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Phenotypic and Molecular Characterization of Rubber Degrading Strain *Streptomyces* Strain E1

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ABSTRACT

Streptomyces sp. strain E1 isolated from soil sample beside the fuel station in Qena governorate, Egypt, this strain able to degraded both synthetic *cis*-1,4-polyisoprene rubber and natural rubber and to be translucent halos (clear zones) forming on latex overly agar plates. The clear zones formation on natural rubber latex appears after 4-5 days at 30 ºC of incubation. It was also shown that able to utilize some isopronoid compounds as a carbon source and energy. The isolate strain E1 was aerobic, Gram-positive, slightly acidophilic that grows at pH 5.5 – 8.5 and non-motile. Taxonomic characterization of this isolate by 16S rDNA analysis showed highest similarities to the 16S rDNA of *Streptomyces setonii* (99.8) which is mesophilic *actenomycetes*. Consequently, bacterial isolate was defined as *Streptomyces sp*. strain E1. The mineralization experiments were confirmed the capability of rubber degradation for isolate strain. Degradation of isoprenoid compounds and related compounds namely, squalane, phytol, squalene, acetonylacetone, geranylacetone, citronellal and citronellic acid was recognized indicating that the isolate strain has the metabolic capability to degraded some isoprenoid compounds as sources of carbon.

INTRODUCTION

Natural rubber (NR) is composed of poly(*cis*-1,4-isoprene) and produce by over 2000 types of plant. It has a high molecular weight (about 10^6 Da). Commercially, produced in huge amounts (10^8 tons /year) from the rubber tree *Hevea brasiliensis*. The huge amount of waste rubber material is becoming problem (Liu *et al*., 2000). Consequently, it necessary to trying develops a microbial process for waste natural rubber disposal (Jang, *et al*., 1998). Many microorganisms have been published to degrade both synthetic and natural rubber (Rook,1955; Linos, *et al*., 2000; Arneskoetter et al,2004; Ibrahim *et al*., 2006; Schulte *et al*., 2008; Broeker and Steinbuechel, 2009 and Ibrahim and El-ameen 2013). According to Linos *et al*., (2000), bacteria capable of natural and synthetic rubber utilization are subdivided into two groups. On the one hand, bacteria grow only in direct contact with rubber material. Representatives of this bacteria show strong growth on natural and synthetic rubber. Member of this subdivision belong to the CMN-group (*Corynebacterium*, *Mycobacterium* and *Nocardia*). On the other hand, several *streptomyces* cause a formation of translucent halos (clear zones) if cultivated on latex overlay agar plates by secretion of one or several enzymes.

In this study, isolate strain that is able to utilize natural rubber latex and synthetic poly(*cis*-1,4-isoprene) as source of carbon was isolated and characterized. Furthermore, the mineralization of bacterium isolate on treated or nontreated natural rubber latex gloves was investigated. The potency of the *Streptomyces sp*. strain E1 to utilize synthetic and natural rubber materials and some isoprenoids compounds and related compounds were studied.

MATERIALS AND METHODS

Culture Medium: Cultivation was carried out in flasks containing mineral salts medium (MS medium) as described by Schlegel *et al*., (1961) and rubber as sole carbon source. The synthetic poly(*cis*-1,4-isoprene) rubber (IR) and natural rubber (NR) latex were added in concentration of 0.5% (w/v). The other carbon sources like hexadecane, glucose, acetate and fructose were added to liquid MS medium in concentration of 0.2% (wt/v), the entire media was autoclaved. Natural rubber latex gloves were added in a small pieces either untreated or after extraction with chloroform or acetone in concentration of 0.5% (w/v). All culture was inoculated with cell obtained from 4-5 days precultured in nutrient broth. For preparation of solid media, 105% (w/v) agar was added to the nutrient solution.

