

# 16S rRNA characterization of a *Bacillus* isolate and its tolerance profile after subsequent subculturing

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Ola M. Gomaa\* and Osama A. Momtaz\*\*

\* National Center for Radiation Research and Technology (NCRRT),  
PO Box 29, Nasr City, Cairo-Egypt.

\*\* Agricultural Genetic Engineering Research Institute (AGERI), 9 Gama St. Giza, Cairo- Egypt.

The isolation of microorganisms from different highly contaminated environments offers novel bacteria of unique functionality and potential applications in different biotechnological processes. It also entails a variety of genes responsible for microbial tolerance or defense against extreme conditions or xenobiotics present in the media. In a previous work, *Bacillus* spp. isolated from textile wastewater was exploited in terms of characterization, tolerance to pH, salinity, cold temperature and hydrogen peroxide, and the mechanism of resistance against hydrogen peroxide (Gomaa and Momtaz, 2006). This isolate was phenotypically identified as *Bacillus maroccanus*/*Bacillus simplex*. In this study, the primary aim was to characterize this strain using 16S rRNA partial gene amplification and sequencing. It was assigned as *Bacillus simplex* TWW-04. DNA-DNA hybridization showed 96% relatedness to *Acinetobacter*. The tolerance profile of this strain was examined in regard to the four traits previously mentioned. A 36-month subculturing of this strain showed no change in tolerance to pH, salinity and cold temperature, whereas the tolerance to hydrogen peroxide was lost. A sample, frozen for the same duration, showed no change in any of the tested traits. The tolerance alleviation was evident after 24 months of subculturing. Testing the *KatA* gene responsible for hydrogen peroxide tolerance in the two samples showed higher band intensity in the frozen sample when compared to the subcultured sample. This indicates that the hydrogen peroxide tolerance trait is extrachromosomal and has been lost during successive cell division, while the other traits are chromosomal.

Genbank/NCBI/accession number for this 16S rRNA strain EF025542.

**Key words:** 16S rRNA, phylogenetic identification, extreme conditions, tolerance.

\*Corresponding Author: Ola M. Gomaa, email: ola\_gomaa@hotmail.com

## INTRODUCTION

The presence of microorganisms in extreme stress conditions or contaminated environments facilitates their use in different biotechnological applications, as their enzymatic systems are encoded by genes which could be up-regulated for use in different industries or transferred to agricultural plants for use in developing

genetically modified strains. Obtaining such newly modified strains would add traits to plants and enable them to grow in high salinity, to acclimate to different temperatures or to cope successfully with any unexpected environmental variations. In addition, the use of such highly tolerant naturally occurring strains in wastewater bioremediation, is a current demand.

*Bacillus spp.* is widely distributed in the natural environments. Their unique nature lies in their ability to inhabit a variety of extreme and contaminated environments. Their tolerance to stresses are attributed to external shield, which is the cell membrane and internal enzymatic system, besides its spore coat which protects it against physical and chemical agents (Popham *et al.*, 1995; Nicholson *et al.*, 2000; Riesenman and Nicholson, 2000). There have been a number of reports on isolation, identification and characterization of microbial communities from different environmental sources. *Vibrio spp.* was isolated from drain pool of a fish product processing plant; the fish pool contained extremely high concentrations of hydrogen peroxide. The isolate was found to exhibit extraordinary catalase activity (Yumoto *et al.*, 1998); this strain was further assigned and characterized using 16S rRNA gene amplification and sequencing (Yumoto *et al.*, 1999). The right taxonomic position of an environmental isolate could be accurately assigned to identify its novelty, this could only be done by the use of advanced molecular techniques (Yumoto *et al.*, 2001). In some cases, phenotypic identification alone did not verify the right taxonomic position and seemed truly necessary in case of *Bacillus spp.* and relatives (Guinebretiere *et al.*, 2001), and in identifying novel species within a genera (Watabe *et al.*, 2004). For example, antractic coastal marine samples yielded novel *Psychrobacter* (Bozal *et al.*, 2003), this would not have been known if not for the use of phylogeny and DNA-DNA hybridization. Novel eubacteria were isolated from spent mushroom compost (SMC). This study was important to ascertain the ecological consequences involved in the disposal of SMC waste on agricultural land (Watabe *et al.*, 2004). The isolation from waste such as pasteurized zucchini purees revealed a number of bacteria giving an insight to the microbial

