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Biotechnological Production of Kojic Acid Synthesized by Endophytic Fungi, *Aspergillus oryzae* Isolated from *Euphorbia peplis*.

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ABSTRACT

Six endophytic fungal species belonging to genera, *Aspergillus, Alternaria, Penicillium,* and *Fusarium,* as identified morphologically, were isolated from healthy leaves, stems, and roots of the medical plant *Euphorbia peplis* collected from Damietta governorate of Egypt. One Fungal strain morphologically identified as A. oryzae, was selected as the most potent producer of Kojic acid, that produced in this study by agricultural waste fermentation using sugarcane bagasse, corn cobs, and rice straw. The bagasse agricultural waste fermentation using endophytic *Aspergillus oryzae* strain was optimized as a source of Kojic acid in a cheap, fast, and environmentally beneficial way at agitation state rather than static and pH 6.0 after 20 days of incubation at 28 °C. Ethyl acetate extract of filtrate of *Aspergillus oryzae* showed promising antioxidant activity against DPPH free radical, recording IC50 = 5.7. Also, the E A extract was separated using Gas Chromatography technique recording RT: 31.95 min. and MW: 142.The study aims to the fermentation biotechnology used to reduce the cost of industrial KA production through optimization of environmental conditions of the biosynthesized KA with low toxicity and high antioxidant efficiency.

INTRODUCTION

Endophytes microorganisms in internal tissues of nearly all plant species are a proven source of novel organic natural molecules, maximizing the borders of drug discovery. Next to the clinically acknowledged endophyte research has yielded potential drug compounds with antimicrobial, antioxidant, antiviral, antidiabetic, anti-Alzheimer's disease and immunosuppressant, and many other diseases.These evidences raises hopes to combat incurable diseases, drug resistance and other challenges related to human health. Plant and fungi are recognized as asource of natural products. Medicinal plant harbour endophytic fungi and their host produce similar photochemical. The endophytes are known to protect their host from infectious agents and provide strength to survive in harsh conditions. The potential of finding new drugs that may be effective candidates for treating newly developing diseases in humans is great (Shukla *et al.*, 2014).

Endophytes provide a broad variety of active secondary metabolites with unique structure, including alkaloids, glycosides, benzopyranones, flavonoids, phenolic acids, quinones, steroids, xanthones, terpenoids, tetralones, and others (Kaul *et al*., 2012).

Euphorbia peplus is a species of Euphorbia, native to most of Europe, northern Africa, and western Asia, where it typically grows in cultivated arable land, gardens, and another disturbed land. It is an annual plant and is toxic to rapidly replicating human tissue, and has long been used as a traditional remedy for common skin lesions. (Siller *et al.*, 2009)

Skin color disorders may be caused by various factors, such as excessive exposure to sunlight, aging, and hormonal imbalance during pregnancy, or taking some medications (Saeedia *et al*., 2019). Kojic acid (KA) is an organic chelation agent produced as a natural secondary metabolite by several microorganisms such as *Aspergillus* genus, *Penicillium*, Mucor, and about 58 different strains used for its production (Chaudhary *et al*., 2014). Kojic acid (KA) is produced by fungi during aerobic fermentation of various substrates. KA has the Japanese common name koji which was derived from "Koji", the inoculum of fungus starter used in food fermentations for many centuries (Ammar *et al.*, 2017). KA has various applications in several fields such as the cosmetic industry, medicine, food industry, agriculture, and chemical industry. Nowadays, KA plays a crucial role in cosmetics (Rosfarizan *et al.*, 2010) especially skincare products that prevent exposure to UV radiation. It has been used in the production of skin whitening creams, skin protective lotions, whitening soaps, and tooth care products, and it acts as an ultraviolet protector. KA suppresses hyperpigmentation in human skin by restraining the formation of melanin through the inhibition of tyrosinase formation, the enzyme that is responsible for skin pigmentation [Ohyama and Melanosis (1990), Noh *et al.*, 2009]. In addition, KA interferes with the oxygen required for enzymatic browning leading to the reduction of o-quinones to diphenols and prevents the formation of melanin pigment (Mohamad *et al.*, 2010). Moreover, KA has economic importance in the medical field where it can be used as an anti-inflammatory drug and painkiller.

There is a risk in the synthesis of KA by chemical methods represented in free radical production in the living cell (Hazra *et al.*, 2008). Many attempts were carried out to select an alternative method for KA production to avoid this. KA production by microorganisms has been used as an alternative non-toxic and safe method. Different types of raw materials, which include various synthetic carbon sources like glucose, sucrose, maltose, xylose, and alcohols, were used by the earlier researchers to obtain better yields of KA [2]. Agro-waste by-products considered the cheapest source was used as for production of KA such as industrial wastes, fruit wastes, vegetable wastes, etc. [Abd El-Aziz (2013); Nurashikin *et al.*, (2013); Chaudhary *et al*., (2014); El-Kady *et al.*, (2014)].

