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A Potential 17- β Estradiol degrader Bacterium Isolated from Sewage water

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ABSTRACT

A successful 17 β estradiol (E2) degrading bacterium was isolated from waste water of Saft Elhena drain in Egypt. Molecular identification of the isolated bacteria was carried out using DNA (RAPD), the amplification was made by restriction analysis of amplified 16s rDNA and sequencing. Alos, the bacterium was incubated in saline media under different levels of 17 β-estradiol anaerobically. Levels of CO_2 production were examined by GC and residual 17 β-estradiol was tested by HPLC to measure biodegradation capacity. Identification of the genetic element responsible of the gene resistance in the bacteria was tested. The sequence analysis of 16S rRNA gene for 17 β estradiol degrader bacterium showed that its affiliations to phylum Enterobacteriaceae, and it belonged to the genera *Klebsiella sp.* Results revealed that *Klebsilla* was cabable of degrading estradiol and that the responsible resistance gene was mostly the chromosomal gene. However, further studies are still going on to locate and examine the gene responsible for E2 resistance.

Keywords: 17 β-estradiol, degradation, curing, carbon production

INTRODUCTION

The occurrence and fate of pharmaceutically active compounds in the aquatic ecosystems has been recognized as one of the emerging issues in environmental chemistry. 17 β-estradiol (E2) is an important compound of these pharmaceuticals which belong to endocrine disrupting chemicals (EDCs), it is found as residues in medicinal products, as pesticides, and as additives in animal feed. Even though that these compounds are found in very small amounts in the environment but the effect of their traces is very huge on marine animals and humans.

17 β Estradiol has been a concern of investigation since the 1930s (Cook *et al*., 1934; Tawfic, 2006), however, when 17 β Estradiol ,excreted from the human body and live stocks (Narender and Cindy, 2009), in addition to the synthetic estrogenic chemicals, added in animal feeding, and/ or contraceptives are discharged into marine environments as waste waters or sewage, they would occur with concentration levels in the range of nanograms /L (Koh *et al*., 2008).

Yet, even concentrations less than 1 ng/L in the aquatic environment can cause infertility and reduce estrogenic activity, decrease reproductivity of fish females (Burton & Wells, 2002), change behavioral habits which may change reproductive physiology of fish (Denslow *et al.*, 2007), and reduce release of growth hormone from the pituitary (Ng *et al*., 2001). When these chemicals reach the marine environment, they may be biotransformed and/or bioconcentrated (Lai *et al*., 2002), and/or accumulate in marine animals (Gomes *et al.*, 2004; Lai *et al*., 2002) through the food chain. Eventually, the environmental safety, the health of marine living organisms and human health might be threatened.

The attention is directed towards developing new methodologies for bioremediation of water to remove (Sofue, *et al*., 2007) degrade (Takashi *et al*., 2004; Scherr *et al.*, 2009) or eliminate these compounds from the water bodies. Therefore, this research aimed to isolate bacteria from the waste water and aquatic systems and test their ability to resist 17 β estradiol in order to develop a practical methodology of bio-remediation using these bacterial isolates to eliminate 17 β estradiol from water.

MATERIALS AND METHOD

Isolaton of bacteria:

The isolation was made from water samples collected from the main end points at which the main sewage discharges of Delta is released. The samples were collected from Saft Elhenna drain. A volume of 100 ml was collected at 50 cm depth. Samples were used immediately after collection for effective bacteria isolation.

The collected water samples were filtered on a sterile filter paper 0.2 mm in a vacuum filtration to insure harvesting a high yield of microorganisms. The filter papers were cultivated on a minimal salt medium (MSM) as described below by (Sambrook J. *et al*., 1989), 17 β-estradiol $C_{18}H_{24}O_2$ was added to the medium as the only carbon source in the media in concentrations between 20-30 µg/ml.

Each sample was inoculated directly into medium. The samples were cultured at 32°C for 48 hours. The bacteria isolates were cultivated on MS agar medium where 17 β-estradiol was added in different concentrations (5-300 µg/ml). Successful colonies grown on the highest concentrations were isolated and plated separately on Luria-Bertani (LB) broth for 24 hours to prepare them for the molecular identification process.

Bacterial identification by 16S rRNA gene sequence analysis:

One colony was picked and inoculated on Luria-Bertani (LB, Sigma) broth for rapid growth of bacteria in 15 ml tubes for 24 hours at 37ºC. Cells were precipitated from liquid medium by centrifugation at 3000 rpm for 10 min at 4ºC. Then they were re-suspended in 0.5 ml of 10% sucrose 50mM tris HCl. Then, genomic DNA of the isolated bacteria was carried out as described by (Sambrook J. *et al.*, 1989).