communities under conditions like refrigeration to enable reliable data on the actual microbial presence in stored food (Guinebretiere *et al.*, 2001). The structural and functional composition of soil bacterial community inhabiting anoxic rice paddy soil was revealed accurately using the same technique of 16S rRNA and comparison with numerical abundant culturable bacteria (Hengstmann, *et al.*, 1999). 16S ribosomal DNA (rDNA)-based molecular identification could achieve identification, for reasons including its universal distribution among bacteria and the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large public-domain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates (Drancourt *et al.*, 2000).

In the present study, the taxonomic position of *Bacillus spp.* is determined through 16S rRNA phylogenetic identification and the stability of its characteristics was tested after subsequent subculturing through a period of 36 months compared to a sample frozen for the same period.

## MATERIALS AND METHODS

### Source of textile wastewater bacteria

A bacterium was isolated from textile waste water of factory at the 10<sup>th</sup> of Ramadan district, 40 Km from Cairo, as reported by Gomaa and Momtaz (2006). Samples were purified and stored in +4°C and subsequently subcultured over a period of 36 months, while another sample was stored at -80°C under glycerol for the same period.

### DNA extraction

Genomic DNA was extracted from pure bacterial cultures, 24 hr grown in Lauria-Bertani (LB) media at 30°C, centrifuged for 2 min. Bacterial lysis was performed using 10% SDS and 20 mg/ml proteinase K in TE buffer and incubated for 1 hr at 37°C. NaCl (5M) and CTAB/NaCl were added and incubated for 10 min at 65°C. DNA was extracted using chloroform/isoamyl alcohol, then phenol/chloroform/isoamyl alcohol. Aqueous phase was transferred to a fresh tube and DNA was extracted with isopropanol. Finally, DNA was resuspended in 100 µl of TE buffer. The amount of DNA extracted was electrophoresed on 0.8% agarose gel and the results obtained were compared with *HindIII* digest of λ DNA.

### PCR amplification of bacterial 16S rDNA

Oligonucleotide primers were used to amplify 16S rRNA. PA forward: (AGAGTTTGATCCTGGCTCAG) and PH reverse: (AAGGAGGTGATCCAGCCGCA). 16SrRNA was amplified from the obtained DNA in a reaction mixture of PCR conditions were as follows: 10x*Taq* buffer, 1.25 U *AmpliTaq* Gold DNA Polymerase, 2mM dNTP mixture, 25 mM MgCl<sub>2</sub>, 0.7 µg DNA, double-distilled water mixed in a final volume of 50 µl. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and extension at 72°C for 7 min, +4°C∞. Amplification was done using Perkin Elmer GeneAmp PCR system 2400. Amplicons were visualized by Electrophoresis on 1% agarose gel after staining with ethidium bromide (Hengstmann, 1999).

### Cloning and sequencing

16S rDNA PCR product was extracted from gel using gel extraction kit QIAquick Qiagen and was cloned using TA cloning kit (Invitrogen, San Diego, Calif.) as recom-

mended by the manufacturer. DNA sequencing was conducted using ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit according to instructions of manufacturer (PE Applied Biosystems) ABI Prism™ 377XL DNA Sequencer (Perkin Elmer).

### Phylogenetic analysis

The 16S rDNA sequences obtained were added to publicly available bacterial 16S rRNA sequences, the sequences were integrated into the database with the automatic alignment tool. Phylogenetic tree was generated by performing distance matrix analysis using the NT system. Database search and comparisons were done with the BLAST search using the National Center for Biotechnology Information (NCBI) database.

### Nucleotide sequence accession number

The 16S rDNA sequence was deposited in the NCBI Gene Bankit nucleotide sequence database under accession number EF025542.

