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# Biodegradation of Natural Rubber Latex of *Calotropis procera* by Two Endophytic Fungal Species

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**Abstract**

In this study eight species related to *Aspergillus* (3 species), *Fusarium* (1), *Penicillium* (1), *Emericella* (1), *Nigrospora* (1) and *trichoderma* (1) were isolated from leaves and latex of *Calotropis procera*. Only *P. chrysogenium* and *A. niger* were able to grow on natural rubber but other species were not. The degrading ability of (*Penicillium chrysogenum* and *Aspergillus niger*), isolated from latex of *calotropis procera* was assessed. The degradation of rubber latex was determined by measuring the increase in protein content of the fungus (mg/g dry wt), reduction in molecular weight (g/mol) and inherent viscosity (dl/g) of the latex. Moreover, the degradation was also confirmed by observing the growth of these species strain using scanning electron microscopy.

**Keywords:** *Calotropis procera*; *Penicillium chrysogenum*; *Aspergillus niger*; Biodegradation; Natural rubber; Endophytic fungi

## Introduction

*Calotropis* is a small genus belonging to family Asclepiadaceae, *Calotropis gigantea* and *C. procera* are the two most common species in this genus used in traditional medicine, and only these two species are usually reported in literature [1], and are distributed in tropical and subtropical Africa, Asia, and America [2-5].

*Calotropis procera* (Ait) WT Aiton is a small wild-growing tropical plant, known by different names like sodom apple, usher, dead sea apple, swallow wort, and milk weed [6,7]. All parts of this plant have the ability to produce large quantities of latex when cut or broken [7-9].

The plant latex could be separated after centrifugation into three layers: top layer containing Natural Rubber; middle layer of serum, and the bottom layer of lipids [9,10]. The chemical composition of latex is very complex, it contains (wt/wt) natural rubber poly (cis-1, 4-isoprene) 25 to 35%, which consider the main constituent of Natural rubber latex, and it is consisting of isoprene units $C_5H_8$ in the cis-configuration, a highly unsaturated hydrocarbon, with an average molecular weight about $10^6$ Da [11-13].

Natural Rubber is a basic material for manufacturing tires, latex gloves, condoms, seals, balloons, balls for sports and many other things [14]. Degradation potential of the microorganism is dependent upon colonization on natural rubber and accompanied by a loss in the weight of the rubber hydrocarbon and a decline in the relative viscosity of the polymer solution [13].

Many other reports have been published on the biodegradation of natural rubber hydrocarbon as a sole carbon source by bacteria [15-22], fungi [13,23,24]. Several microbes were used to biodegrade *Calotropis Procera* latex [25,26].

The aim of this work is to assess the ability of mesophilic endophytic fungi isolated from *Calotropis procera* leaves and latex on sugar-free Czapek agar and to test their ability to metabolize and degrade rubber latex of *C. procera* as a sole carbon source.

## Materials and Methods

### Sampling, fractionation and preparation of natural rubber latex

The latex of *Calotropis procera* plant was obtained by taping method. Fresh latex was centrifuged at 17,000 rpm for 20 min at 4°C in SR4000 Prolabo centrifuge (France). latex was separated into three layers: a sticky top layer containing Natural Rubber; clear middle fraction of serum, and a small quantity of lipids at the bottom of the tube (Figure 1).

The natural rubber fraction was separated and washed three times by deionized water to remove impurities and dried at 30°C for 24 h, A known weight (20 mg) of the prepared natural rubber was dissolved in 2 ml of tetrahydrofuran (THF), then its molecular weight was determined by gel permeation chromatography (GPC) [9,13].

Isolation of endophytic fungi from *C. procera* leaves and latex, calculation of colonizing frequency and Identification of endophytic fungi were done by the current authors according to these papers [13,21,22].