The quantity and purity of the precipitated DNA were assessed using NanoDrop ND-1000 full spectrum UV spectrophotometer at 260 and 280 nm, respectively.

Approximately 1500 bp of the 16S rRNA gene fragment was PCR-amplified according to (Singer *et al*., 2006; Tikko *et al*., 2001) amplification reaction mixture was vortexed and placed in PCR (Eppendorf) and subjected to 10 min of denaturation at 94ºC, followed by 35 cycles of denaturation at 94ºC for 30 seconds. Annealing of primers 5'-AGCGCCATTG-3' was made at 43ºC for 30 seconds, and primer extension at 72ºC for 90 seconds. Finally, PCR fragments were then tested by sequencing.

Plasmid identification and curing in the selected isolates

Isolation of plasmid DNA was carried out by extraction with a phenol-

chloroform mixture (1:1). For this purpose 25 ml of the growth mixture was centrifuged at 10,000 rpm for 10 minutes and the cells were re-suspended in 200 µL of sterile distilled water. An equal volume of phenol chloroform mixture was added sand the mixture was emulsified by vortex. The emulsion was broken by centrifugation at 10,000 rpm for 10 min. The supernatant solution was carefully withdrawn by sterile tips of micropipette and the process was repeated three times.

Curing of plasmids was carried out according to (Ansari & Khatoon, 1997) by using ethidium bromide (EBr). The bacterial isolates were cultured in MSM plus 40 µl E_2 containing diluted concentrations of (EBr), and they were incubated in 37ºC for 48 hours. A control without EBr, and another one containing EBr without isolates were both incubated over night. The contents of the control tube and the EBr-containing tubes were plated on MSM and MSM-E2 to check for the absence of E2 resistance genetic element.

Degradation activity E2:

Bacterium was inoculated in MSM-E2 at 32ºC, and the concentration of estradiol was measured every 6 hours in the containing media using HPLC-FLD on a C18 column with gradient elution with Acetontrile. Carbon dioxide detection was measured according to (Parawira *et al*., 2008) using GC. Helium was used as the carrier gas at a flow rate of 30 ml/min. The column temperature was 70ºC. The injector temperature was set a 110ºC, and detector temperature was set at150ºC.

RESULTS AND DISCUSSION

Isolation and identification of bacteria:

One successful species bacterial isolate was able to grow in the presence of 17 β-estradiol. DNA was successfully isolated from these bacterial isolate and subjected for PCR amplification. The results of PCR reactions amplifying 16S rRNA gene fragment from the DNA of the bacterial isolate are showed in Fig 1. 16S rRNA gene sequencing revealed bacterium with phylogenetic affiliations to phyla Enterobacteriaceae. It was identified as *Klebsiella* sp.

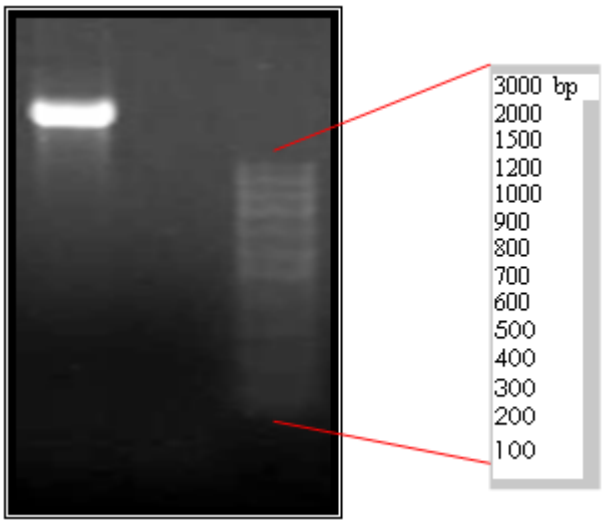

Fig 1: PCR identification of the isolates using 16 S DNA.

Degrading activity of 17 β-estradiol:

Klebsiella sp. showed a good estrogen degrading activity when it was inoculated in estradiol anaerobically Fig. (2). E2 concentration was degraded down to 13% within 24 hours. Also, the measured % CO_2 production of the bacteria (Fig. 3) suggests that *Klebsilla*

was degrading estradiol as it was the only source of carbon for the bacteria. It produced carbon dioxide by 13 % within 24 hours. The carbon percentage produced by each bacterium was correlated to the amount of estardiol added to the medium; results showed that *Klebsilla* was produced 0.9% of added estradiol in 48 hours.

