \times \text{Dilution Factor.}$$

Quantity assay of Pleurotin

For quantity assay of Pleurotin, colorimetric method given by [14] was followed, briefly, 0.1 ml of purified Pleurotin (dissolving in D.W. 1:5 W:V) add to KCN, incubated at 5 min in 25°C then absorbance was measured by Spectrophotometer at 300 nm. For determination of Pleurotin, standard curve Figure-1 was used.

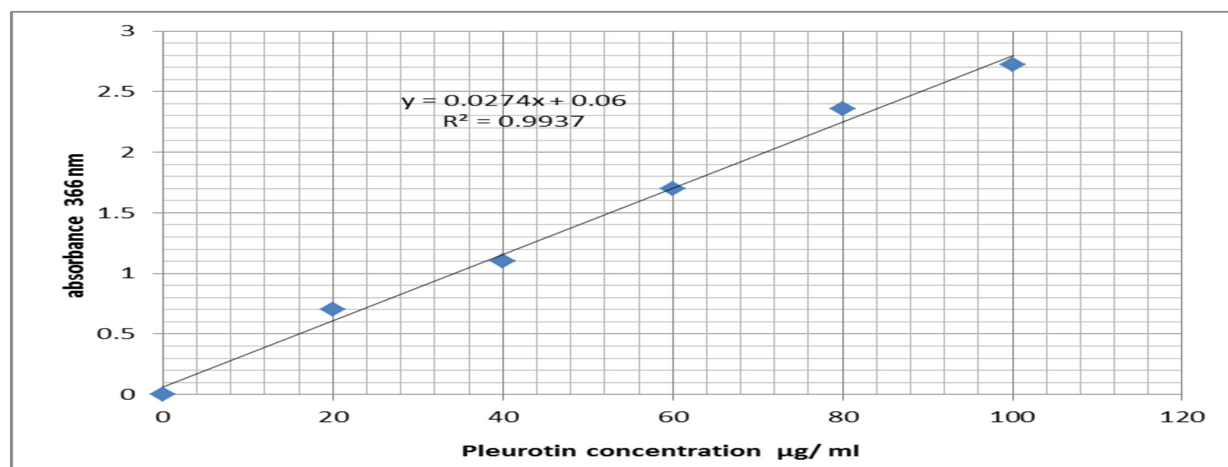

Figure 1- standard curve of pleurotin determination by Spectrophotometer at 300 nm [14].

Characterization of Pleurotin using UV- Spectroscopy

UV- spectroscopy measurement of purified Pleurotin at (50µg /ml) was carried out on a UV spectrophotometer.

Optimum conditions for Pleurotin production

Fungal Isolates

The ability of six *Pleurotus* spp. fungal isolates for pleurotin production was evaluated using Czapek-Dox liquid media supplemented with 5% saw dust, 25°C, pH 6 and Incubation period 4 weeks the Pleurotin purified by ethyl acetate and chloroform then determined by colorimetric method.

Culture Media

After screening of *Pleurotus* spp., various type of liquid media were tested for determination the most suitable one that give higher Pleurotin. PDB,MEB and Czapek-Dox liquid media were used. Purification of Pleurotin and quantity assay as above.

Types of Fibers

After screening of media, various types of fibers (Corn powder/*Zea mays*, Sawdust/ Various sp., Dry Cane/ *Phragmites* spp., Rice husks/ *Oryza sativa*, Palm Leaf/ *Phoenix dactylifera* and Barley crushed/ *Hordeum vulgare*) were tested for determination of most suitable substrate that give higher Pleurotin. Purification of Pleurotin and quantity assay as above.

Concentration of suitable fiber

The fiber that gives maximum pleurotin was used at rate 1, 2, 3, 4, 5% in the suitable medium. Purification of pleurotin and quantity assay as above.

Temperature

Pleurotus sp. that give higher pleurotin incubated at 15, 20, 25, 30, 35°C then Purification of Pleurotin and quantity assay as above.

Incubated Period

The incubation period including 7, 14, 21 and 28 days in suitable medium and fiber then Purification of Pleurotin and quantity assay as above.

pH

Pleurotus sp. that give higher pleurotin inoculated in suitable medium and fiber, then the pH of this medium adjusted at 5, 6, 7, 8, 9 and incubated at 30°C then Purification of Pleurotin and quantity assay as above.

