# Coumarin degrading microorganisms isolated from Egyptian soil

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## 1. Abstract

Coumarins, an old class of compounds, are naturally occurring benzopyrene derivatives. A lot of coumarins have been identified from both natural and synthetic sources. Coumarins have recently attracted intense interest because of their diverse pharmacological properties. However their cytotoxicity and accumulation in the environment have become a controversial and limiting issue. Biodegradation seems as an attractive solution to reduce their toxicity or promote safer derivatives for medical applications. In our study, coumarin degrading bacteria were screened using Minimal salt media with coumarin as a sole carbon source. A total of 23 coumarin decomposing strains were isolated from 41 soil samples previously sprayed with herbicides using 0.1% coumarin. The bacterial isolate *Pseudomonas fluorescens* SZ1 showed the best growth when tested against higher coumarin concentrations (0.1%-0.5%) and was furthermore identified using morphological and biochemical tests.. The bacterial isolate SZ1 was identified as a *Pseudomonas fluorescens* strain and assumed to be a potential strong coumarin degrading strain which may be used in further industrial and medical applications. In conclusion, the aim of the study was to screen potential coumarin degrading bacteria as a biological solution to biodegrade toxic coumarin derivatives that accumulate in the environment.

**Key words:** *Biodegradation, Coumarin, Biochemical identification, Pseudomonas fluorescens.*

## 2. Introduction

Coumarin and its derivatives are toxic polycyclic aromatic compounds from lactone class containing a benzene ring fused to the α-pyrone ring and chemically described as C9H6O2 [1, 2] . Coumarins naturally occur in higher plants such as Rutales, Asterales, Fabales, and Oleales and within plants tissue including seeds, fruits, roots, leaves and are highly concentrated in fruits and flowers [3]with their residues transferred to the soil and persist for a long time[4]. Few microorganisms (bacteria and fungi) also produce coumarin derivatives as secondary metabolites; Aflatoxins are toxic fungal bisfuranocoumarin derivatives produced mainly by toxigenic *Aspergillus flavus* and *Aspergillus parasiticus* strains and contaminate food and feed resulting in huge worldwide economic losses every year [5, 6]. Similarly *Penicillium nalgiovense*, a dominant fungus associated with contamination of hard, semi-hard, and semi-soft cheese produces five toxic isocoumarins; dichlorodiaportin, diaportinol, diaportinic acid, Citreoisocoumarin, and 6-methyl-citreoisocoumarin[7, 8]. However, the novel aminocoumarin simocyclinone D8 was isolated from bacterial cultures of *Streptomyces antibioticus Tii 6040* with

both antimicrobial and cytotoxic activities [9].

Moreover , numerous man made compounds such as fertilizers, paints, sprays, industrial solvents, dyes, pesticides, fragrances, herbicides even food coloring materials and alcoholic beverages and other polycyclic aromatic compounds are coumarin based and extensively contaminate the environment with hazardous effects to both humans and animals due to their persistence and accumulation in natural ecosystems[10, 11]. Coal tar, for example, is considered a primary source for many aromatic compounds including coumarins and about 15 million tons are produced annually causing massive environmental pollution [12-14]. The usage of coumarins as food flavors was also banned from the USA in 1954 based on hepatotoxic reports on both rats and dogs fed coumarin in their diet[15, 16].Recently coumarins have been used in therapeutic applications as they possess anti-inflammatory, antioxidant, anti-allergic, and anticarcinogenic properties[17, 18]but few papers highlighted their cytotoxic effects . So though coumarins are natural compounds with sweetish odor and used in multiple industrial and pharmacological products as mentioned earlier, their toxicity is still a controversial issue and several solutions and validations were proposed[4, 19]

Biodegradation has become a popular eco-friendly solution to remove organic pollutants from contaminated sites as bacteria developed several strategies to degrade, transform and even mineralize numerous organic compounds of both natural and synthetic origin[20].

To this date there are only few studies reporting decomposition of coumarin using microorganisms though coumarin exposure is very common on daily basis; *Cunninghamella elegans NRRL 1392* fungus was reported to efficiently metabolize coumarin into umbelliferone, 3, 4-dihydrocoumarin and transcinnamic acid[21] also similarly *Glomerella cingulate* was reported to metabolize coumarin into its corresponding acid (hydrocoumaric acid) [21], also an *Arthrobacter spp.* was also reported to utilize coumarin as a sole carbon source [22]. Several microorganisms such as *Mycobacterium spp.[*23],*Bacillus subtilis BUM*[24]and *Stenotrophomonas maltophilia*[25]were reported to degrade benzopyrenes but not specifically coumarins. Our study aims to screen soil samples for potential coumarin degraders and identify them [30, 29].

## 3. Materials and Methods

Unless otherwise specified, all experiments were conducted under aseptic conditions and in triplicate .

### 3.1 Reagents and media

Coumarin was purchased from AVI-CHEM laboratories (Mumbai, India). Coumarin medium (CM) contained 2 g L-1 (NH4)2SO4, 0.2gL-1 MgSO4•7H2O, 1.5 g L-1 Na2HPO4•12H2O, 0.001 g L-1 FeSO4•7H2O, 0.01 g L-1CaCl2•2H2O and 1.5gL-1 KH2PO4 plus variable coumarin concentrations in distilled water. The pH of the medium was adjusted to 7.All reagents and bacteriological media were purchased either from Oxoid or Hi-media-India.

