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PRODUCTION OF CHITIN FROM SOME SOIL FUNGI

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ABSTRACT

Crustacean shells constitute the traditional and current commercial source of chitin. Conversely, the control of fungal fermentation processes to produce quality chitin makes fungal mycelia an attractive alternative source. Our study examined the production of chitin from mycelia of different fungal isolatesrecovered from soil samples collected from different places in El-Beheira Governorate, Egypt, during 2021. Among the 21 isolate 9 were identified with standard key and microbial expert and because these isolates are pure isolates.In batch culture, isolates were screened to determine the fastest growing and greatest chitin-yielding fungi. Optimum chitin yields were observed for most cultures at 28°C on Czapek's dox (CD) for 7 days. From the results we observed that; the production of chitin (by a dry weight method) ranged from 15.41 to 54.78 %of the isolate dry weight, the *Fusarium* KYM3 was the best producer of chitin of the isolates examined and

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Penicillium KYM6 was the lowest producer of chitin of the isolates examined.

Keywords: Chitin, fungal isolates, soil samples, El-Beheira Governorate

INTRODUCTION

Chitin is the second most prevalent biopolymer on the earth, after cellulose, and is found in the exoskeletons of a wide range of eukaryotic creatures such as crustaceans, insects, and fungal cell walls. Chitin's biocompatibility, non-toxicity, and flexibility make it ideal for use in a variety of industries (Antoniraj *et al.*, 2020; Bharati *et al.*, 2020).

Chitin is a linear cationic heteropolymer made up of 2-acetamide-2-deoxy-D-glucopyranose (N–acetyl–D–glucosamine) and randomly dispersed units of 2-amino-2-deoxy-D-glucopyranose (D–glucosamine). Chitin, degree of acetylation (DA) can approach 90%, and its molecular weight (MW) can range from 1 $x10^{6}$ to 2.5 $x10^{6}$ Da, corresponding to a degree of polymerization (DP) of 5,000–10,000 (Mourya & Inamdar, 2008). Between linear chitin chains, the presence of acetamide groups allows for the creation of many inter- and intramolecular hydrogen bonds. Furthermore, these groups are close to the hydroxyl groups in the transposition, resulting in strong crystallinity and low solubility in water and organic solvents. Chitin has been discovered to have a variety of unique characteristics, according to research studies. For example, it inhibits the development of bacteria, fungi, and viruses, has excellent chelating properties, and a high affinity for proteins, making it ideal for enzyme immobilization (Synowiecki & Al-Khateeb, 2003; Younes & Rinaudo, 2015). Chitin has a wide range of uses due to its biodegradability, biocompatibility, non-toxicity, physiological inertness, and gel-forming characteristics, which include food, cosmetics, pharmaceuticals, synthetic materials, agriculture, and even electronics for the manufacture of biosensors (Rinaudo, 2006).

Many countries have great interestin the industrial synthesis of chitin from fungal sources in recent years because of the considerable benefits over currently used techniques. The benefits of producing chitin from fungal mycelia over crustacean sources; there are no seasonal or regional restrictions; inorganic materials have low amounts; simple extraction procedure; Physico-chemical characteristics that are consistent; waste management costs are low; Length of homogeneous polymer; deacetylation to a high degree and solubility in comparison to the existing marine source (Adams, 2004; Knorr *et al.*, 1989; Rane & Hoover, 1993; Teng *et al.*, 2001).Manufactured-chitin is currently commercializedin Japan, the United States, India, Poland, Australia, and Norway, with smaller amounts produced in Canada, Italy, Chile, and Brazil (Alves *et al.*, 2019). Chitin production in nature is predicted to be between 1×10^{10} and 1×10^{12} tons per year (Varun *et al.*, 2017) making this biopolymer a cheap and readily available resource.