Statistical Analysis

Dunken multiple range used in optimum condition to pleurotin production when the ($P < 0.05$).

Results and Discussion

Cultivation of *P. ostreatus* in Liquid media

After the inoculation of the modified Czapek-Dox liquid medium with deferent types of natural additions by *Pleurotus ostreatus* for 4 to 8 weeks the growth of *Pleurotus* was fully covered media surface by whit mycelium figure-2 if the development is ideal circumstances. This result is consistent with study of [15] stated that *P. ostreatus* ARC280 was grown on liquid media under static culture condition for thirty-one days of incubation.

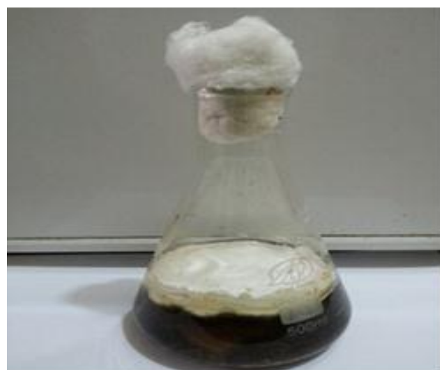

Figure 2- growth of *P. ostreatus* mycelium on modified Czapek-Dox liquid medium after 8 weeks.

Purification of Pleurotin by chloroform and ethyl acetate

After purification by chloroform and ethyl acetate, pleurotin (Amber/ yellow colored) crystals were formed after evaporation of the solvent figure -3, it was determinate by sensitive balance, their weight equal about 110 to 150 mg for each liter. This is agree with [9] in his study found pleurotin fine needle-like yellow crystals were formed as a result of rapid evaporation of the solvents. From 100 to 180 mg. of the crystalline material were obtained per l. of culture fluid. This result disagrees with [10] study when stated that pleurotin typically reaching ~300 mg/l at 5 weeks was shaken in the dark at 215 rpm at room temperature.

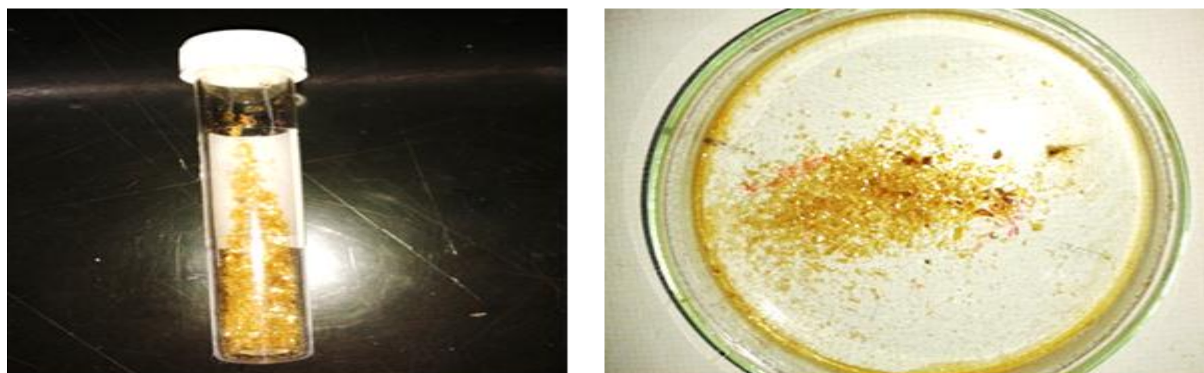

Figure 3- Amber/ yellow colored crystals of Pleurotin were formed after evaporation of solvent.

Detection of Purified Pleurotin

Thin Layer Chromatography (TLC)

In this study Thin Layer Chromatography (TLC) was used to determine a suitable solvent system for detection of pleurotin from the bioactive fraction of *P. ostreatus.* After a few trial and error experiments, it was found that the suitable solvent system was the mixture of hexane and acetone (3ml acetone + 7 ml Hexan) figure -4. TLC performed in this study showed that ethyl acetate extraction and chloroform extract of *P. osteratus* (P.11) contained pleurotin. TLC is used to separate components in a mixture and to identify unknown materials by comparing their separation pattern to that of known reference materials (stander).