### Methods used for measuring the capabilities of endophytic fungi to degrade pure natural rubber Growing of isolates on solid media coated with natural rubber film for 15 days of incubation

After preparation of pure natural rubber (NR), It was dissolved

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in tetrahydrofuran (THF) (20 mg/2 ml THF), and spotted on to agar media (each 50 µl/colony), then left in an oven at 30°C until THF is evaporated and the NR became white in appearance. Fungi were inoculated with tooth pick on the top of pure natural rubber (NR) spot, incubated for 15 days at 28°C. The diameters of colonies were measured, then fungi which grow well in solid media Growing of in liquid media for measuring biodegradation ability after 30 days. At the end of incubation period the rubber pieces and microorganisms were separated by centrifuging at 10000 rpm for 15 min, microorganisms (bottom layer) were subjected for protein determination while natural rubber pieces (top layer) were collected, washed with deionized water, and dried at 30°C and then subjected for analytical methods (molecular weight and viscosity) as well as SEM photography has been described before [13,22].

### Protein determination

The total protein pellet was determined according to the method adopted by Lowry et al. [27].

### Molecular weight determination by Gel Permeation Chromatography (GPC)

A known weight of natural rubber was dissolved in 2 ml THF and passed through a 0.45 µm-pore size filter. Gel permeation chromatogram of the the model Agilent technologies 1100, Germany (present in National Research Center, Doki, Cairo) was used. THF was used as the eluent with a flow rate 1 mL / min. Polymethylmethacrylate and polystyrene standards were used to calibrate the columns. The refractive index detector $10^4$ A°, Colum PL gel Micrometer 100; 10000; 100000. The GPC apparatus was run under the following conditions: flow rate=2.000 ml/min., injection volume=100.000 µl, sample concentration=1.000 g/l. The values of molecular weight were calculated by means of a computer program [13,22].

### Scanning Electron Microscopy (SEM)

The morphological changes during growth of the organism on natural rubber was also studied by scanning electron microscopy. The inoculated samples as well as the control were fixed overnight in glutaraldehyde 5%, then dried at 50°C. The samples were mounted on metal stubs and coated with gold and palladium (Jeol JFC1100E Iosputtering Device). Micrographs were taken with a Joel JSM- 4500 LV electron microscope operating at 15 KV in Assiut University, electron Microscopy unit [13,22].

## Results and Discussion

### Isolation and identification of fungi

Six hundred and seventy-five segments of leaf and 15 latex sample of *C. procera* were processed for the isolation of endophytic fungi. Eight species related to 6 genera were isolated and these were *Aspergillus niger, A. fumigatus, A. flavus, Emericella nidulans, Fusarium oxysporum, Nigrospora oryzae, Penicillium chrysogenum* and *Trichoderma harzianum*. Isolation of endophytic fungi from *C. procera* leaves and latex, calculation of colonizing frequency and Identification of endophytic fungi were done by the current authors (data not shown).

### Growth of mesophilic endophytic fungi on solid media coated with natural rubber film after 15 days of incubation

Eight isolates (representative for the species isolated) were tested for their ability of growing on sugar-free Czapek agar coated with thin film of pure natural rubber (3 replicate agar plates for each isolate). Only 2 isolates related to *Penicillium chrysogenum* and *Aspergillus niger* could grow on pure natural rubber film (Table 1 and Figure 2), while *Emericella nidulans*, *A. fumigatus*, *A. flavus*, *Nigrospora oryzae*, *fusarium oxysporum* and *Trichoderma harzianum* showed only growth on sugar-free Czapek agar supplemented with crude latex [28-32].

### Biodegradation abilities of *Penicillium chrysogenum* and *Aspergillus niger* grown in liquid media after 15 and 30 days of incubation

**Fungal protein, viscosity and molecular weight of *P. chrysogenum*:** Protein content of *P. chrysogenum* grown on natural rubber increased significantly from 2.71 mg/g mycelial dry weight after 15 days to 5.23 mg/g after 30 days of incubation in liquid Cz medium. On the other hand rubber viscosity decreased significantly from 6.0 dl/g after 15 days to 5.5 dl/g after 30 days of incubation.