### Tolerance to salinity, pH, temperature and hydrogen peroxide

One ml of 24 hour culture, taken from 0, 12, 24 and 36 months of cultures subsequently transferred every 3 months on non-inducible LB slants, were transferred to different LB broth media classified into groups for testing tolerance for salinity (0, 0.5, 1, 1.5, 2.5 and 5 M NaCl), pH (3, 5, 7, and 9), incubation temperatures (0, 4, 39, 37 and 50°C) and hydrogen peroxide (145, 442 and 885 mM). Cultures were incubated statically at 30°C, and the growth was monitored at O.D<sub>600</sub> against the control (no H<sub>2</sub>O<sub>2</sub>). The colony forming ability was studied for all cultures by the plate count method on LB agar media under the same culture conditions. The same experiments were performed on textile wastewater samples frozen since isolation (36 months). Samples of all experiments were triplicated.

### PCR amplification of KatA

An 8 Kb plasmid was isolated from *Bacillus spp.* under study using miniprep kit according to manufacturer's instructions. PCR amplification was carried out using primers designed for KatA, primers were **P1** (TGAAGTTGGTCTGCTGACAC) and **P2** (GCCGTTATCATCAAAACGC). PCR conditions were as follows: 10xTaq buffer, 1.25 U Taq DNA polymerase, 2mM dNTP mixture, 25 mM MgCl<sub>2</sub>, 0.7 µg DNA, double-distilled water mixed in a final volume of 50 µl. The program for PCR was as follows: 94°C for 3 min, 20 cycles of 94°C for 1 min, 54°C for 45 sec, 72°C for 2 min and 2 cycles of 94°C for 20 sec, 69°C for 30 sec and 72°C for 3 min, and extension at 72°C for 7 min,+4°C .