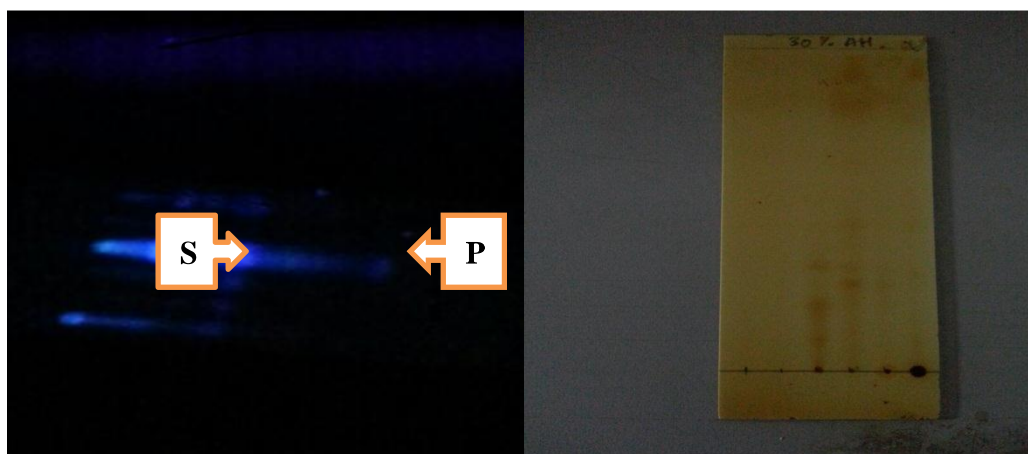

Figure 4- TLC detection of pleurotin (S) refers to stander pleurotin, (P) refer to extracted pleurotin in present study.

High Performance Liquid Chromatography (HPLC)

After 2 months growth of *P. osteratus*, a chloroform extract was made from each whole ferment and examined by reverse phase HPLC with diode array detection and comparison of retention time with an authentic standard for the presence of pleurotin. Pleurotin concentration in chloroform extract was found equal to 6 µg/ml (ppm) compared with standard which equal 10 µg/ml (ppm) Figure-5 showed presence pleurotin peak in chloroform extract from modified Czapek-Dox liquid media after completion growth of *P. osteratus.* Ideally any extract should be defined so that any unknown extracts

can be compared to check that the composition is the same. TLC or HPLC profiles best define extracts chromatographically [16].