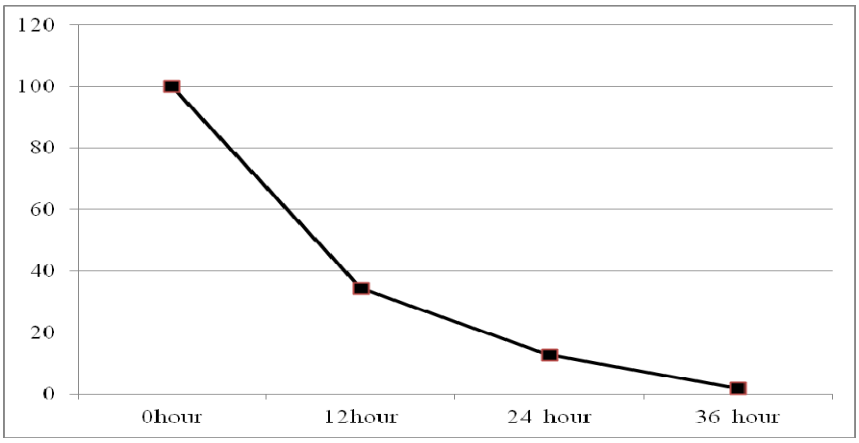

Fig. 2: 17 β-estradiol degrading activity anaerobically

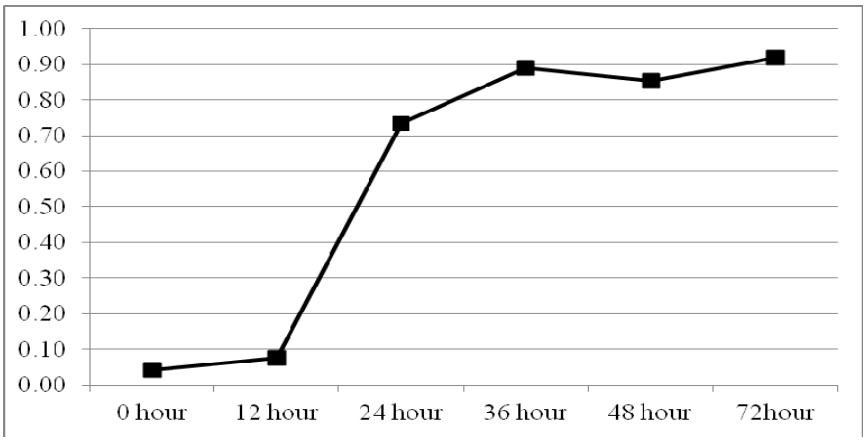

Fig. 3: CO_2 % production by bacteria

However, the capability and the time intervals in which the successful tested bacteria degraded estradiol is more or less acceptable compared to other bacterial isolates previously reported as potential 17*B*-estradiol-degrading bacteria; such as *Rhodococcus zopfii*, which completely degrade 100 mg/ L of 17 β-estradiol plus estrone, estriol, and ethinyl estradiol within 24 hours (Yoshimoto *et al*., 2004). Another bacteria (not named) was reported by (Lee & Liu, 2002) to be able to completely degrade 20 000 μg of E2 within 18 hours. Other species were also found efficient such as G*eneras Aminobacter, Brevundimonas, Escherichia, Flavobacterium, Microbacterium, Nocardioides, Rhodococcus,* and *Sphingomonas* (Yu *et al*., 2007), *Novosphingobium* species. (Fujii *et al*., 2002); ARI-1 and KC8 strains (Roh & Chu, 2010), Sphingomonas sp. and *Rhodococcus (Kurisu et al*., *2010)*.

Generally, It is not yet fully clear how E2 is degraded (Christoph & Juliane, 2009), but it is believed that oxidation of E2 to estrone (E1) at the D ring of E2 is accepted to be the first step of the degradation pathway (Lee & Liu, 2002; Shi *et al.*, 2004). Trimethylsilyl (TMS) derivatives of E1 and five other metabolites (I–V) were also reported by

(Kurisu *et al*., 2010). However, it is commonly stated in different investigations that oxidation of E2 to E1 occurs both in complex culture systems, such as activated sludge (Ternes *et al*., 1999) and in purified bacterial cultures (Chang-Ping *et al*., 2007).

Identification of the genetic element responsible of the gene resistance

The molecular weights of plasmids differes notably which suggests the presence of different plasmids or conformations of the same plasmid (Bertin, 1995). For this purpose plasmid profile determination of the natural isolated bacteria was made in agarose gel electrophoresis from colonies removed from an agar surface. However plasmid determination is suggested to be made by colonies or with extracts from microscale from small volumes of bacterial culture (Mazza, 1986).