Preparation of rubber latex overlay plates: Natural rubber latex was concentrated and purified by centrifuge at 5500 rpm for 3 min and the cream was resuspended in sterile water to give 5% rubber dry weight latex. To prepare the solid plates, 100 ml of MS medium with 1.5 g agar was autoclaved and then poured in Petri plates. After three hours, solid MS medium plate overlaid with 8 ml of mineral salts medium mixed with sterilized latex (0.2% (v/v) rubber dry weight) resulting in a composition overlay agar plates (Jendrossek *et al*., 1997).

Isolation of clear zone forming rubber degrading bacterium: *streptomyces sp*. strain E1 used in this study was isolated from soil sample beside the fuel station in Qena governorate, Egypt. One gram of soil sample were diluted with 50 ml sterile saline solution and vortexes for 2-3 min. 100 µl of the dilution was spread to the surface of the mineral agar plates supplemented with natural rubber as carbon source for growth and incubated at 30 ºC. Colonies with translucent halos were purified by alternating transfers to latex overlay plates and complex media.

Polymers: In this investigation, three types of poly(*cis*-1,4-isoprene) were used: The first type was synthetic *cis*-1,4-polyisoprene (IR) with molecular weight about 600.000g/mol was obtained from Aldrich. The second type was purified natural rubber latex fro *H. brasiliensis* was a gift from Weber and Schaer (Germany). While, the third type of polymer was NR-latex gloves bought from Roth (Karlsruhe, Germany).

Mineralization of the rubber substrate: Mineralization of all rubber material was determined by a method described by Linos and Steinbuchel, (1998).

Extraction of antimicrobial substances from NR-latex gloves: In order to extract antimicrobial substances from natural rubber latex gloves, the NR-latex gloves were treated with chloroform or acetone according method described by Linos and Steinbuechel, (2000). Mineralization of natural rubber latex gloves and the effect of extract antimicrobial substances with organic solvent by

Streptomyces sp. Strain E1 were determined according to the method described by Linos and Steinbuchel, (1998).

DNA Extraction and determination of 16S rDNA gene:

To prepare genomic DNA from isolated strain cells used method described by Pospiech and Neumann, 1995. PCR-mediated amplification of 16S rDNA genes, using the primers 1525r (5-AGAAAGGAGGTGATCCAGCC -3_) and 27f (5-GAGTTTGATCCTGGCTCAG-3_) and *Taq* DNA polymerase. PCR products were carried out using procedures described by Rainey, *et al*., (1996).

16S rDNA gene sequences and phylogenetic analysis:

Purified PCR products were sequenced using the automatic sequencer (LI-COR 4000L (LI-COR, Alabama, USA). The sequencing reaction was carried out by the use of SequiTherm Long Read Cycle Sequencing kit (Epicenter Technologies, Madison, WI, USA) according to the protocol supplied by the manufacturer. The purified 16S rDNA gene fragment was direct sequenced. DNA sequences were detrmined with the primers, 1525r(5-AGAAAGGAGGT GAT CCAGCC-3), 357f(5-TACGG GAGGCAGCAG -3), 343r(5-CTGCTGCCTCCCGTA-3), 907r(5-CCGTCA ATT CATTTGAGTTT-3), 536f(5-CAGC (C/A)GCCGCG GTAAT(T/A)C-3), 803f(5-ATTAGATA CCCTGGTAG-3), 519r(5-G(T/A)ATTACCGCGGC(T/G) GCTG-3), 1385r (5-CGGTGGTGT(A/G)CAAGGCCC-3), 1114f(5-GCAAC GAGCGCAACCC-3 and 27f(5-GAGTTTGATCCT GGCTCAG-3). The 16S rDNA sequence obtained was aligned manually with published sequences from representative *streptomyces* obtained from EMBL. BlastN was used to determine the percentages of nucleotides identical to 16S rDNA sequences in Genbank databases.