Molecular weight of natural rubber (that was previously inoculated with *P. chrysogenum*), decreased from $8.08 \times 10^4$ (in control medium) to $4.88 \times 10^4$ g/mol after 30 days (degradation rate of *P. chrysogenum* after 30 days=40.0% comparable with the control (Table 1; Figure 3).

**Fungal protein, rubber viscosity and molecular weight of *A. niger*:** Protein content for *A. niger* grown on natural rubber increased significantly from 1.71 mg/g mycelial dry weight after 15 days to 4.22 mg/g after 30 days. On the other hand rubber viscosity decreased significantly from 6.2 dl/g after 15 days to 5.9 dl/g after 30 days of incubation.

Molecular weight of separated natural rubber inoculated with *A. niger*, decreased from $8.08 \times 10^4$ to $5.82 \times 10^4$ g/mol after 30 days (degradation rate of *A. niger* after 30 days=28.0% comparable with the control (Table 1, Figure 3).

1. Values in brackets represent percentage of degradation rate of natural rubber by the endophytic fungus in case of viscosity and molecular weight after 30 days.
2. Figures in the table are means of three replicates ± standard deviation.
3. *Values Significant at PC 0.05 level.

In this respect, Ismail et al. [13], showed high degradation rate of poly (cis-1, 4-isoprene) rubber (Figure 4) by *Aspergillus terreus* 31.04% and *A. flavus* 28.73% after 30 days and comparable with the control. The ability was also determined by measuring the increase in protein content of each fungus, reduction in molecular weight and inherent viscosity and observing the growth using scanning electron microscopy (SEM).