In fungi, however, the cell wall is made up of branching 1–3 and 1–6 glucan, while chitin is made up of 1–4 glucans. As a result, the technique for isolating chitin from fungal sources varies, but it can result in yields of 22–44 % with less reliance on chemicals (Mane et al., 2017). Extraction with alkali, 1 M NaOH at 60–120°C for 0.5–12 h, deacetylation, 2–10 % acetic acid at 50–95°C, and precipitation are common chitin extraction processes from fungus (with alkali up to 2 N NaOH). Decoloration or bleaching is used in several extraction methods utilizing marine and fungal sources to eliminate colors that are naturally present in the organism (Abo Elsoud & El Kady, 2019).

In comparison to crustacean-derived chitin, fungal chitin is readily accessible in the form of nanofibrils that can be easily separated from the original matrix by low-energy mechanical mixing, enabling for the creation of nanopapers that are similar to cellulose nanopapers (Nawawi *et al.*, 2019). The quantity of chitin in the fungal cell wall varies depending on the species, the habitat, and the age of the fungus. In yeast and Euascomycetes, the chitin concentration of the dry fungal cell wall can range from 2 to 42 %. Many researchers are working to increase the output of fungal chitin

while lowering manufacturing costs in order to compete economically with crustacean shell chitin (Batista *et al.,* 2018).

Current study examined the production of chitin from mycelia of different fungal isolates with high amount and high quality, to increase the output of fungal chitin whereas lowering manufacturing costs with the aim of compete economically with crustacean shell chitin.

MATERIALS AND METHODS

Chemicals and media

All reagents and chemicals were of analytical grade and used without further purification. Acetic acid and sodium hydroxide were from Merck (Merck KGaA, 64271Darmstadt, Germany). Double distilled water with conductivity 0.05 μS/cm was used to prepare all solution. Czapek's dox (CD) was used for isolation and purification of fungal isolates and potato dextrose (PD) was used for the production of chitin. The both were obtained from Sigma- Aldrich Co.

Czapek's dox (CD):consists of: 30 g sugar cane, 1 g potassium phosphate, 0.5 g magnesium phosphate, 0.5 g potassium chloride, 0.01 g iron sulfate, 2 g sodium nitrate, 2 g peptone and adding 15 g agar (if need solid media) in 1000 ml distilled water.

Potato dextrose (PD): consists of: 200 g potato, 20 g dextrose and adding 15 g agar (if need solid media) in 1000 ml distilled water.

Soil samples collection

The soil samples used for this work were collected from different places in El-Beheira Governorate, Egypt. Soil samples were collected from the soil surface and taken with the help of a sterile shovel and used to collect about 100 g of soil. The samples were labeled according to the site of collection as S1, S2, S3, S4, S5, S6, S7, S8 and S9. The samples were transported in polyethylene bags in ice pack to the laboratory. When samples could not be processed immediately, they were stored at 4 ^{o}C for no longer than 18 to 24 h. The samples locations and their contents were described as shown in Table 1.

Table 1. The locations of samples and their contents

Code	Site	Sample description
S1	Beheira water and drainage company	Clay soil mixed with sand soil
S2	Karakas village	Agriculture soil mixed with manure
S3	Zawet Ghazal village	Clay soil
S4	Zawet Ghazal village	Clay soil mixed with manure
S5	Damanhour city	Soil sample from dust
S6	Sheaer village	Clay soil
S7	Dametuoh village	Clay soil
S8	Eflaka village	Clay soil
S9	Saad village	Clay soil mixed with manure

Isolation and purification of fungal isolates

The serial dilution technique was used to the isolation of fungi from the soil (Kutateladze *et al.,* 2016). Five grams of the soil samples were mixed with a solution 95ml of 1% peptone water (Choudhary & Jain, 2012, Mohanasrinivasan *et al,* 2012) with some modification. Five screw cap test tubes with nine ml distilled water were autoclaved and arranged in the laminar flow hood for further processing, and serial dilutions were made from 10^{-1}, 10^{-2}, 10^{-3},10^{-4} and 10^{-5}were made. Then one milliliter (1ml) of soil suspension from the fifth dilution was taken and spread on the CD agar media plates. The plates were incubated at 27°C for5 days (Choudhary & Jain, 2012; Oyeleke *et al.,* 2010). A mixture of various soil-borne fungi was grown on CD agar medium, and one colony of each fungus transferred to

new CD agar medium plate by a sterilized needle for isolation of pure culture. After ensuring purity, the cultures were sub cultured on CD agar slants and allowed to grow for a period of time (5-7 days) and subsequently stored at 4ºC as stock cultures (Cappuccino & Sherman, 2008; Mohanasrinivasan *et al.,* 2012).