This study aims to reduce the cost of industrial fermentation for KA production and increment through the optimization of environmental conditions and using different cheapest agriculture raw materials. In addition, it aims to confirm KA application by studying the antioxidant activity of fungal filtrate and its relation to KA production at all conditions.

MATERIALS AND METHODS

Chemicals and Solvents:

Potato dextrose agar (PDA), yeast extract, malt extract, peptone, glucose, sucrose, and all other constituents of culture media. TLC silica gel 60 F254 Plates were obtained from merck, Germany, while, Formic acid, dichloromethane, n-Hexane, and Ethyl acetate were obtained from Sigma-Aldrich, USA. Kojic acid standard, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), methanol, ferric chloride, and hydrochloric

acid. All obtained from Al - Nasr Chemical Company, Egypt.

Plant Samples Collection:

In this study, healthy (showing no visual disease symptoms) and mature medicinal plant parts were carefully chosen for sampling processes. Leaves, stems, and roots from plants were randomly collected from Damietta governorate, Egypt. The samples then brought to the laboratory in sterile bags and processed within a few hours after sampling.

Culture Media:

The experimental strains were isolated and preserved on different culture media for estimation of KA through this study.

Malt Extract Agar (MEA) medium. This medium was used for cultivation, maintenance, propagation, and counting all fungal strain. It has the following composition (g/L): Malt extract (20.0), Peptone (1.0), Glucose (20.0) and Agar-agar (25), these ingredients were completed to 1000 ml of distilled water and the pH was adjusted to 5.5, then autoclaved at 121°C for 15 min.

Yeast-extract sucrose (YES) liquid medium. This semi-synthetic medium was formulated by Scott *et al*. (1970), for extracellular secondary metabolites production. The YES medium contains (g/L); Yeast extract, 20.0; sucrose, 150.0; MgSO4.H2O, 0.5; distilled water to 1.0 L. The pH was adjusted to 6.5 ±0.2.

Potato Dextrose Agar (PDA) medium. The medium contains (g/L): Agar-agar, 20.0; glucose, 20.0; potatoes infusion from 300.0 g potatoes. The infusion from the potatoes was prepared by using 300.0 g potatoes that were peeled and diced. Then 500 ml of distilled water was added to potatoes heated gently to boil for 30 min. The potatoes infusion was then filtered, the glucose was then added and mixed thoroughly and the volume was completed to 1.0 L. by distilled water. The medium was then heated gently to boiling (**Paterson and Bridge, 1994**).

Isolation of Endophytic Fungi and Identification of Fungal Strains:

Isolation of endophytic fungi from plant parts was done according to using a modified method described by Arnold *et al.* (2002). First, the plant material was rinsed in tap water to remove the dust and debris then cut into small pieces by a sterilized blade under aseptic conditions. Each sample was surface sterilized by 70% ethanol for 30 sec and 0.1% mercuric chloride ($HgCl_2$) solution for 2 min. The samples were rinsed in sterile water for 1 minute and then allowed to surface dry on filter paper. After proper drying 4 pieces of plant parts were inoculated in PDA plate supplemented with an antibiotic (Chloramphenicol) and incubated at 28 ±10C for 5 to 7 days. The purity of each fungal culture was achieved by examination of colony morphology. After purifying the isolates several times as mention above, the final pure cultures were transferred on PDA slant. The fungal strains in the pure culture were preserved on potato dextrose agar (PDA) slant at 4 to 5^{o}C with proper labeling and were sub-cultured from time to time.

Uses of TLC for the Detection & Estimation of KA:

The thin layer chromatography was employed successfully for the separation of KA compound (Frost, 1966; Dedio *et al*., 1969). The samples were applied on thin-layer chromatography TLC plates (silica gel G-60 F254 aluminum sheet, Merck, Germany) using CAMMAG LINOMAT 5 application system (TLC scanner unit at RCMB, Al-Azhar University) against KA standard. Developing processes were carried out using toluene, ethyl acetate, formic acid (5:4:1, v/v/v) (TEF) system for separation of KA and other secondary metabolites.

KA Production Using Agricultural Raw Materials:

Different agricultural raw materials including corn cob, rice straw, and sugarcane bagasse, were selected as cheap sources for the production of KA by astrain of *A. oryzae*. The agricultural raw materials were dried in the oven at 60 °C, and then grinded and sieved. 10 g of each agricultural material was suspended in 100 ml distilled water (El-Kady *et al.*, 2014). All flasks containing raw materials were sterilized for 20 min at 121 °C. Sterilized flasks were then inoculated with 1ml. spore suspension of each strain and

incubated at the optimum conditions. After the incubation period, the fungal growth was observed and measured.