RESULTS AND DISCUSSION

1-Isolation and characterization of rubber degrading bacterium: strain E1 was isolated from soil sample beside fuel station in Qena governorate, Egypt and produced clear zone if cultivated on latex overly agar plates. After 4-5 days incubation, the clear zone occurred on latex overly agar plates. It also able to degraded both synthetic and natural rubber. Cells of strain E1 stained Gram positive, slightly acid fast and non-motile. The bacterium was catalase and oxidase-positive. The E1 strain was able to utilize a lot of isoprenoud compounds as source of carbon such as hexadecane, acetate, glucose, starch, fructose and skim milk (Table 1). The E1 strain grows at 10, 20, 30, and 37 ºC, but appeared no growth at 45 ºC. The almost complete 16S rDNA sequence isolate E1 was sequenced as described in Material and Methods. The 16S rDNA gene sequence strain E1 showed highest similarities to the 16S rDNA gene sequence of *Streptomyces setonii* (Takeuchi *et al*., 1996) and *Streptomyces sp*. VTT (Suutari *et al*., 2002)(99.8% and 99.6% respectively), which is mesophilic *actenomycetes*. Fig. 1 shows the phylogenetic position of isolate strain within the genus *streptomyces* and could be classified among the second group of bacterium that appeared translucent halos on latex overly agar plates (Jendrossek *et al*.,1997 and Linos *et al*., 2000). Since then the isolate E1 has become called *Streptomyces sp*. strain E1.

Table1. Phsiological characterization of *Streptomyces sp.* strain E1.

Characteristic	*Gordonia alkanivorans* strain E1
Gram staining	+
pH range	5.5 - 8.5
LAAP -test	-
KOH -test	-
Spor forming	+
Fructose	+
Glucose	+
Gluconate	+
Na-citrate	+
Na-succinate	+
Hexadecane	+
Na-acetate	+
Pentane	-
Hexane	-
Skim milk	+
Casein	-

Growth was qualitatively estimated as follows:(+) growth; (-) no growth

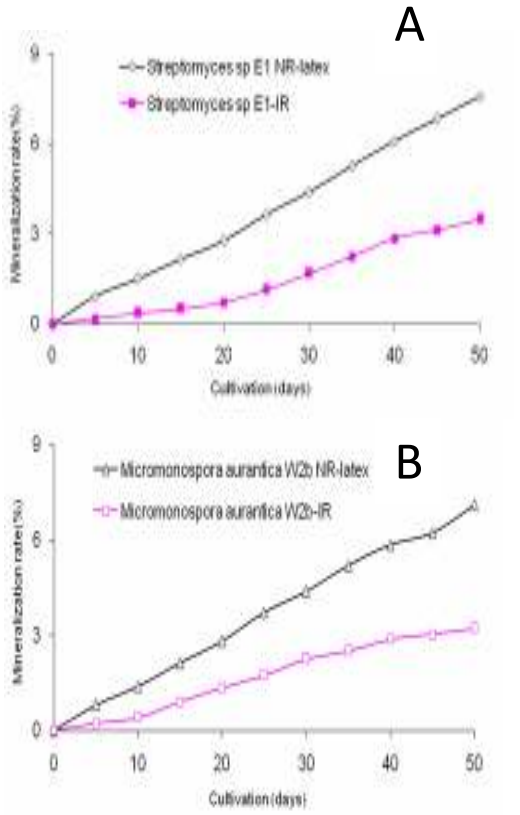


Fig. 1. Mineralization of natural rubber latex and synthetic *cis*-1,4- polyisoprene. (A) *Streptomces sp* strain E1 and (B) *Micromonospora aurantica* W2b

2-Mineralization of Natural and synthetic Rubber: The capability of *Streptomyces sp*. strain E1 to utilized both synthetic *cis*-1,4- polyisoprene rubber and natural rubber latex was investigated in this experiment. Determination was showed in Material and Methods. The results showed in(Fig. 2A) Appear that *Streptomyces sp*. strain E1 were able to mineralize natural rubber latex and synthetic poly(*cis*-1,4-isoprene) demonstrating the metabolic conversion of polymer to carbon dioxide. They were compared to the mineralization values formally obtained from *Micromonospora aurantica* W2b which classified as rubber degrading bacterium belongs to the translucent halos growing group (Linos and Steinbuechel, 2000). As expected, the *cis*-1,4- polyisoprene contained in natural rubber latex served as a more favorable carbon source than