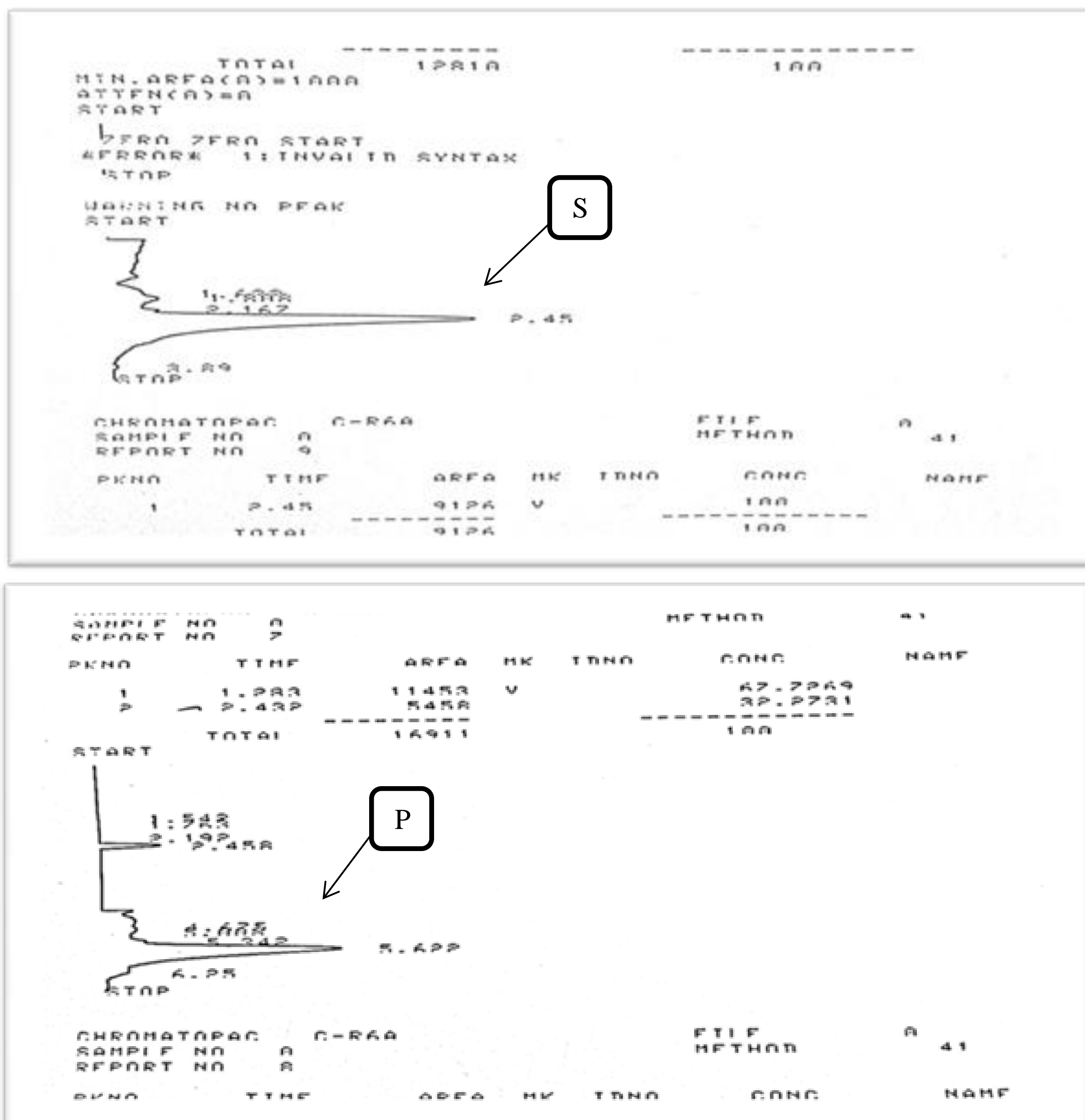

Figure 5- HPLC analysis of pleurotin standerd (S) and chloroform extract of *P. ostreatus* shows the pleurotin peak in this extract (P).

Quantity assay of Pleurotin

Characterization of Pleurotin using UV- Spectroscopy

Pleurotin was characterized by UV- spectroscopy. The UV-Visible absorption spectra of the pleurotin were measured in the range of 200-300 nm using a UV- spectrophotometer. A peak located at 250 nm was observed Figure- 6. The maximum absorption of pleurotin at 250nm regard a suitable tool proves for the pleurotin identification in compared with the related studies UV- spectroscopy is an important and valuable technique for the characterization of pleurotin [9], in addition to fix the maximum wavelength for determination of pleurotin in the colorimetric method. The reason of maximum absorption of pleurotin at 250 nm may be due to pleurotin structure compose of hexacyclic framework which absorbed at uv-spectrum more than others [17].