Optimization of Kojic Acid Production:

Sterilized triplicate flasks, containing 100 ml of sugarcane bagasse medium were inoculated with 1ml. freshly prepared spore suspension of 5 days old of *Aspergillus oryzae* and incubated for different incubation periods (6, 8, 10,12, 14, 16, 18, 21 and 23 days) and another set of flasks at different incubation temperatures (10, 15, 20, 25,28, 30, 35, and 37 °C). At the end of each incubation period, the dry biomass and KA amount were estimated. To evaluate the optimum pH values for maximum production of KA, the pH of the media broth medium was adjusted. Also, static and shaking (160 rpm) condition was also investigated in triplicate.

Antioxidant Activity (free radical scavenging activity) of Ethyl Acetate Extract of Endophytic Fungus, *Aspergillus oryzae*:

The antioxidant activity of the extracts and metabolites dissolved in methanol was determined on the basis of their scavenging activity of the 1, 1- diphenyl-2-picryl hydrazyl (DPPH) free radical according to the method described by Melo *et al.* (2008). The reduction of DPPH by antioxidant extracts or metabolites results in a loss of absorbance. Thus, the degree of discoloration of the solution indicates the scavenging efficiency of the added substance. When DPPH reacts with an antioxidant compound, which can donate hydrogen atoms, it is reduced and its colour changes from deep-violet to light-yellow.

Procedure:

Antioxidant activity of metabolites was determined by the method described by Lee and his associates (Lee *et al.*, 1996), 1, 1-Diphenyl-2picrylhydrazyl (DPPH) was prepared in ethanol (300M solution), test samples were prepared by dissolving in dimethylsulfoxide (DMSO), Reaction mixtures containing 10 L of test samples and 90 L of PPH (final concentration of test sample was 500 g/mL and 300 a mol of DPPH was added in 96 - well microtiter plates. Plates were incubated at 37°C for 30 minutes, Absorbance was measured at 55 nm using a spectrophotometer, Percent inhibition by sample treatment was determined by comparison with a DMSO treated control group.

Gas Chromatography of Ethyl Acetate Extract of Endophytic Fungus, *Aspergillus oryzae*

Analyzed using GC/mass system: Thermo scientific trace 1310 Gas chromatography attached with ISQ LT single quadrupole mass spectrometer at the Regional Center for Mycology and Biotechnology. The gas chromatographic column was a fused capillary column (DB1 J&W; 30 m length; 0.25mm Inner diameter; 1.5 um film thickness), which chemically bonded dimethylpolysiloxane.

The GC temperature program was started at 40°C (1 min), then elevated to 250°C (2 min) at a rate of 5°C/min and then elevated to 310°C (2 min) at a rate of 5°C/min. The detector and injector temperature were set at 300°C. WILEY Mass Spectral Database was used in the identification of the separated peaks.

RESULTS AND DISCUSSION

Fungal Isolates Identification:

Association between fungal endophytes and their host plant is due to the result of unique adaptations that enable the endophytes to harmonize their growth with that of their host (Verma *et al*., 2012).

In this study, six fungal isolates were isolated from *Euphorbia peplis* medicinal plant and purified until having a pure culture. The preliminary identification of these isolates was referring to three *Aspergillus sp.*, *Alternaria sp.*, *Fusarium sp.* and *Penicillium sp.* (Fig.1).These results were close to that of Kamel *etal* 2019, they isolate 22 isolates from *Euphorbia geniculate* plants with genus *Aspergillus* was the most common fungus isolate. Also, this result agrees with the observations of Khan *et al*. (2013) who reported the isolation of large numbers of endophytes from medicinal plants. Nalini *et al*. (2014) reported the diversity in endophytic fungi could be attributed to the environmental conditions in which the host plants live.