Identification of fungal isolates

The isolated fungi were sub cultured on CD agar and allowed to grow and sporulation. Then from their colony and morphological characteristics, the fungi were identified. Lactophenol cotton blue stain used as the mounting fluid. The slides observed under the microscope then fungi were identified by following the mycological literature (Mohanasrinivasan *et al.,* 2012) with the help of standard fungal identification manuals. The following morphological characteristics evaluated the colony growth (length, width, presence or absence of aerial mycelium, colony color, a presence of wrinkles and furrows, and pigment production) (Tarman *et al.,* 2011).

Production of chitin by fungal isolates

Actively growing mycelia were obtained from a newly prepared CD agar media plate culture after being incubated for 7 d at 28 °C. The pre-inoculums were prepared as follows: about 1 cm × 1 cm of mycelia was inoculated into a 250 ml Erlenmeyer flask, which contained 60 ml of PD broth medium. The inoculated Erlenmeyer flask was incubated at 28 °C for 7 d, without any disturbance. After 7 d of incubation, the mycelia of fungal isolates were separately filtered through Whatman No. 1 filter paper. The mycelium mat was washed with sterilized distilled water until getting a clear filtrate. The mat was dried at 60 °C to get a constant weight for the extraction of chitin.

Chitin extraction

Demineralization and deproteinization are the key stages in the chitin extraction from crustacean exoskeletons. And to remove pigments, different organic and inorganic solvents such as glacial acetone (Soon *et*

al., 2018) and sodium hypochlorite (Srinivasan et al., 2018)are utilized. The calcium carbonate and calcium chloride, the major inorganic components of crustacean exoskeletons, are removed during the demineralization by strong inorganic acids HCl, HNO_3, and H_2SO_4 (Gadgey & Bahekar, 2017), as well as strong organic acids HCOOH and CH_3COOH (Baron *et al.*, 2015), are utilised. When chitin is harvested from fungus, this step is skipped.

The dried fungal cell mass was finely homogenized and subjected to alkali treatments to extract soluble alkali material like glucan and protein. Fungal mycelia mat was subjected to alkaline treatment with 1M NaOH in the ratio of 1:30 (v/v). Then the mixture was mixed, and this was incubated in 45 °C for 2 h. Then this suspension was centrifuged at 8000 rpm for 10 min, and washed with deionized water. Decolorization was done with 10 g /l potassium permanganate for 90 min, and then this was treated with 10 g /l oxalic acid for 1 h. After decolorization technique again centrifuged and washed until getting neutral ph. This was dried at 45 °C for 12 h. The amount of chitin was determined by a dry weight method (Abo Elsoud & El Kady, 2019).

RESULTS AND DISCUSSION

Isolation and identification of fungal isolates

The pure isolates were labeled as follows: KYM1, KYM2, KYM3, KYM4, KYM5, KYM6, KYM7, KYM8 and KYM9 and the plates were examined under the light microscope to identify the characteristics of each isolates. Colony morphology of fungal isolates after incubation at 28°C for 7 days on plates of CD medium shown in Figure 1. The morphological characteristics and microscopic characteristics of fungal isolates were described as shown in Table 2.