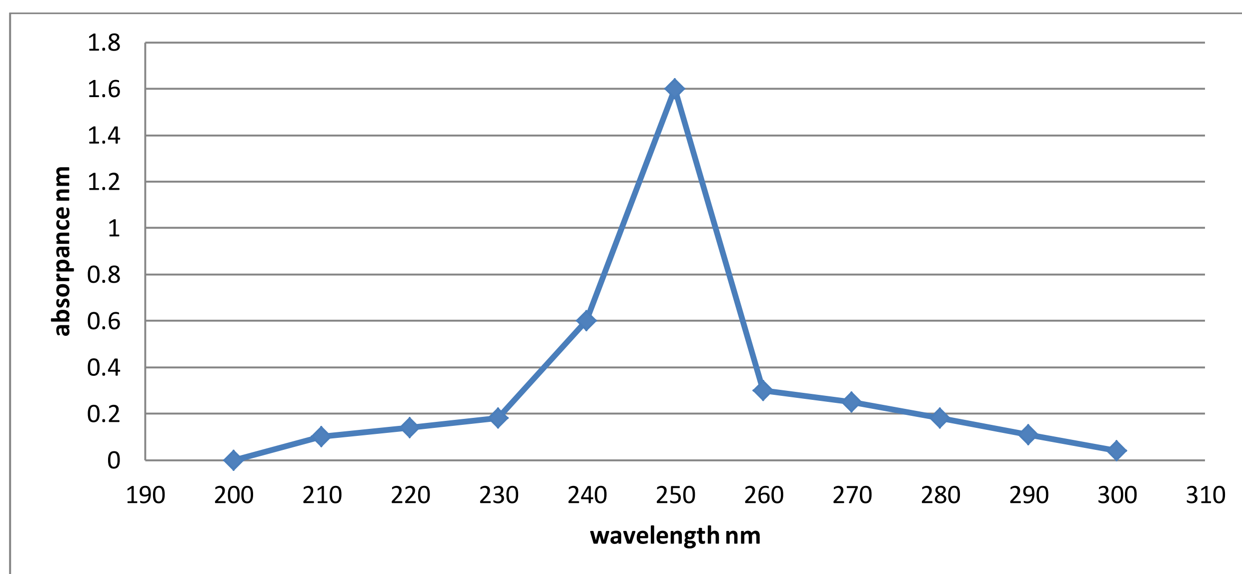

Figure 6- UV- absorption spectra of pleurotin (50 µg /ml) purified from *P. ostreatus.*

Optimal Conditions for the Production of Pleurotin

Table-1 shows that screening of *Pleurotus* spp. grown on modified Czapek-Dox liquid media supplemented with /without 5% sawdust for pleurotin production, Incubation period 4 weeks, pH 6, incubation temperature 25°C. In this study the local isolate of *P. osteratus* (P.11) showed significant highest pleurotin production 14.6 µg/ml pleurotin concentration when was grown on the modified Czapek-Dox liquid media supplemented with 5% sawdust, while the same fungus gave 0.2 µg/ml pleurotin when grown on the modified Czapek-Dox liquid media without 5% sawdust. *Pleurotus eryngii* gave a trace amount of pleurotin 0.01 µg/ml when was grow on the modified Czapek-Dox liquid media supplemented with/ without 5% sawdust in same condition. Results in the same table-1 showed that all the *Pleurotus* spp. had the ability to produce pleurotin when were grow on modified Czapek-Dox liquid media supplemented with 5% sawdust, the local isolate *P. ostreatus* (*P.* 11) gave the highest pleurotin concentration, when was grow on this media, thus it was selected for further steps of this study. [10] Had study a shredded wood product made of aspen and used as a filtration aid in the food processing industry, or without adding of wood fiber. From flasks lacking the wood fiber, no pleurotin was detected, but when wood fiber was present during fermentation, pleurotin was found consistently at concentrations of 40–60 mg/l.

Table 1- Evaluation of *Pleurotus* spp. grown on Czapek-Dox liquid media supplemented with /without 5% sawdust for Pleurotin production, Incubation period 4 weeks, pH 6, incubation temperature 25°C.

Fungus	Addition	Pleurotin concentration (µg/ml)
Pleurotus eryngii (King oster-KING)	With saw dust	0 e
	Without saw dust	0 e
Pleurotus djamar (Pink oster-PK)	With saw dust	0.2 d
	Without saw dust	0 e
Pleurotus ostreatus *P.*o. 2	With saw dust	0.5 d
	Without saw dust	0 e
Pleurotus ostreatus (localy)	With saw dust	14.6 a
	Without saw dust	0.2 d
Pleurotus ostreatus (Bluel grey-BG)	With saw dust	2.3 c
	Without saw dust	0 e
Pleurotus ostreatus (White oyster-Whi)	With saw dust	7.8 b
	Without saw dust	0 e

Evaluation of liquid media

Table-2 summrized the media culture effects of the pleurotin production, they were view modified Czapek-Dox liquid media gave significant highest concentration of pleurotin when the *P. osteratus* supplemental (*P.* 11) grown on this media with sawdust 14.6 µg/ml, and without sawdust 0.2 while when the same fungus was grow on Malt Extract Broth (MEB) with the same condition, incubation period 4 weeks, pH 6, incubation temperature 25°C given the low concentration of pleurotin 0.5 µg/ml with sawdust and didn't give any pleurotin without sawdust.

Perhaps the reason is due to the chemical factors in the modified Czapek-Dox liquid media such as Carbon Source, Nitrogen Source. Pleurotin It's a secondary metabolity substance, [18] showed Nitrogen is a critical factor in the synthesis of some fungal enzymes involved in both primary and secondary metabolism.

Table 2- Effects of Media Culture types supplemented with /without 5% sawdust for pleurotin production from the localy *P. ostreatus* (P.11), Incubation period 4 weeks, pH 6, incubation temperature 25°C.