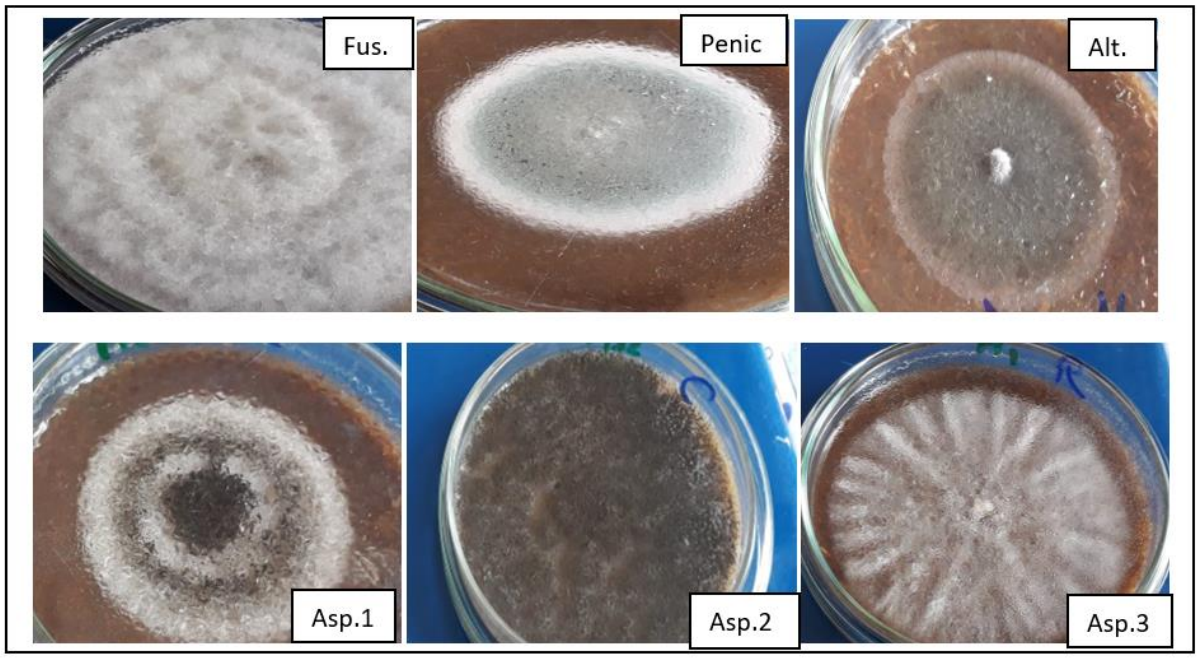

Fig. 1: Fungal isolates pure culture isolated from *Euphorbia peplus*. Where Asp. = *Apergillus sp., Fus*. =*Fusarium sp.,* Penic = *Penicilliumsp.*and Alt = *Alternaria sp*

Kojic acid production:

100 ml of each PDA culture broth of the isolates, were extracted, concentrated, separated, screened for KA production, and quantified according to KA standard using TLC scanner system, as shown in fig. (2). It is elevated that *Apergillus* sp.3 was the most potent as KA producer. Kojic acid concentration (mg/L) of each fungal culture is illustrated in fig. (3).Tauhidur *et al*. (2018) reported the isolation of Kojic acid as the main secondary metabolite present in the fungal extract.

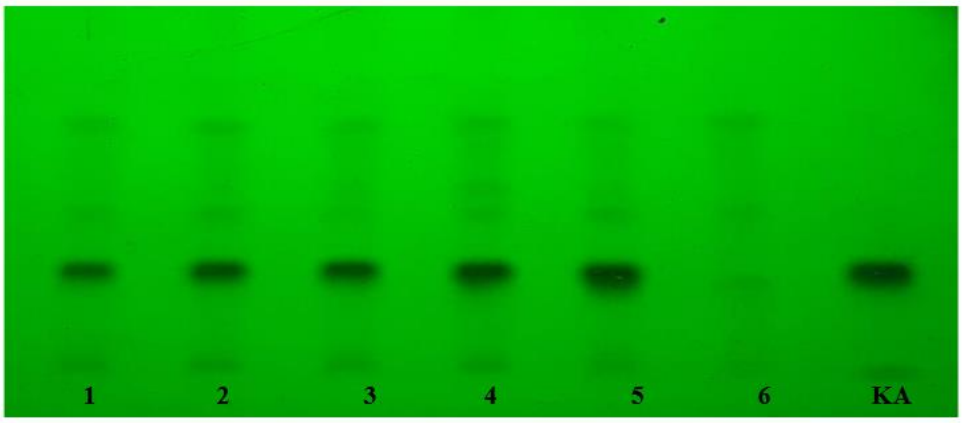

Fig. 2: Fungal filtrates of endophytic fungal isolates separated on TLC under 254 nm UV lamp. Where, No. 1-6: number of the fungal isolates listed in table (1), where KA is kojic acid standard.

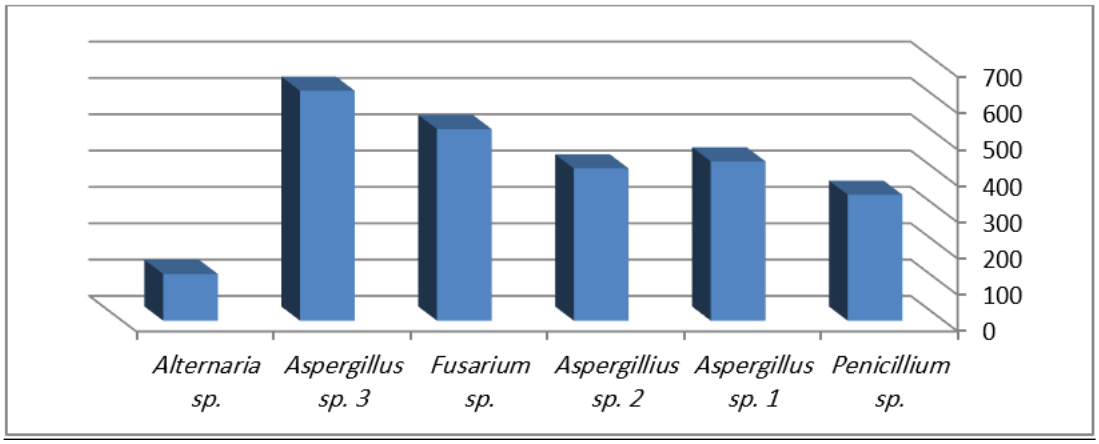

Fig. (3): Kojic acid concentration (mg/L) of each fungal culture

Biosynthesis of Kojic Acid by fungal fermentation of some agricultural wastes:

In this part, the producer fungi were cultivated on agricultural wastes (sugarcane bagasse, corn cobs, and rice straw) with agar only as a solid medium. The ability of the producer fungi to grow on the agricultural wastes was summarized in table (1).