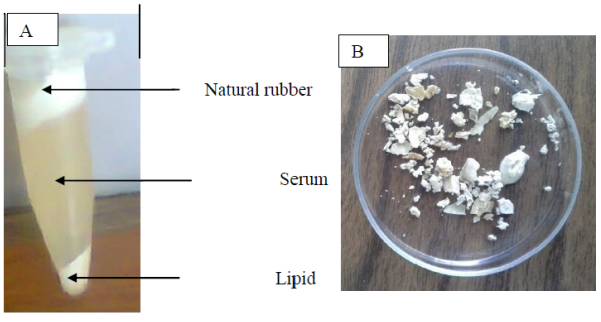


**Figure 1:** Fractionation's of *C. procera* latex: Natural rubber fraction (A), and after preparation (B).

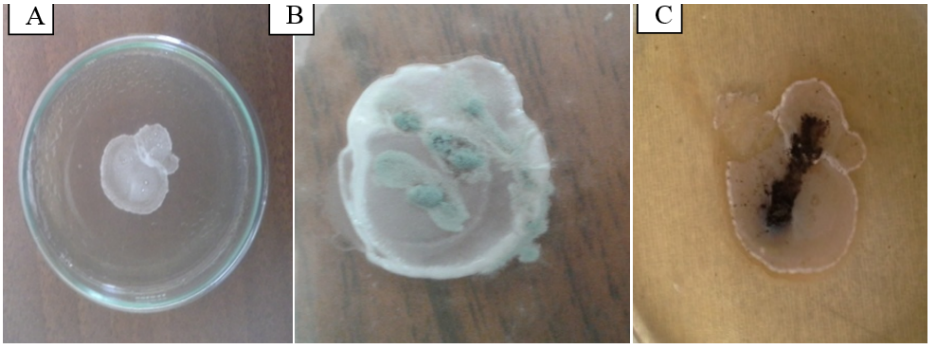


**Figure 2:** Film of pure natural rubber (A), *P. chrysogenum* and *A. niger* on NR (B, C), respectively.

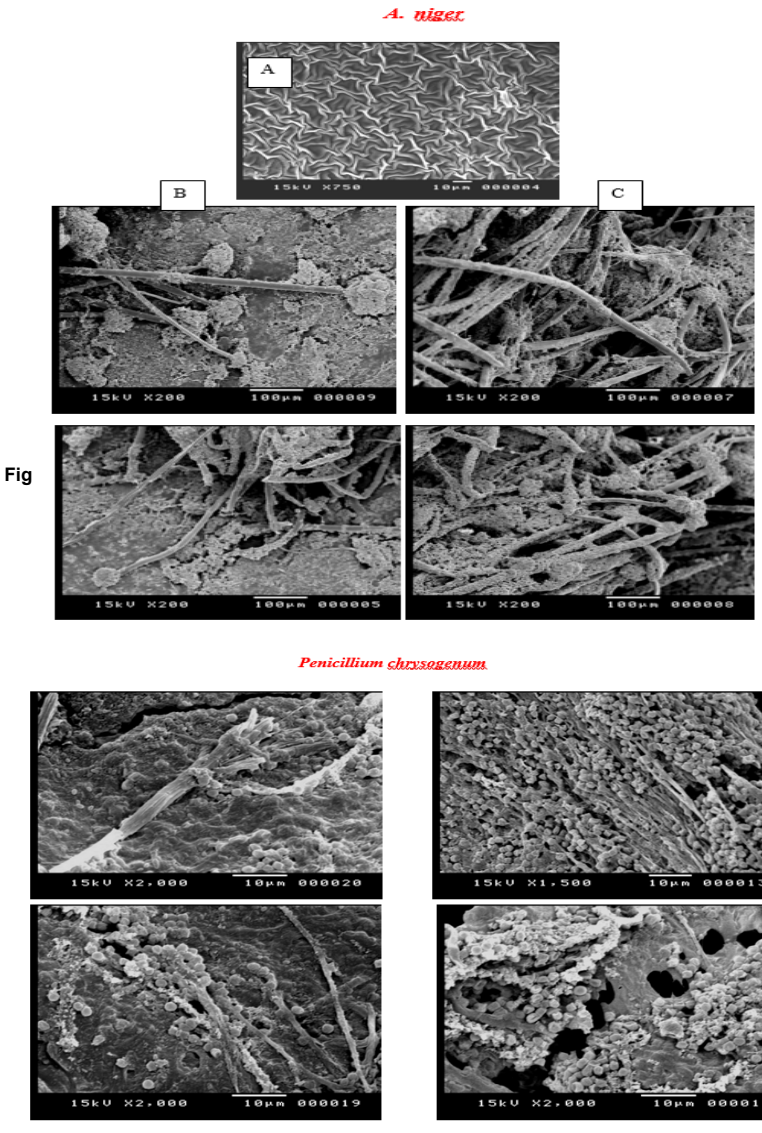


**Fig** ely.

**Figure 3:** Degradation of *Calotropis* latex rubber (20 mg/25 ml Cz small medium under shaking 150 rpm at 25°C for the endophytic fungi, *A. niger* and *P. chrysogenum* as determined by Scanning Electron Microscope (SEM): uninoculated rubber (A), inoculated rubber latex after 15 days (B) and 30 days (C) with *A. niger* or *P. chrysogenum*.

Roy et al. [17] stated also that significant decrease in the molecular weight of the natural rubber was treated with *Aspergillus* sp. after 20 weeks (MW $2.70 \times 10^5$ in the control to $1.90 \times 10^4$). Indicating that *Aspergillus* sp. was able to degrade the natural rubber.

Also Nayanashree and Thippeswamy [24] found that *Aspergillus niger* and *Penicillium* species effectively degraded *Hevea brasiliensis* rubber (Figure 4).

## Scanning Electron Microscopy (SEM)

The 15 and 30 days old inoculated natural rubber samples with *P. chrysogenum* and *A. niger* were photographed using scanning electron microscopy (SEM). These SEM microphotographs clearly indicate the colonization of the fungus on the natural rubber surface (Figure 3). In this respect, the colonization and/or growth on the rubber surface using by SEM micrographs have been observed for *Aspergillus terreus*, *Aspergillus flavus* and *Myceliophthora thermophila* [13], *Aspergillus* sp. and *Pseudomonas* sp. [17].