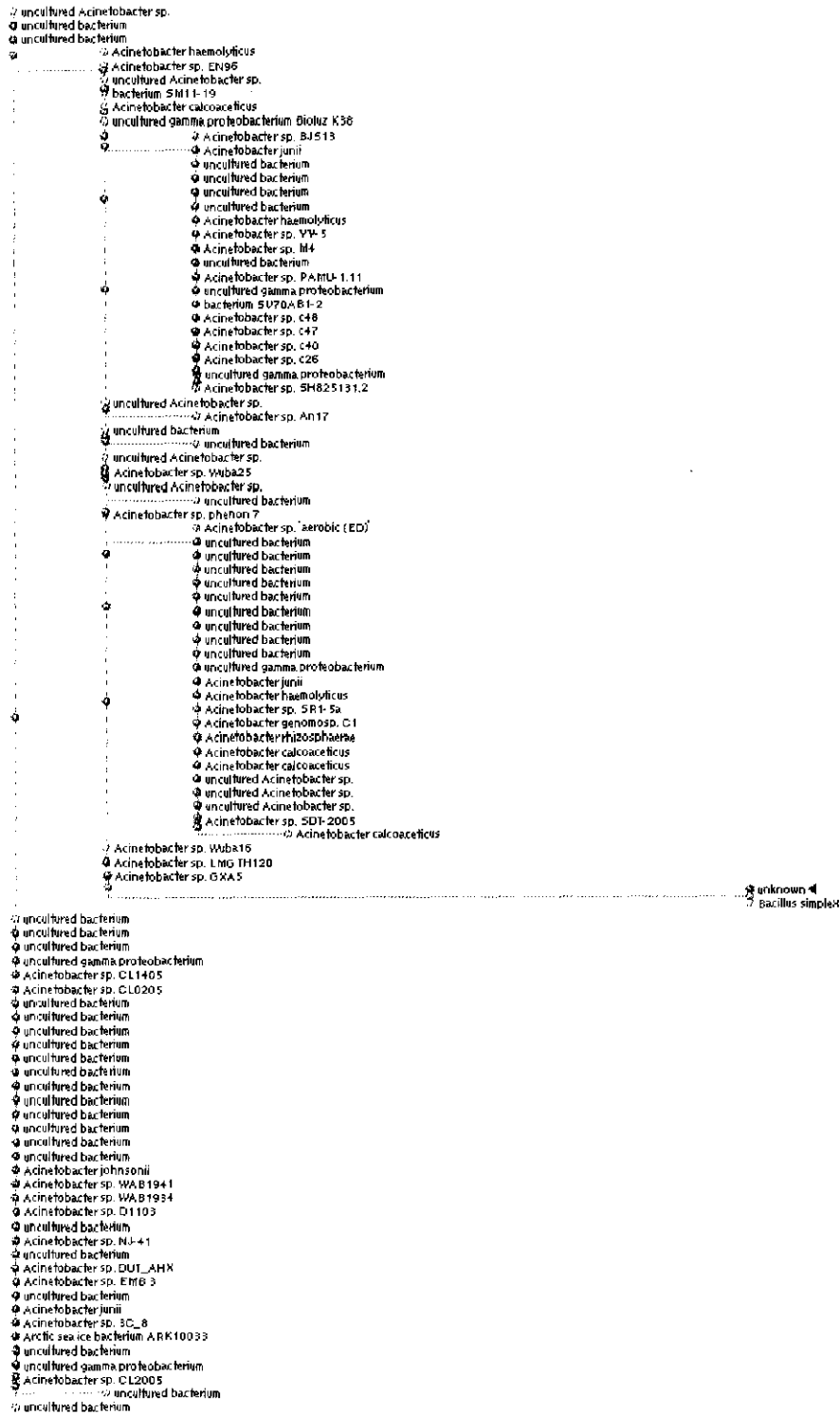
### RESULTS AND DISCUSSION

In biotechnological applications, the key is always the choice of the appropriate microorganism, it is important to select the suitable microorganism to carry out a desired biotechnological process. The isolation of microorganisms from extreme conditions or contaminated sites offers microorganisms with unusual properties and activities. Studies undertaken to examine the identification and characteristics of environmental samples revealed the true diversity of microorganisms and their unique functionality which arise from their biological system that produce enzymes to make them tolerate or adapt to their environments. The use of molecular techniques adds more precision and accuracy to the phylogenetic identification and also to the true reflection of microbial diversity.

An isolate of *Bacillus spp.* obtained from textile waste water was studied for its potential in hydrogen peroxide tolerance in combining

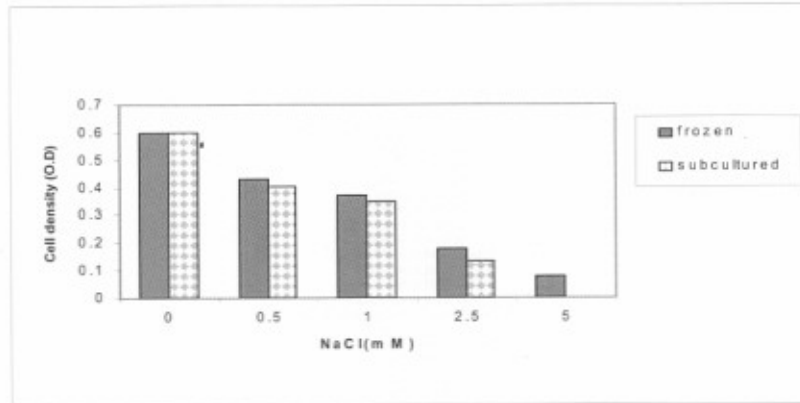
Advanced Oxidation Processes (AOP) and biological processes in textile wastewater treatment (Data not shown). This isolate was further studied to determine its taxonomic position and characteristics; it was phenotypically identified as *Bacillus maroccanus* and was given the description of extremely halotolerant, facultative psychrophilic and facultative alkalophilic, it also exhibited tolerance to hydrogen peroxide up to 442 mM above which it showed no colony forming ability (Gomaa and Momtaz, 2006).

The first part of this study was dedicated to find the precise position of this isolate via phylogenetic identification. DNA-DNA relatedness showed no relevance to *Bacillus subtilis*, but there was closeness of 96% to *Acinetobacter*. Comparisons with *B. thuringensis*, *B.cereus*,*B. anthracis*,*B. subtilis subsp subtilis*, and *B. licheniformis* showed less relation. Comparison to the whole bacterial datpabase showed more relevance to *Acinetobacter* and another relation to *Psychrobacter*, especially *Psychrobacter arcticus* and *Psychrobacter cryohalolent*. It cannot be accepted that the isolate be assigned as any of the Gram negative bacteria such as *Acinetobacter* or *Psychrobacter*, because of other tests done on this strain, but it is plausible that this bacterium, due to its different characteristics, could hold some of the traits found in other strains, especially that this strain is a spore forming bacteria and its known that spores here protective shields against extreme conditions. Therefore, this bacterium was assigned as *Bacillus simplex* TWW-04. *Bacillus maroccanus* is a synonym for *Bacillus simplex* as indicated by Pubmed taxonomists (Fig.1).