Media Culture	Addition	Pleurotin concentration (µg/ml)
Czapek-Dox liquid media	With sawdust	14.6 a
	Witout sawdust	0.2 c
Patoto Dextrose Broth (PDB)	With sawdust	9.3 b
	Witout sawdust	0 d
Malt Extract Broth	With sawdust	0.5 c
	Witout sawdust	0 d

Values within column followed by different letters differ significantly according to Dunken multiple range (P < 0.05).

Evaluation Extraction Methodes

The results indicated that there is variation in the two extraction methods of pleurotin; it was observed that ethyl acetate is more efficient in pleurotin concentration 14.6 µg/ml when the *P. ostreatus* (P.11) was grown on the modified Czapek-Dox liquid media, incubation period 4 weeks, pH 6, incubation temperature 25°C, while the pleurotin concentration extracted by chloroform was 9.8 µg/ml. This result refers to the ethyl acetate extraction better than chloroform extraction. [10] Used ethyl acetate in pleurotin extraction.

Evolution of Fiber types

Result in table-3 shows when the *P. osteratus* (*P.* 11) grown on the modified Czapek-Dox liquid medium with all natural addition (Corn powder, Sawdust, Dry Cane, Rice husks, Palm Leaf and crushed Barley) had the ability to produce pleurotin when incubation period 4 weeks, pH 6, incubation temperature 25°C, but pleurotin was increased significantly in present rice husks, Palm leaves dry reed Cane resulting in 21.2, 19.7 and 19.3 µg/ml respectively while the least pleurotin was in Corn powder and Barley crushed 9.7 and 7.8 µg/ml respectively, at the same experiment conditions.

The study of [19] revealed that nutritional composition of mushroom (*Pleurotus florida*) depends on the composition of substrates (nutrient source of mushroom) and so differs significantly when grown in different substrates. For this reason, in percent study the production of pleurotin in media with rice husk more than other natural additions because rice husk contain the essential nutrient source for the mushroom to growth and pleurotin production.

Table 3- Effect of various Fiber type at 5% on pleurotin production by *P. ostreatus* (P.11) grown on the modified Czapek-Dox liquid media, pH 6 incubation for 4 weeks at 25C.

Fiber type 5%	Pleurotin concentration (µg/ml)
Corn powder	9.7 cd
Sawdust	14.6 b
Dry Cane	19.3 a
Rice husks	21.2 a
Palm Leaf	19.7 a
Crushed Barley	7.8 d

Values within column followed by different letters differ significantly according to Dunken multiple range (P < 0.05).

Determination of concentration of suitable fiber

Result in table-4 shows the positive relationship between the rice husks percentage and the pleurotin concentration. When the *P. ostreatus* (P. 11) grown on the modified Czapek-Dox liquid media with different rice husks percentage, incubation period 4 weeks, pH 6, incubation temperature 25°C, the pleurotin concentration significant increased when the rice husks increase from 0.7 µg/ml up to 29.6 µg/ml when the rice husks increased from 0% to 10% from the media.

Perhaps the reason in this result was due to the major components that make up the husks of rice, which leads to the well growth of mushrooms to produce the pleurotin.

Table 4- Effect of various Rice husks percentage on Pleurotin production by *P. ostreatus* (P.11) grown on the modified Czapek-Dox liquid media, pH 6 incubation for 4 weeks at 25C.

Rice husks (%)	**Pleurotin concentration (µg/ml)**
0	0.7 d
2.5	12.8 c
5	21.2 b
7.5	29.4 a
10	29.6 a

Values within column followed by different letters differ significantly according to Dunken multiple range ($P < 0.05$).

Determination of optimum temperature

After the choice of modified Czapek-Dox liquid medium as the most suitable medium for *P. ostreatus* (P.11) mycelia growth and pleurotin production, the fungus incubated at different temperature for 4 weeks. Table-5 showed the concentration of pleurotin significantly increased when the temperature increased from 15°C to 30°C, in this temperature fungus gave the highest pleurotin concentration 33.2 µg/ml then the concentration of pleurotin decreased 11.8 µg/ml in 35°C.

The reason was that the fungus grows well in temperatures ranging from 25°C to 27°C. This was confirmed by [20] in his study on the optimum temperature for the growth of *P. ostreatus*, Where proved the heaviest dry weight of mycelia growth in liquid media was 7.12 gm at 25ºC followed by 5.83 gm at 30ºC, in comparison with 15 and 20°C given the least dry weight of fungal growth 2.09 and 4.76 gm, respectively.