Table 2. Morphological identification of fungal isolates

Isolate code	Morphological characteristic	Microscopic characteristic	Genera
KYM1	Color: yellowish white Reverse: orange Pigment: yellow Growth and Texture: colony diameter from 1 to 10 cm. filamentous-downy to powdery	Hyphae are septate and hyaline. Conidia surface very rough and irregular. Conidial head uniserrata. Conidiophore: long, smooth, colorless or brown and shape is globose	*Aspergillus*
KYM2	Color: blue green, yellowish with weight margin. Reverse: pale white to yellowish weight. Growth: velvety to powdery. Growth rate: moderately growth. colony diameter from 2 to 3 cm.	Hyphae: septate and hyaline. Conidiophore: simple – branched. Phialides: Flask-shaped. Conidia: Globose, Ellipsoid unicellular, hyaline, coarsely roughened.	*Penicillium*

KYM3	Color: whitish and concentric rings of aerial mycelium. Reverse: white red and some pale orange. Growth: dense cottony. Aerial mycelia were abundant, densely floccose to fluffy. Growth rate: moderately rapid growth. Colony diameter: range from 6.5 to 9 cm.	Hyphae: septate. Conidiophore: simple. Conidia: Macroconidia is abundant, slender almost straight, curved apical cell and foot-shaped basal cell and 3 to 5 septa. Microconidia: microconidia were 0-septate, oval, club shape with flattened. Chlamydospores were 8 to 15 µm in diameter, smooth, intercalary and single .	*Fusarium*
KYM4	Color :White to creamReverse: Brown Growth: velvety to powdery. Growth rate: rapid growth. Colony diameter from 1 to 2 cm	Hyphae: septate and hyaline. Conidiophore: simple – branched. Phialides: Flask-shaped. Conidia: Globose, Ellipsoid unicellular, hyaline, coarsely roughened.	*Penicillium*
KYM5	Color: yellow to brown. Reverse: white to wood brown Growth and Texture: colony diameter from 2 to 3	The hyphae are septate and they branch at acute angles. Conidial heads typically radiate Conidiophore uncolored, coarsely roughened Vesicle Subglobose	*Aspergillus*

	cm. filamentous-downy.		
KYM6	Color: Green Reverse: White to cream Growth: velvety to powdery. Growth rate: moderately rapid growth. colony diameter from 0.5 to 2 cm	Hyphae: septate and hyaline. Conidiophore: simple – branched. Phialides: Cylindrical. Conidia: Globose, Subglobose unicellular, round to ovoid,coarsely roughened	*Penicillium*
KYM7	Color: whitish to pink. Reverse: whitish to orange. Aerial mycelia were abundant, densely floccose. Growth rate: moderately. Colony diameter: range from 7 to 8 cm	Hyphae: septate. Conidia: macroconidia were sparse, often lacking, fusoid and variable. Microconidia: were abundant and hyaline Chlamydospores were hyaline, spherical with no septa (one celled), produced either singly or in chain and 3.0 – 8.5 µm in diameter	*Fusarium*

KYM8	Color: orange to light violet. Reverse: pale orange. Aerial mycelia were abundant, densely floccose to fluffy. Growth rate: moderately growth. Colony diameter: range from 3 to 4.5 cm	Hyphae: septate. Conidiophore: simple. Conidia: Macroconidia is abundant, curved apical cell and foot-shaped basal cell and 3 to 4 septa. Microconidia: microconidia were 0-septate and oval. Chlamydospores were 8 to 15 µm in diameter, smooth and single.	*Fusarium*
KYM9	Color: white, black margin Reverse: pale white Growth: cottony Growth rate: very rapid growth	Hyphae: broad and non-septate. Rhizoids and stolons are present. Sporangiophore: brown color –smooth- ovoid. Sporangia: round.	*Rhizopus*