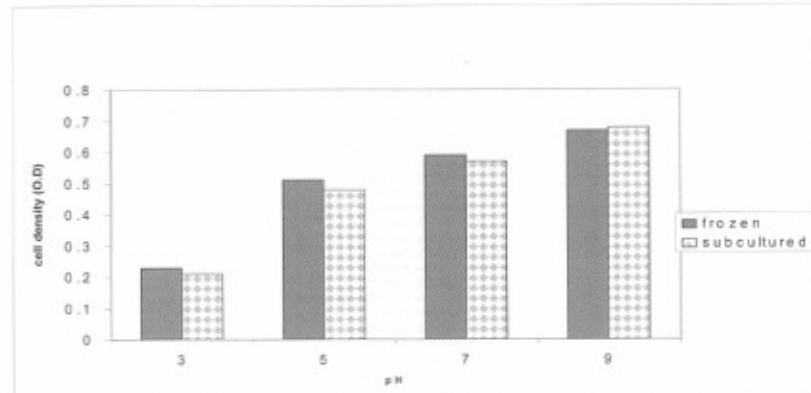


**Fig. (1):** Phylogenetic tree representing the strain under study compared to the whole bacterial database.

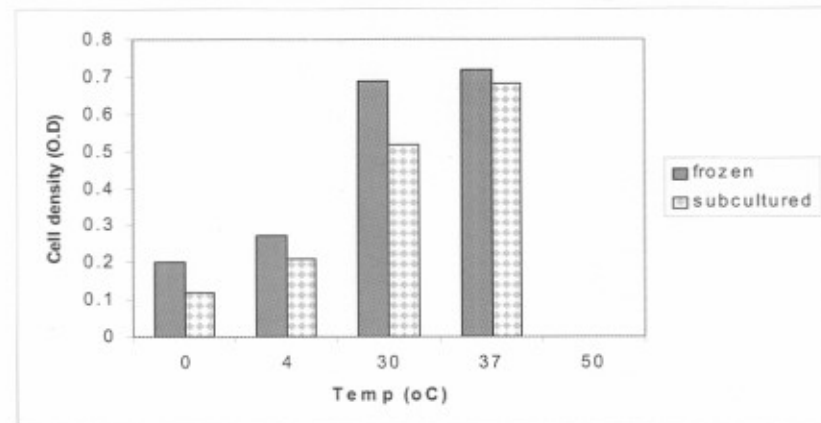
**Fig. (2):** Tolerance to NaCl after 36 month subsequent subculturing of *Bacillus simplex* compared to the frozen sample.



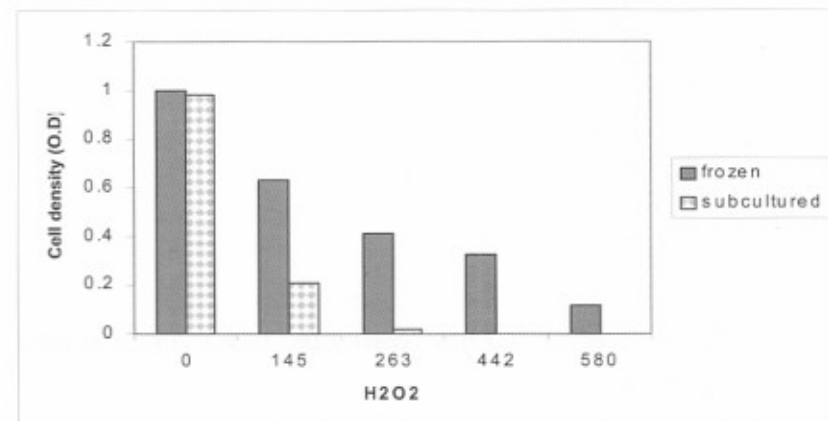
**Fig. (3):** Tolerance of 36 month subcultured to *Bacillus simplex* at different pH compared to the frozen sample.



**Fig. (4):** Tolerance of 36 month subcultured *Bacillus simplex* to different temperatures compared to the frozen sample.