synthetic *ces*-1,4- polyisoprene as indicated by higher mineralization values obtained, which might be due to the fact that the main composition of natural rubber latex consists of about 90% of poly(*cis*-1,4-isoprene)(Mw 10^6 Da) and about 10% of non rubber materials (Subramaniam, 1995). It was appeared that the mineralization level of natural rubber latex by *Streptomyces* sp. strain E1 and *Micromonospora aurantica* W2b was approximately the same (Fig 2B). However, *Streptomyces sp*. strain E1 mineralized about 3.11% (w/w) of synthetic poly(*cis*-1,4-isoprene) and about 7.51% of poly(*cis*-1,4-isoprene) contained in natural rubber latex after 50 days at 30 ºC. Similarly rang was obtained by values mineralization rates with cultures of clear zone forming bacterial strains (Jendrossek *et al*., 1997; Gallert, 2000 and Rose *et al*., 2005). Finally, isolate strain belong to the translucent halos forming group and metabolize the polyisoprene by secretion of one or several enzymes. In comparison to adhesive growing group, this group show relatively weak growth on natural rubber and synthetic *cis*-1,4-polyisoprene rubber (Jendrossek et al., 1997)

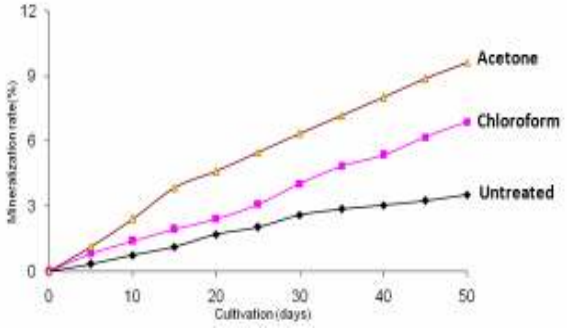
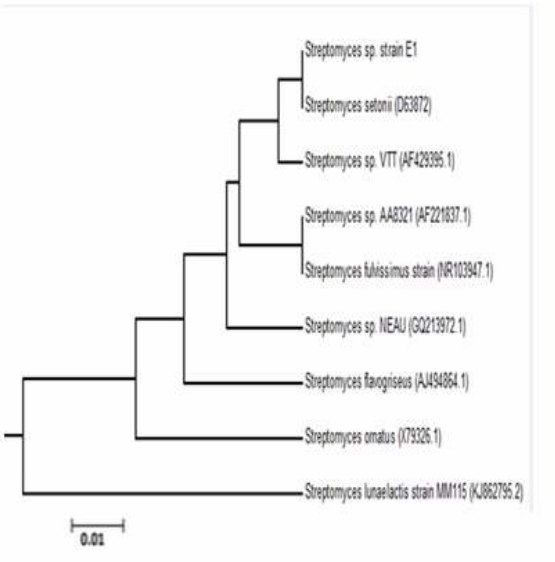


Fig. 2. Mineralization of natural rubber latex gloves by *Streptomyces sp* strain E1.

Mineralization of treated and untreated natural rubber latex gloves: Manufacture of rubber products from synthetic rubber (IR) and raw natural rubber are usually accompanied by the addition of compounding ingredients. A lot of these materials are known to prevent microbial growth (Zyska, 1981). No information about these ingredients in natural rubber latex gloves was allowed from the manufactures. Interestingly, extractions of natural rubber gloves show the possibility to improve the biodegradation process with respect to potential biotechnological treatment of the rubber wastes. Beside the potency of *Streptomyces sp* strain E1 to mineralize synthetic poly(*cis*-1,4-isoprene) rubber and natural rubber latex, the degradation of vulcanized rubber products such as natural rubber latex gloves was investigated. In addition to the degradation of natural rubber latex gloves after removal of additives by extraction with organic solvents such as chloroform or acetone was also investigated. For this proposal, the capacity of the *Streptomyces sp*. strain E1 to degrade natural rubber gloves was described in Materials and Methods by measuring the CO_2 released during growth on untreated or treated natural rubber latex gloves as a source of carbon and energy. Results appeared in Fig. 2 demonstrated the mineralization of natural rubber latex gloves after treatment of the glove material with chloroform or acetone. This treatment led to increases of mineralization of rubber expressed by carbon dioxide released during growth two to three times. Fig. 3 showed that the strain recorded 3.53, 6.89 and 9.64% CO_2 release during growth on untreated or treated with chloroform or acetone respectively. The extraction of antimicrobial