Curing plasmid DNA profile was made in order to point the exact genetic element linking the bacteria to the degradative activity. The bacterium was cultivated on a new petri dish containing 17 β-estradiol after curing of plasmid. No growth on either MSM or MSM-E2 was observed. Also, plasmid profile was analyzed, and no plasmid was shown on the electrophoratic gel analysis (Fig. 4). This gives a clue that the genetic element responsible of the 17 β-estradiol gene resistance is mostly the chromosomal gene. This characterization of the genetic elements responsible of estradiol degradation will help to design an efficient system for its biodegradation.

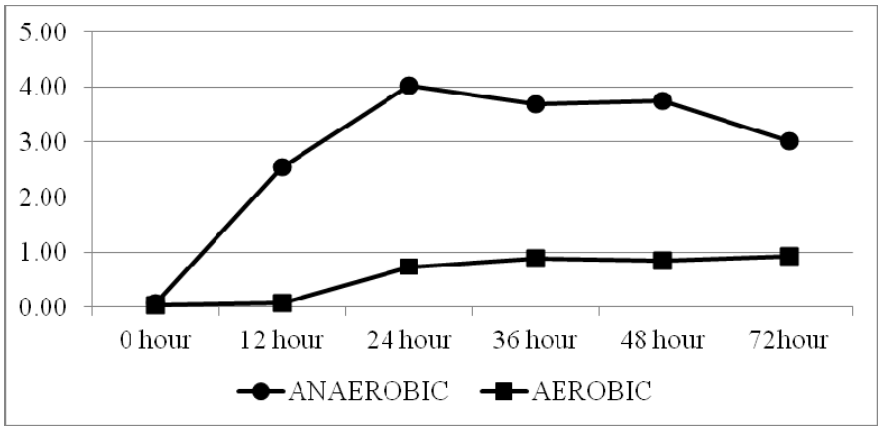

Fig. 4: Carbon % produced aerobically from the added 17 β-estradiol

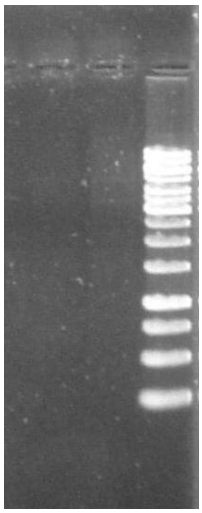

Fig. 5: DNA-Plasmid identification and curing confirmation

CONCLUSION

An isolated E2-degrading bacterium identified by 16S rRNA gene sequence analysis as Klebsilla was detected from the sewage water. Application of HPLC-FLD and GC showed that the bacterium was able to degrade E2 within 48 hours.

It is strongly suggested to locate and examine transferring the gene responsible for E2 resistance in *Klebsilla* into friendly bacteria in future investigations.

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ARABIC SUMMARY

عزل سلالة بكتريا من مياه الصرف الصحي قادرة على مقاومة لمادة 17 بيتا استراديول

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هدفت هذة الدراسة الى عزل سلالة بكتيرية قادرة على استهلاك مادة 17 بيتا استراديول من مياه الصرف الصحي في مصر. وقد جرى التحديد الجزيئي للبكتيريا المعزولة باستخدام الحمض النووي، وفحص التسلسل sequencing. وتم تحضين هذة البكتيريا في بيئة خاصة فى وجود تركيزات مختلفة من 17 بيتا استراديول في ظروف لاهوائية. ولقياس قدرة التحلل البيولوجي تم متابعة إنتاج ثاني أكسيد الكربون من البكتيريا و دراسة المتبقي 17 بيتا استراديول باستخدام جهاز الفصل الكروماتوجرافى. بالاضافة الى دراسة العنصر الجيني المسؤول عن مقاومة البكتيريا المعزولة للاستراديول.

وكشفت نتائج الدراسة أن البكتيريا المعزولة القادرة على تحليل الاستراديول تنتمي إلى عائلة Enterobacteriaceae وهى تحديدا البكتيريا الكلبسيلة. كما وصل إنتاج ثاني أكسيد الكربون في الظروف الاهوائية من البكتيريا بعد 24 ساعة الى 13 % واستهلكت البكتيريا 0.9 % من الاستراديول. واظهرت اختبارات تحديد العنصر الجينى المسؤول عن استهلاك ومقاومة الاستراديول وجود العنصر على الكروموسومات.

مزيد من الدراسات ما زالت جارية لتحديد وفصل الجين المسؤول عن مقاومه الاستراديول والتوصل الى سلالات اخرى لها قدرة اعلى مشابهة على استهلاكة.