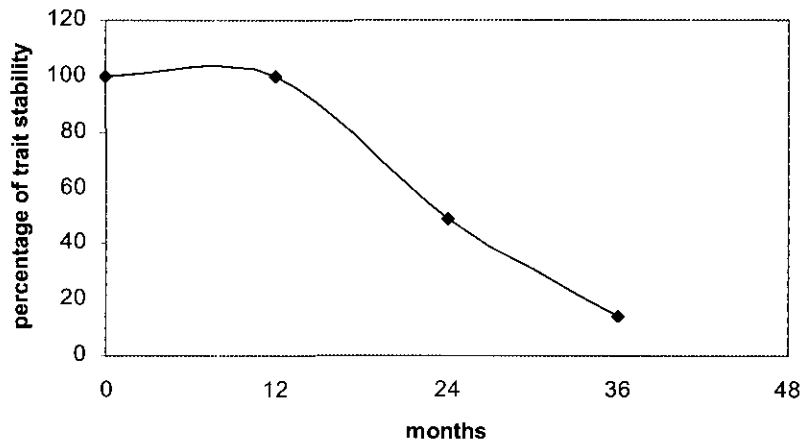
**Fig. (5):** Tolerance to 36 month subcultured *Bacillus simplex* to different hydrogen peroxide concentrations compared to the frozen sample.



The second part of this study was related to the stability and loss of some important traits such as the salinity, pH, temperature and hydrogen peroxide tolerance. Figs. 2-5 show the effect of different temperatures, NaCl, pH and hydrogen peroxide on the subcultured and frozen samples. The results clearly demonstrate the continuation of tolerance for all tested traits, except for hydrogen peroxide. The tolerance profile of this strain showed the production of catalase as the major enzyme responsible for counteracting the damaging effect of hydrogen peroxide on the cells during

the first 2 hours of incubation with hydrogen peroxide, it exhibited high catalase activities as an initial defense against hydrogen peroxide compared to other bacterial strains (Gomaa and Azab, 2007). Over the period of study done on this strain, the cells started to show less tolerance to the hydrogen peroxide, therefore it was decided to study the characteristics of this strain after subsequent subculturing compared to samples that were kept at  $-80^{\circ}\text{C}$  with glycerol for the same period.

**Fig. (6):** The percentage of *Bacillus simplex* tolerance to hydrogen peroxide over 36-month duration of subsequent subculturing for the 263 mM Hydrogen peroxide.



In fact, *Bacillus subtilis* displays an adaptive response to hydrogen peroxide through peroxide induction to a number of genes which encode several enzymes (Herbig and Helmann, 2001). It was therefore suggested that KatA, the gene encoding catalase would be detected in this newly identified strain. KatA was examined in the present study in a 36-month sample after subsequent subculturing and in a 36 month frozen sample. In this study, screening of KatA gene was performed for total DNA and repeated on an isolated plasmid. An 8 Kb plasmid was detected and was used as DNA template in PCR amplification for the KatA

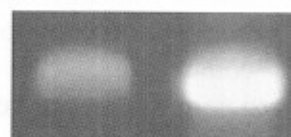
gene, a product was obtained which suggests that KatA gene is located extrachromosomally in this strain. This might explain the difference in band intensity for KatA PCR product for the strain after subculturing and for the frozen sample resulted in a very bright band for the latter when compared to the faint band of the subcultured bacterial sample. This is an indication that subsequent subculturing under non-specific conditions results in decrease in the number of copies of KatA gene. Plate (1) shows the intensity of the signal for the frozen sample indicating that there might be a substantial loss in extrachromosomal DNA fragments, or that KatA is carried on a

transposon which moved freely and was lost after successive subculturing. While for the other characteristics, pH, salinity and temperature, they were chromosomal and were maintained during the period of study. It is known that induction could be attained by manipulating the above characteristics, but in the case of hydrogen peroxide, it was difficult to preserve the strain under hydrogen peroxide inducing conditions, it is known that hydrogen

peroxide is easily broken and its half-life is very short (Hess, 1995).

Fig. (6) shows the changes in tolerance to hydrogen peroxide every 12 months for the subcultured *Bacillus* compared to the frozen sample. As expected, subculturing over a prolonged period resulted in a marked decrease of the trait due to excessive cell division.