## Conclusion

The degradation process of natural rubber of *C. procera* by *P. chrysogenum* and *A. niger* were proven by the increase in protein content, decrease in both molecular weight and inherent viscosity, and by observing the fungal growth on the surface of natural rubber using scanning electron microscopy (SEM). The current results demonstrate that the degrading fungal isolates could be used as promising tools for the removal of disposal of rubber products.

| Analysis parameters | Growth diameter (cm) | Protein content mg/g dry wt. | | Viscosity measurements dl/g | | Molecular weight g/mol × $10^4$ |
|---|---|---|---|---|---|---|
| | | X ± SD | | X ± SD | | |
| **Days of incubation** | 15 | 15 | 30 | 15 | 30 | 30 |
| **Control (natural rubber)** | | 0 | | 6.3 ± 0.1 | | 8.08 |
| ***P.chrysogenum*** | 2.83 ± 0.1 | 2.71* ± 0.1 | 5.23* ± 0.1 | 6.0 ± 0.10 | 5.5* ± 0.1 | 4.88* (40.0) |
| ***A.niger*** | 2.3 ± 0.1 | 1.71* ± 0.1 | 4.22* ± 0.1 | 6.2 ± 0.20 | 5.9* ± 0.2 | 5.82* (28.0) |

**Table 1:** Degradation of *C. procera* rubber fraction by *P. chrysogenum* and *A. niger* through determining protein content (mg/g mycelial dry wt); viscosity (dl/g) and molecular weight (g/mol) after shaking incubation for 15 and 30 days at 25°C respectively.

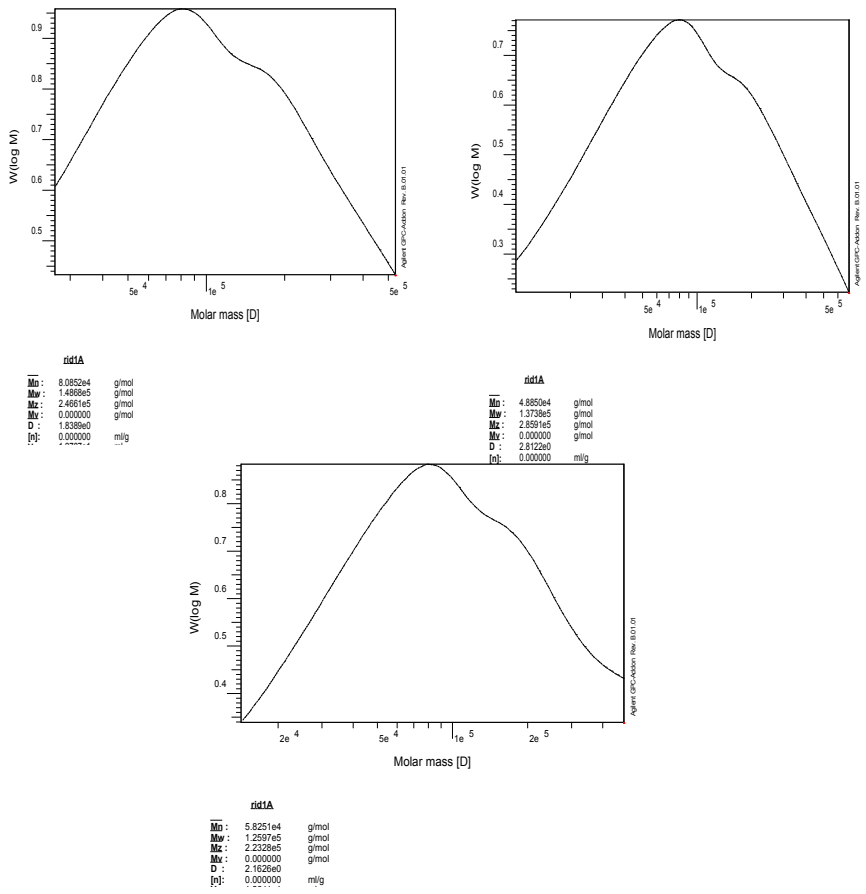


**Figure 4:** Molecular weight of natural rubber of *C. procera* (A), natural rubber after treated with *P. chrysogenum* and *A. niger* (B, C).

## Conflict of Interest Statement

We declare that we have no conflict of interest.

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