materials from natural rubber gloves was also done by Tsuchii *et al*., (1996) and Ibrahim and El-ameen (2013) in order to remove chemicals with microbicidal activities.

Fig. 3. Phylogenetic dendrogram, obtained by distance-matrix analysis, showing the position of *Streptomyces sp.* strain E1 among members of the genus *Streptomyces*. Bar, 0.01 substitutions per nucleotide position.

Degradation of structurally analogous isoprenoid compounds: To study the capability of *streptomyces sp*. strain E1 to utilized structurally analogous isoprenoid compounds and other related material was studied. The results presented in Table 2 showed that the keto and acidic forms of the isoprenoids compounds such as geranylacetone, citronellic acid, acetonylacetone, and geranic acid were easily utilized by Streptomyces sp. strain E1. Similarly, good growth on saturated-branched hydrocarbons (squalane) and moderate growth on unsaturated-branched isoprenoid compounds squalene and phytol was also recorded. In the contrast, no growth was appearing when Citronellal was used as carbon source for growth. Berekaa and Steinbuechel (2000) investigated the analyzing of similar compounds by rubber degrading bacterium Mycobacterium fortuitum NF4. Finally, all this results showed that the isolate strain has the metabolic capability to degrade some isoprenoid compounds as carbon source for growth.

Table 2. Growth of *Streptomyces sp.* strain E1 on isoprenoid compounds and related compounds.

Compound tested	Growth of *Gordonia alkanivorans* strain E1 on the respective carbon source
Squalene	++
Squalane	+++
Geranylacetone	+++
citronellic acid	+++
Geranlic acid	+++
Phytol	++
Acetonylacetone	+++
Citronellal	-

Growth was qualitatively estimated as follows: (-) no growth, (++) moderate growth and (+++) good growth

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التوصيف المظهرى والوراثى لسلالة *Streptomyces sp.* Strain E1 ذات المقدرة على تحليل المطاط

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تم عزل *Streptomyces sp.* Strain E1 ذات المقدرة على تحليل كلا المطاط الطبيعى والصناعى ولها المقدرة على تكوين هالة حول المستعمرات عند نموها على اطباق بترى تحتوى على بيئة الحد الادنى مضاف لها المطاط الطبيعى وتظهر الهالة حول المستعمرات بعد 3-5 أيام. ومن خصائص هذة السلالة بأنها تنمو هوائيا وموجبة الجرام وتستطيع ان تنمو فى بيئة حامضية Ph لها من 5,5 – 8,5 وهذه السلالة ليس لها المقدرة على الحركة ولها المقدرة على تكوين جراثيم لونها اصفر رمادى ومتصلة مع بعضها فى صورة سلاسل . والتوصيف الوراثى لهذه البكتيريا بواسطة تحليل الـ 16S rDNA أوضح انها على درجة عالية من التشابة مع بكتيريا *Streptomyces setonii* (99,8%) لذلك سوف نطلق عليها *Streptomyces sp.* Strain E1 . ومقدرة هذه البكتيريا على تحليل المطاط تم دراستة بواسطة تجارب الـ Mineralization على المطاط الطبيعى والصناعى. كما أظهرت السلالة ان لها المقدرة على تحليل بعض مركبات الايزوبرونيد والمركبات المشابهة لها تركيبيا مثل الاسكوالان والاسكوالين الاسيتيل اسيتون والجرانيول اسيتون والفيتولواستخدامها لتلك المركبات كمصدر للكربون والطاقة.