*Plate (1): Intensity of KatA gene in 36 month subcultured Bacillus simplex sample compared to frozen sample.*



## CONCLUSIONS

The use of isolates with enzymatic activities of potential advantage and biotechnological applications is considered important now that environmentalists are concerned with the safety which might be endangered by the release of genetically engineered microorganisms (GEMs) in the environment. Studies which target the diversity of microbial communities in contaminated sites or those which highlight their applications in bioremediation or any other biotechnological processes are of constant need due to the ever-increasing problems related to increase in pollution and depletion in natural resources. Partial sequencing of 16SrRNA gene is a good alternative for problematic phenotypic identifications and placing isolates in their right taxonomic position; it seems necessary in the case of environmental samples and is indispensable in ecological studies, to match relatedness of different species, and gain insight to their diversity in characteristics. Also, the stability of the bacterial traits reveals more about their practicality in use in biotechnology, and if classical techniques in

preservation are to be used, especially in developed countries. The screening of environmental microflora should extend to their gene stability prior to their storage by classical techniques.

This work is complementary to a number of existing studies devoted to the use of this bacterium in treatment of textile wastewater as an initial step for removing hydrogen peroxide prior to biodegradation of dyes. Further study of this gene integration in the bacterial chromosome and potentiality of overexpression of this gene is needed.

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### الملخص العربي

## استخدام 16S rRNA للتعريف الجيني لبكتريا الباسيلس المعزولة من مياه صرف مصانع النسيج و دراسة خصائص التحمل بعد الأستزراع المتتالي

\*علا محمد جمعة و \*\* أسامة أحمد ممتاز

\*المركز القومي لبحوث و تكنولوجيا الاشعاع , ٣ ش أحمد الزمر,مدينة نصر  
\*\* معهد الهندسة الوراثية الزراعية,مركز البحوث الزراعية,٩ ش الجامعة,الجيزة

يعد عزل و تعريف الميكروبات من مياه الصرف من الأساليب الهامة لمعرفة أنواع جديدة من الميكروبات ذات القدرة علي تحمل ظروف قاسية و تلوث مرتفع و ذلك نظرا لوجود نظام بيولوجي و جيني خاص فسي هذه الميكروبات. و لهذه الميكروبات استخدامات عديدة في مجالات التكنولوجيا الحيوية و لكن لكي يتم استخدامها يجب أن تكون الصفات الجينية ثابتة. من دراسة سابقة تم عزل بكتريا من مياه صرف مصانع النسيج و كان قد تم تعريفها بالشكل الظاهري (Phenotypic identification) على أنها سلالة *Bacillus maroccanus* و تم دراستها من حيث قدرتها على تحمل بعض الصفات القاسية. في هذا البحث تم دراسة التقسيم البيولوجي الجيني لهذه السلالة باستخدام 16S rRNA partial gene amplification and sequencing و أشار ال DNA-DNA hybridization الى تعريف هذه السلالة على أنها *Bacillus simplex* و مقارنة هذه السلالة في الصفات من سلالة ال *Acinetobacter sp.* بنسبة ٩٦% . و لاستخدام مثل هذه السلالة في مجالات التكنولوجيا الحيوية كان يجب دراسة الثبات الجيني فتم استزراع متتالي على مدي ٣٦ شهر و بدراسة الصفات و وجد أن قدرة السلالة على تحمل الملوحة و الالاس الهيدروجيني pH و درجات الحرارة المنخفضة كانت ثابتة بينما انخفضت صفة تحمل فوق أكسيد الهيدروجين خلال مدة الدراسة و كان الانخفاض ملحوظا بعد ٢٤ شهر من الاستزراع المتتالي و بدراسة الجين المسؤول عن تحمل هذه الصفة في هذه السلالة (Kata) و وجد أنه لا يزال موجود و لكن بنسبة ضئيلة مقارنة بعينة كان قد تم تجميدها عند -٨٠ درجة مئوية مع الجليسرول لنفس المدة (٣٦ شهر) و بذلك دلت الدراسة على ان حفظ السلالة بنظام الاستزراع المتتالي أفقد السلالة الصفة الخاصة بتحمل فوق أكسيد الهيدروجين و ذلك لوجود هذا الجين خارج الكروموسوم البكتيري مما أدى الى فقد الصفة مع تكرار عمليات أنقسام الخلايا بينما كانت الصفات الأخرى محمولة على الحمض النووي الكروموسومي.