Table 5- Effect of various temperature values on Pleurotin production by *P. ostreatus* (P.11) grown on the modified Czapek-Dox liquid media supplemented with 10% Rice husks, pH 6, incubation period 4 weeks.

Temperature °C	Pleurotin concentration (µg/ml)
15	18.5 c
20	27.3 b
25	29.4 b
30	33.2 a
35	11.8 d

Values within column followed by different letters differ significantly according to Dunken multiple range ($P < 0.05$).

Determination of optimum pH

Through studying the effect of pH modified Czapek-Dox liquid medium on pleurotin production when the *P. osteratus* grown on this media, incubation period was 4 weeks, incubation temperature 30°C, the fungus gave the maximum significantly pleurotin concentration 33.4 in pH 7 followed in little difference by 33.2 in pH 6. The minimum pleurotin 10.5 in pH 8 table-6.

Most studies on this type of mushroom employed this pH to obtain the highest growth of mycelia in submerged liquid fermentation (SLF) [21]. The result of this study agree with many studies such as [20- 22] were state that most of fungi and bacteria give the utmost growth at pH ranged 6-7.

Table 6- Effect of various pH values on Pleurotin production by *P. ostreatus* (P.11) grown on the modified Czapek-Dox liquid media, supplemented with 10% Rice husks, incubation for 4 weeks at 30 °C.

pH	Pleurotin concentration (µg/ml)
4	19.1 c
5	28.5 b
6	33.2 a
7	33.4 a
8	10.5 d

Values within column followed by different letters differ significantly according to Dunken multiple ranges ($P < 0.05$).

Determination of optimum incubated time

During studying the effect of incubation period on the growth of *P. ostreatus,* (*P.* 11) and pleurotin production found that it has a significantly large effect on the pleurotin concentration table-7 showed the optimum period to produced maximum concentration of pleurotin 38.5 µg/ml in 5 weeks incubation followed by 36.2 µg/ml in 6 weeks incubation, comparison with the lowest value of pleurotin 3.6 µg/ml in 1 week incubation. Pleurotin concentration was drop in 7 and 8 weeks incubation to reach to 20.6 µg/ml and 14.3µg/ml respectively. The findings achieved from this experiment in agreement with the finding [10] in his study Pleurotin was not detected at 1 or 2 weeks. At 3, 4, 5, and 6 weeks pleurotin concentration increases, typically reaching _300 mg/l at 5 weeks.

Table 7- Effect of various Incubation period (week) on Pleurotin production by *P. ostreatus* (P.11) grown on the modified Czapek-Dox liquid media, supplemented with 10% Rice husks, pH 7, incubation temperature 30°C.

Incubation period (week)	Pleurotin concentration (µg/ml)
1	3.6 f
2	7.9 e
3	19.4 c
4	33.4 b
5	38.5 a
6	36.2 ab
7	20.6 c
8	14.3 d

Values within column followed by different letters differ significantly according to Dunken multiple range ($P < 0.05$).

References

1. Sivrikaya, H., Bacak, L., Saracbasi, A., Toroglu, I. and Eroglu, H. **2002**. Trace elements in *Pleurotus sajorcaju* cultivated on chemithermomechanical pulp for bio-bleaching. *Food Chem*. 79, pp: 173-176.
2. Oyetayo, V. O. and Ariyo, O. O. **2013**. Antimicrobial and Antioxidant Properties of Pleurotus ostreatus (Jacq: Fries) Cultivated on different tropical woody substrates. *Journal of Waste Conversion, Bioproducts and Biotechnology***.** 1(2), pp: 28–32.
3. Papaspyridi, L. M., Aligiannis, N., Topakas, E., Christakopoulos, P., Skaltsounis, A. L. and Fokialakis, N. **2012**. Submerged fermentation of the edible mushroom *Pleurotus ostreatus* in a batch stirred tank bioreactor as a promising alternative for the effective production of bioactive metabolites. *Molecules*. 17, pp: 2714-2724.
4. Elisashvili, V. **2012**. Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). *International Journal of Medicinal Mushrooms.* 14(3), pp: 211–239.
5. Patel, Y., Naraian, R., and Singh, V.K. **2012**. Medicinal properties of *Pleurotus* Species (Oyster Mushroom): a review. *World Journal of Fungal and Plant Biology*. 3 (1), pp: 01-12.
   20. 21.
6. Kulshreshtha, S., Mathur, N. and Bhatnagar, P. **2014.** Mushroom as a product and their role in mycoremediation. *AMB Express*. 4, pp:29-36.