From the data of table (1) and figure (4) it was found that most of the cultures have detectable growth, where the highest growth was recorded in the case of *Apergillus sp*.3and the best agricultural waste for fungal growth was bagasse. The ability of fungi for growth on this media indicates that they can utilize these wastes and can produce Kojic Acid.

Ammar *et al.*, 2017 shows that orange peel and rice straw were the most suitable materials for maximum production of KA by strains *A. flavus* HAk1-M2 and *A. oryzae* . This indicates that the component of culture medium and type of sugars play a significant role in biosynthesis of KA by *Aspergillus* spp. Our results agree with Wan *et al.* (2004) and Rosfarizan and Ariff (2006), who reported that *Aspergillus* spp. has the ability to produce invertase enzyme for the hydrolysis of sucrose to glucose and fructose for subsequent transformation into KA. Rasmey *et al.* (2016) reported that the amounts of KA excreted in the culture medium depend significantly on the type of fermentable sugars consumed by fungal strains.

Identification of the most potent isolate, *Apergillus sp.*3;

Colonies of *Apergillus sp.3* on Czapek's agar at 25ºC were found to reach a diameter of 5.3 cm. Colonies appeared in light brownish green shades and in radiate shape. Conidiophores were hyaline, roughened and 0.88 mm in length. Vesicles were globose with a diameter of 36 µm. The dimensions of the phialides and metulae were 8.1x4.5 and 7.6x3.7 µm, respectively. Conidia were echinulate, of globose shape, and having a diameter of 3.4 µm. According to the previous data, the fungus is *Aspergillus oryzae* (Fig.5).

Table (1): The ability of fungal isolates to growing on each agricultural waste

NO	Fungal isolates	Growth Diameter (mm)		
		Rice straw	Corn cob	Sugarcane bagasse
1	*Penicillium sp.*	52	55	20
2	*Aspergillus sp.* 1	90*	90*	90*
3	*Aspergillius sp.* 2	90*	90*	90*
4	*Fusarium sp.*	31	49	21
5	*Aspergillus sp.* 3	90*	90*	90*
6	*Alternaria sp.*	59	64	34

* Full plate growth

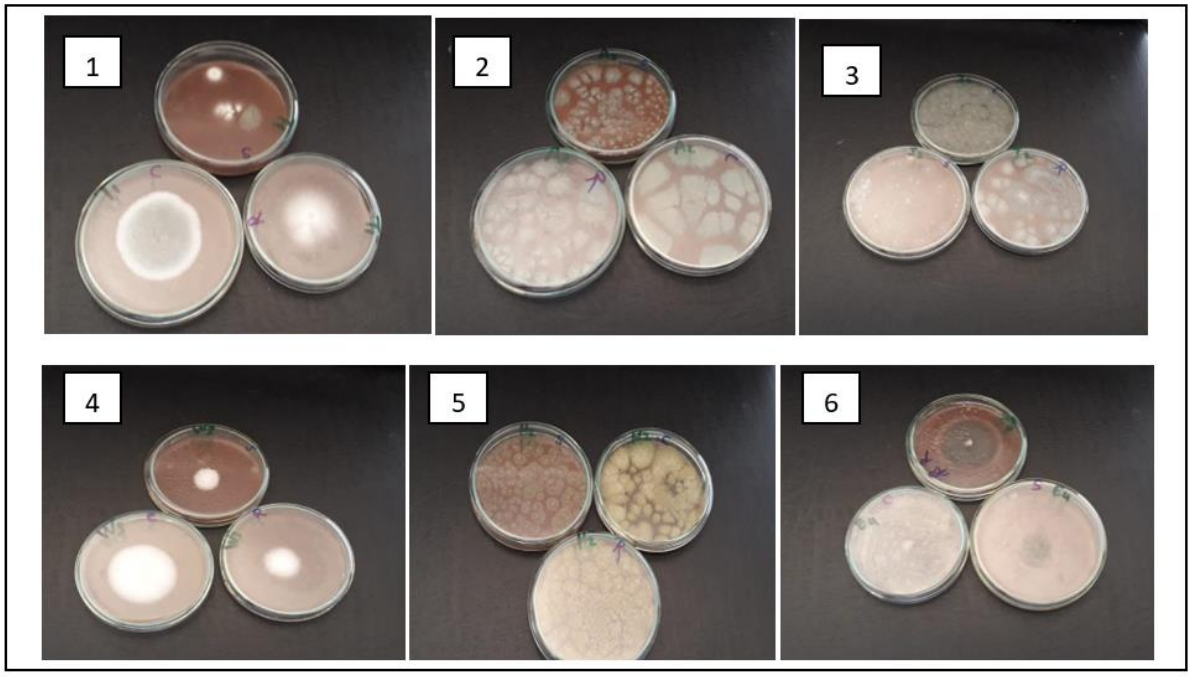

Fig. 4: Endophytic fungal isolates grown on the agricultural wastes. Where, **No. 1-6:** number of the fungal isolates listed in table (1).