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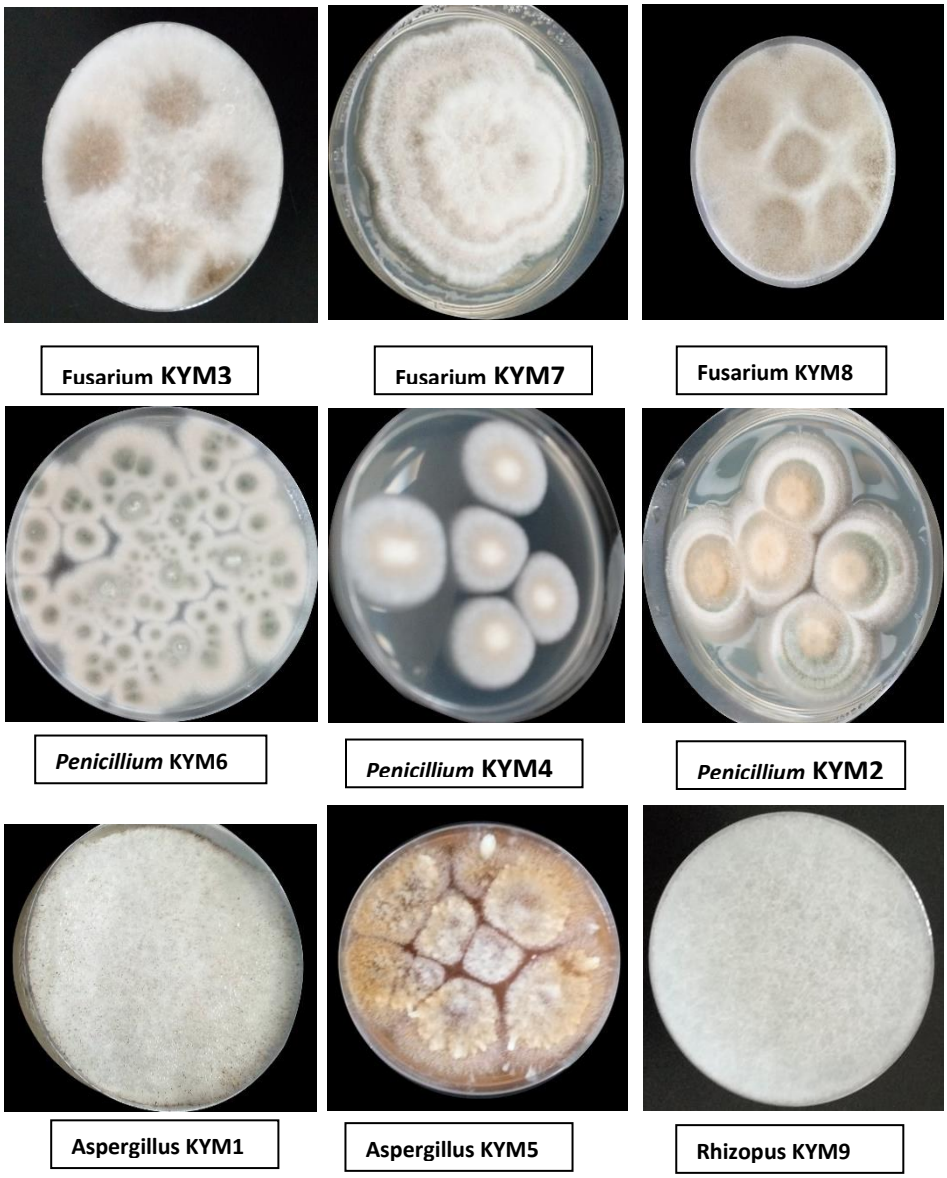

Figure 1. Colony morphology of fungal isolates

Among the 21 isolate 9 were identified with standard key and microbial expert and because these isolates are pure isolates. The 9 isolate were subcultured on CD agar plate and allowed to grow, then the plates were examined on the basis of cultural, microscopic and morphological characteristics. From the soil fungal isolates the most of the species belonging to the genera Penicillium, Fusarium, and Aspergillus were dominant as shown in Figure 1.

Production of chitin by fungal isolates

Screening of fungal isolates for production of chitin were done to distinguish the fungus which produce the highest percentage of chitin and the other fungi which produce the lowest percentage of chitin at pH 8, 30°C after 7 days of incubation on CD media. As shown in Figure 2, it can be seen clearly that the KYM3 (Fusarium sp.) is the fungal isolate which produce the highest percentage of chitin (54.78 %) and the KYM6 (Penicillium sp.) is the fungal isolate which produce the lowest percentage of chitin (15.41 %).