7. La Guardia, M., Venturella, G and Venturella, F. **2012**. On the chemical composition and nutritional value of *Pleurotus* taxa growing on umbelliferous plants (Apiaceae). *J. Agr. Food Chem*. 53, pp: 5997 – 6002.
8. Smiderle, F. R., Olsen, L. M., Ruthes, A. C., Czelusniak, P. A., Santana-Filho, A. P., Sassaki, G. L., Gorin, P. A. J. and Iacomini, M**. 2012**. Exopolysaccharides, proteins and lipids in *Pleurotus pulmonarius* submerged culture using different carbon sources. *Carbohydrate Polymers*. 87, pp: 368– 376.
9. Robbins, W. J., Kavanagh, F. and Hervey, A. **1947**. Antibiotic substances from basidiomycetes I. *Pleurotus griseus. Proc Nat Acad Sci. U.S.A. Bot.* 33, pp:171–176.
10. Shipley, S. M., Barr, A. L., Graf, S. J., Collins, R. P., McCloud, T. G. and Newman, D. J. **2006**. Development of a process for the production of the anticancer lead compound pleurotin by fermentation of *Hohenbuehelia atrocaerulea*. *J. Ind. Microbiol. Biotechno*l. 33, pp: 463–468.
11. Nurestri, A. M., Sim, K. S. and Norhanom, A. W. **2009**. Phytochimical and cytotoxic investigation of *Pereskia grandifolia* Haw. (Cactaceae) leaves. *Journal of Biological Sciences*. 9 (5), pp: 488-493.
12. Lee, G. S. **2011**. Bioactivity and chemical investigation of *Curcuma Mangga* and *Curcuma Inodora.* Ph. D. Thesis, Faculty of Science, University of Malaya, Kuala Lumpur.
13. Abdul Rahman, S. N., Abdul Wahab, N. and Abd Malek, S. N. **2013**. In *Vitro* morphological assessment of apoptosis induced by antiproliferative constituents from the rhizomes of *Curcuma zedoaria*. *Evidence-Based Complementary and Alternative Medicine*. Article ID 257108, 14 pages.
14. Kavanagh, F. **1947**. Chemical determination of pleurotin, an antibiotic substance from *Pleurotus oriseus*, *Arch. Biochem*., pp: 15-95.
15. Elsayed, M. A., Hassan, M. M., Elshafei, A. M., Haroun, B. M. and Othman, A. M. **2012**. Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. *British Biotechnology Journal*, 2(3), pp: 115-132.
16. Houghton, P., Fang, R., Techatanawat, I., Steventon, G., Hylands, P.G.and Lee, C.C. **2007**. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods*, 42, pp: 377–387.
17. Elzner, S. **2006**. Studies toward the total synthesis of noelaquinone and pleurotin. Research Topic Seminar. Wipf Group. Part 2: pleurotin. pp: 4-17.
18. Elisashvili, V. **2012**. Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). *International Journal of Medicinal Mushrooms*. 14(3), pp: 211–239.
19. Khan, M. D., Tania, M., Amin, R. M., Alam, N and Uddin, Md. N. **2008**. An investigation on the nutritional composition of mushroom (*Pleurotus florida*) cultivated on different substrates. *Bangladesh J. Mushroom.* 2(2), pp: 17-23.
20. Abdulla, L.Q. **2013**. Biochemical study and application of lipoxygenase produce from *Pleurotus ostreatus*. M. Sc. Thesis. College of Science, University of Baghdad, Baghdad, Iraq.
21. Omemu, A. M., Bankole, M. O. and Adegbesan, A. M. **2008**. Effect of different processing and supplementation on maize cob as microbiological growth medium for fungi, *World J. Agric. Sci.* 4(5), pp: 600-604.
22. Hala, M.A. and Ghazi, M.A. **2010**. Determination of the optimum conditions for the production of agarase from local isolate *Bacillus* sp. H12. *Iraq J. Biotecno*. 9(1), pp:56-70.