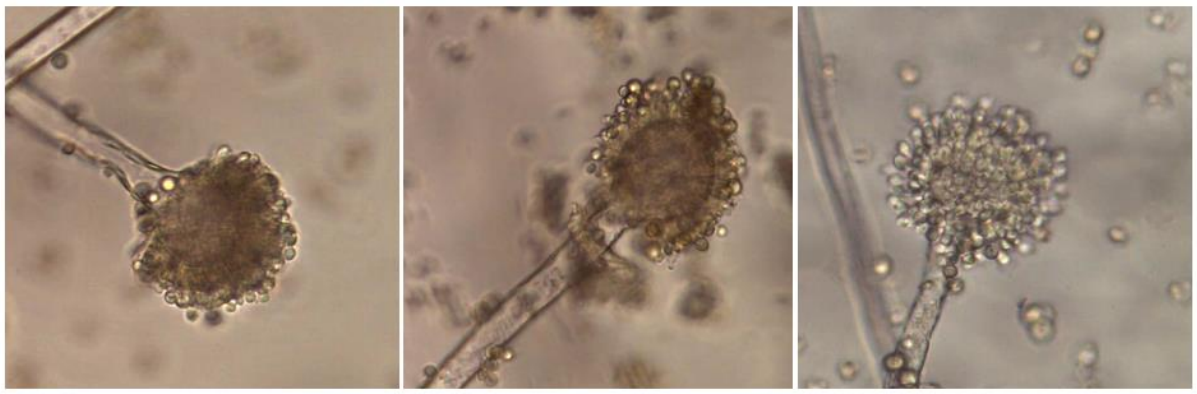

Fig. 5:*Aspergillus oryzae* under 40 × light microscope

Optimization of Kojic Acid Production:

For the applicable production of the highest yield of Kojic acid by *Aspergillus oryzae* liquid fermentation of sugarcane bagasse, the following environmental conditions were studied; method of cultivation, hydrogen ion concentration, incubation temperatures and incubation periods.

The effect of different cultivation methods on the production of Kojic acid by *Aspergillus oryzae* was studied. For this purpose, the fungus was cultivated in both static and shaking conditions and it was found that the shaking cultivation was the best condition (Table 2 and Fig. 6(2)). The effect of different initial pH values on the production of Kojic acid by *Aspergillus oryzae* was studied. For this purpose, the growth medium was adjusted at different initial pH values covering arange of 4.5 - 8.5 and incubated for 15 days. It could be concluded from the results of (Table 2 and Fig. 6(2)) that, the optimum initial pH value capable of promoting KA production by *Aspergillus oryzae* was found to be at the value of 6.0. The experiment was devoted to detecting the suitable incubation period needed for the production of the highest yield from Kojic acid by *Aspergillus oryzae*. The K

A productivity was detected at different time intervals of 6, 8, 10, 12, 14, 16, 18, 20, and 22 days on growth medium. Data in (Table 2 and Fig. 6(1)) showed the relation between K A productivity and incubation time. The level of the K A yield increased gradually with increasing the incubation period up to the end of 20 days. Then, it started the decline phase. For the detection of a suitable incubation temperature for maximizing Kojic acid production by *Aspergillus oryzae*, it was allowed to grow on growth medium adjusted at pH (6.0) and incubated for 20 days at different temperature degrees covering the range of 10 - 37 ºC. Data represented (Table 2 and Fig. 6(3)) showed that the optimum temperature capable of promoting K A production was 28 ºC.

The highest amounts of KA resulted was agree with that reported by Hassan *et al*. 2014; Durgadevi*et al*. 2015 are produced in acidic medium with pH values ranged from 4 to 6. The optimum incubation temperature for kojic acid production has resulted at 28°C. The optimum temperature for kojic acid production by fungi in different studies was found to be 25 - 30°C (Gad 2003, El-Kady *et al*. 2014). Davis, (1963) reported that Optimum conditions for kojic acid production were: pH, 7.0 to 8.0; temperature, 30 to 35°C on kojic acid production by *Aspergillus flavus* growing on peanut oil.

Table (2): Parameters controlling KA production from liquid fermentation of sugarcane bagasse using endophytic fungal isolate *Aspergillus oryzae*.