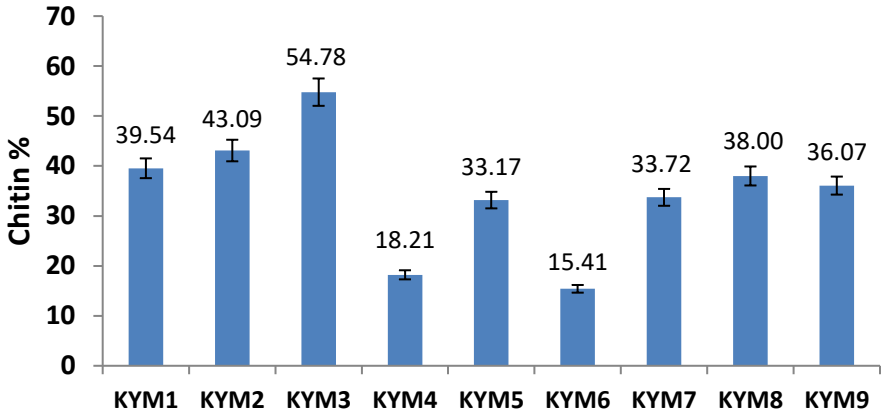

Figure 2. Screening of fungal isolates for production of chitin (%).

DISCUSSION

According to the Figure 2 it was noticed that KYM6 and KYM4 were excluded because their production of chitin (%) is weak and the isolates KYM1 and KYM2, KYM5, KYM7, KYM8 and KYM9 have moderate production of chitin (%) and we observed that the KYM3 isolate has the highest percentage of chitin content so it was selected as the best sp. for production of chitin (%).

CONCLUSION

The results described in this study have verified that *Fusarium* isolates were of the highest chitin production which ranged between 33.72% and 54.78% while *Penicillium* isolates and *Aspergillus* isolates showed lower production rate being 15.41- 34.09% and 33.17-39.54%, respectively. The *Rhizopus* isolate, however, showed 36.07% of chitin.So chitin can be successfully extracted from soil fungal isolates easily and economically. The highest yield of chitin (54%) from fungi and the other benefits enhancethe researchers to more study on the production of chitin from fungi to increase the output of chitin whereas lowering manufacturing costs with the aim of compete economically with crustacean shell chitin.

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الملخص العربى

انتاج الكيتين من بعض فطريات التربة

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الملخص

تم جمع عينات من التربة من أماكن مختلفة بمحافظة البحيرة بمصر. عن طريق التخفيف المتسلسل ، تم زراعة خليط من الفطريات التي تنتقل عن طريق التربة على اطباق تحتوى على وسط غذائى مناسب (CD) ، وتم نقل مستعمرة واحدة من كل فطر إلى اطباق CD جديدة بواسطة إبرة معقمة لعزل المستعمرات النقية. تم تصنيف 9 فطريات نقية معزولة من KYM1 إلى KYM9 وفحصت على أساس الخصائص والميكروسكوبية والمورفولوجية. كانت معظم العزلات الفطرية في التربة هي السائدة من أجناس *Penicillium* و *Fusarium* و *Aspergillus*. ومن خلال فحص الكتلة الحيوية الفطرية لانتقاء الفطريات التي تنتج نسبة عالية من الكيتين وجدنا أن عزلات *Fusarium* كانت من أعلى إنتاج للكيتين حيث تراوحت بين 33.72٪ و 54.78٪ بينما عزلات *Penicillium* وعزلات *Aspergillus* أظهرت معدل إنتاج أقل من 15.41 - 34.09٪ و 33.17 - 39.54٪ على التوالي. بينما أظهرت عزلة *Rhizopus* انتاج 36.07٪ من الكيتين. سلالة KYM3 *Fusarium* هي أعلى سلالة في إنتاج الكيتين (54.78٪). لذلك يمكن استخلاص الكيتين بنجاح من عزلات التربة الفطرية بسهولة واقتصادية مع جودة عالية. أعلى محصول من الكيتين (54٪) من الفطريات والفوائد الأخرى تعزز الباحثين لمزيد من الدراسة حول إنتاج الكيتين من الفطريات لزيادة إنتاج الكيتين مع خفض تكاليف التصنيع بهدف التنافس اقتصاديًا مع الكيتين المنتج من القشريات.