Culture parameters							
Different Temp.		**Different pH**		**Different incubation time**		**Static and shaking con.**	
Temp. °c	KA Con. (mg/l)	pH values	KA Con. (mg/l)	days	KA Con. (mg/l)	Factor	KA Con. (mg/l)
10°c	388.4	4.5	340.2	6	334.8		
15°c	414.5	5.0	435.8	8	346.4		
20°c	418.2	5.5	452.1	10	364.7	Static	429.3
25°c	420.2	6.0	460.6	12	485.7		
28°c	514.7	6.5	426.8	14	514.9		
30°c	491.5	7.0	411.5	16	519.8		
35°c	341.1	7.5	396.3	18	519.7	shaking	499.4
37°c	058.6	8.0	366.4	20	617.9		
		8.5	302.9	22	520		

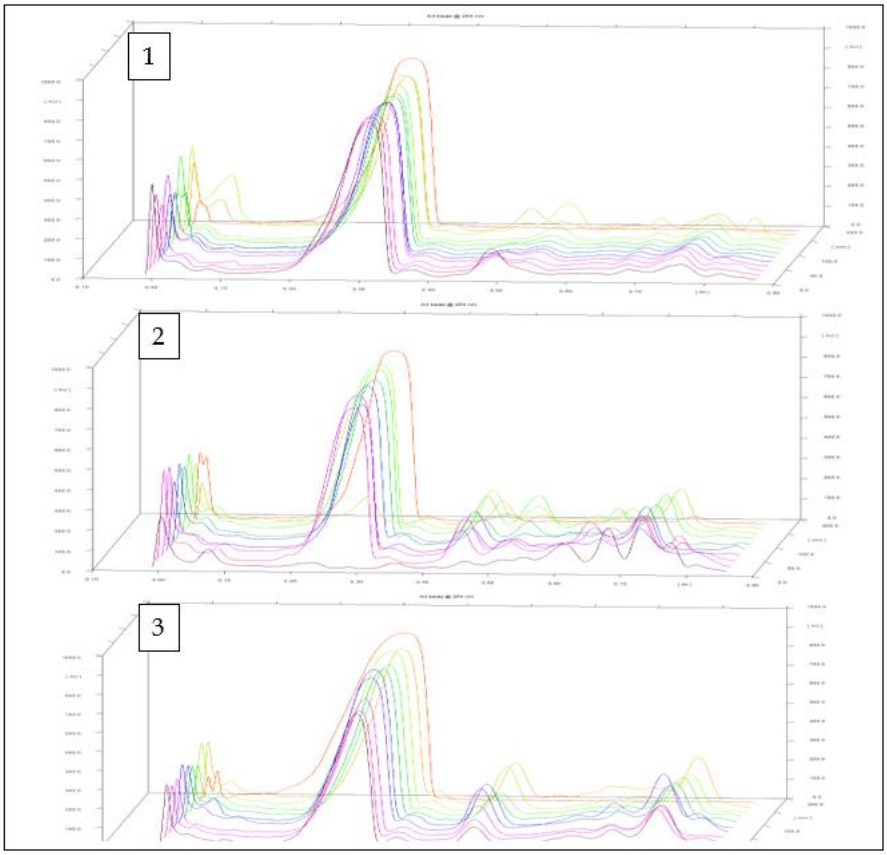

Fig. 6: Total Chromatogrames of TLC plates that determined parameters controlling KA production from liquid fermentation of sugarcane bagasse using endophytic fungal isolate *Aspergillus oryzae*. Where, 1: deferent incubation periods, 2: deferent initial pH values & method of cultivation and 3: deferent incubation temperature.
*All results represented at the same pattern as in table2.

Ethyl acetate extract of *Aspergillus oryzae* showed promising antioxidant activity against DPPH free radical, recording IC_{50} = 5.7 Fig. (7). Also,the E A extract was separated using Gas Chromatography technique recording RT: 31.95 min. and MW: 142 as shown in Fig. (8).

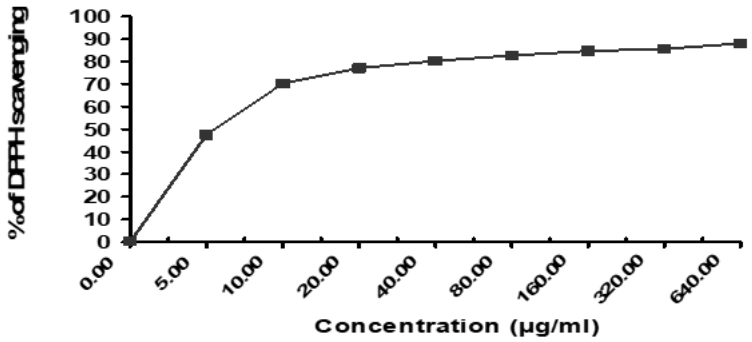

Fig (7): Antioxidant (DPPH scavenging) activity of ethyl acetate extract of endophytic fungus, *Aspergillus oryzae*.

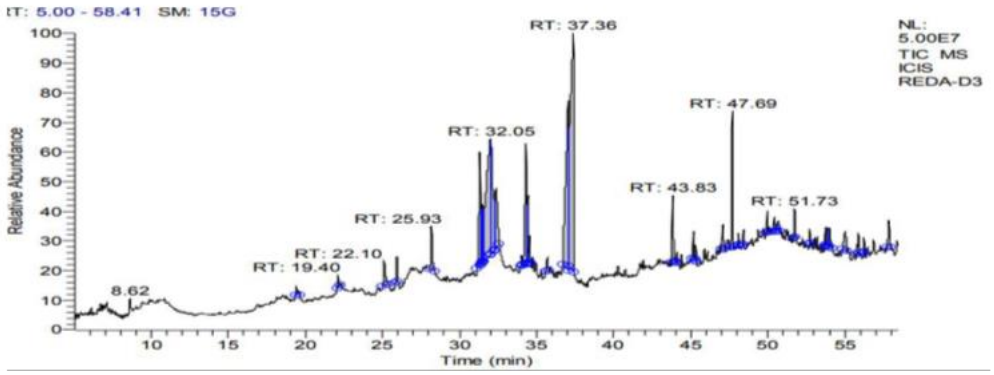

Fig. (8): GC chromatogram of ethyl acetate extract of endophytic fungus, *Aspergillus*

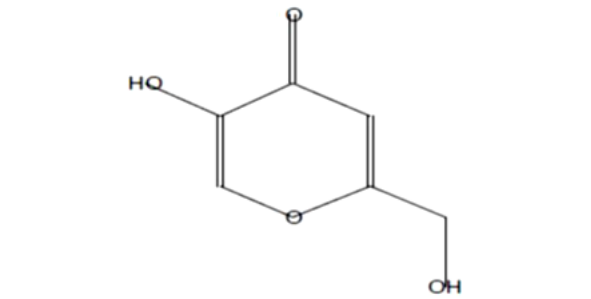

Fig. (9): RT: 31.55 and 31.95 min., Formula: $C_6H_6O_4$, MW: 142

Properties and Chemical Structure of KA:

Knowing the structure of Kojic acid is important in the determination of some chemical and physical properties it possesses. The structure is determined as 5-hydroxy-2-hydroxymethyl-δ-pyrone (Fig. 9) (Yabuta ,1924), And has Molecular formula C6H6O4 and the molecular weight is 142.11g/mol (Uchino, 1988).

Chemical properties of Kojic acid soluble in polar substances like water, ethanol, ethyl acetate, etc. On the contrary, it is less soluble in chloroform, ether. (Nakajima *et al.*, 2001).

Conclusion:

The mode of action of Kojic acid is by blocking tyrosine from forming, which then prevents melanin production. Melanin production is very important for skin coloration so, its inhibition mayhave a lightening effect on the skin. Kojic acid is most commonly used in cosmetic products, such as serums,creams, and lotions. Production of Kojic acid by microorganisms and its uses needs more investigations.

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ARABIC SUMMARY

الإنتاج التكنولوجي الحيوي لحمض الكوجيك المُصنَّع عن طريق الفطر الداخلي أسبيرجللس *اورايزا* المعزول من نبات *ايفوربيا بيلبس*

رضـا شحاتة[1]– شـــادية صبري[2]

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تم عزل ستة أنواع فطرية داخلية تنتمي إلى الأجناس*اسبيرجيللس*،*الترناريا* ، *بنسليـوم* و*فيـوزاريوم* ، و التي تم تحديدها تبعا للشكل الظاهري ،تم عزلها من الأوراق السليمة والسيقان والجذور للنبات الطبي *ايفوربيابيلبس* الذي تم جمعه من محافظة دمياط في مصر. تم اختيار أحد السلالات الفطرية التي تم تحديدها شكليًا على أنها *A. oryzae* ، باعتبارها المنتج الأكثر فاعلية لحمض كوجيك ، الذي تم إنتاجه في هذه الدراسة عن طريق تخمير النفايات الزراعية باستخدام تفل قصب السكر وكيزان الذرة وقش الأرز. تم تحسين تخمير تفل قصب السكر باستخدام سلالة *Aspergillus oryzae* الداخلية كمصدر لحمض كوجيك بطريقة رخيصة وسريعة ومفيدة بيئيًا باستخدام المزارع المتحركةوعند درجة الحموضة 6.0 لمدة 20 يومًا من الحضانة عند 28 درجة مئوية. أظهر مستخلص إيثيلأسيتات من *Aspergillus oryza*e نشاطًا مضادًا للأكسدة واعدًا ضد الشوادر الحرة DPPH، مسجلاً IC_{50} = 5.7. أيضا، تم استخدام تقنية كروماتوجرافيا الغاز فصل مستخلص الايثيلاسيتاتمسجلة RT: 31.95 دقيقة. و MW: 142. تهدف الدراسة إلىتصنيع حمض الكوجيك حيويا وتقليل تكلفة إنتاجه من خلال تحسين الظروف البيئية للإنتاج من مواد رخيصة الثمن مع سمية منخفضة وكفاءة عالية كمضاد للأكسدة.