### 3.2 Samples collection

A total of 41environmental samples were collected from various contaminated sites in Egypt. These samples were soil samples previously sprayed with herbicides and insecticides collected from three Governorates (Giza, Cairo and Sharikya). They were collected in sterile plastic bags and preserved in a cool box till transferred to the laboratory to be cultured on media.

### 3.3 Screening for coumarin degrading microorganisms

Microorganisms were isolated from soil using coumarin as the sole carbon source in the media as reported by Guan and his

colleagues[6] with slight modification. One gram of each sample was transferred to 50 mL of nutrient broth medium (pH 7.0) for 24 h at 30 ∘C. Then after centrifugation (14 000 × g for 10 min), the pellet obtained was incubated in 50 mL of CM (0.01% coumarin) (pH 7.0) at 30 ∘C. After a week, the strains that could grow in CM were further inoculated in fresh CM (pH 7.0) for rescreening; this procedure was repeated three times. To obtain purified strains, the liquid culture (0.3 mL) was spread on CM plates and incubated at 30 ∘C until visible colonies appeared[26]. The successful candidates showing growth on 0.01 % CM were tested against higher levels of coumarin using gradual increase in concentrations from 0.05%, 0.1%, 0.2%, and 0.3% to 0.5%.

### 3.4 Morphological, cultural and biochemical characterization of bacterial isolate SZ1 strain

The bacterial isolate was studied for it's colony morphology, Gram staining, pigment production, spore production, cultural and biochemical properties according to Bergey's Manual of Determinative Bacteriology [27] (Holt et al., 1994).

#### 3.4.1 Morpho-cultural characterization of bacterial isolate SZ1

Isolate SZ1 was inoculated on nutrient agar (NA) for preliminary morphological identification which included colony morphology, Gram staining, and motility testing. Potassium hydroxide test (3% KOH test) is a simpler method than Gram stain to identify Gram- negative bacteria as they produce viscous strings using 3% KOH on the bottom end of loop. Subsequently, cultural characterization of bacterial isolate SZ1 using several selective media such as MacConkey agar, Pseudomonas base agar and Kings B medium were used as the isolate was suspected as Pseudomonas spp. Florescent pigmentation test was performed using sterilized Kings B medium and the isolate SZ1was incubated for five days and observed. Bluish green fluorescent pigment observed under UV light (365 nm) indicated positive results. Otherwise mentioned incubation of the isolate SZ1 on all media plates was at 37 °C for 24h.

#### 3.4.2 Biochemical characterization of *Pseudomonas fluorescens SZ1*

Different biochemical tests were performed for characterization and identification of bacterial isolate. Different biochemical tests such as oxidase test, Catalase test, Starch hydrolysis, Triple sugar iron agar (TSI), citrate test, gelatin hydrolysis , nitrate reduction test, Indole production, Methyl red test (MR test) and Voges-Proskauer test (VP Test) were conducted to identify the selected isolate.

## 4. Results

### 4.1 Screening for coumarin degrading microorganisms

23 bacterial strains were isolated from 41 soil samples using coumarin media (0.01%) after incubation at 30 °C for 7 days. Only one isolate (SZ1) grew in all tested concentrations of CM (0.01%-0.5%) while ten isolates showed growth on 0.2% CM and only 8 on 0.3% CM (**Table 1**)

### 4.2 Morphological, cultural and biochemical characterization of bacterial isolate SZ1 strain

Since isolate SZ1 displayed the highest degradation % to coumarin, it was furthermore identified and characterized using traditional media and biochemical tests. Isolate SZ1 was grown on NA and incubated at 37°C for 24h to study various morphological characters. It showed light green shiny circular colonies. Gram stain

showed Gram negative short rods and gave viscous strings with 3% KOH. Motility test was positive. Yellow non lactose fermenter colonies were seen on MacConkey agar, while green colonies appeared on using pseudomonas base agar assuming a *Pseudomonas* strain. The colonies showed green fluorescence on King B media under UV light. The following biochemical characterization of isolate SZ1 was performed and included oxidase test, Catalase test, Starch hydrolysis, Triple sugar iron agar (TSI), citrate test, gelatin hydrolysis , nitrate reduction test, Indole production, Hydrogen sulphide production, Methyl red test (MR test) and Voges-Proskauer test as seen in **table 2.** Positive catalase, oxidase, nitrate reduction, citrate, gelatine liquefaction and florescent pigmentation test is characteristic for Pseudomonas fluorescens .

## 5. Discussion

Coumarin is the main structure in many compounds including those of high toxicity as aflatoxins and those of low toxicity as several therapeutic agents and food additives [19]. Biodegradation of coumarin using microorganisms has become a popular solution to reduce the toxicity of those toxic coumarinic derivatives and even promote safer, better alternatives for those of low toxicity used in medicine [4, 29]. In our present study, 23 bacterial strains were successfully isolated using a media with 0.1% coumarin as a sole carbon source. In agreement, Wang and his colleagues isolated Fusarium sp.WCQ3361 and two other isolates which could grow very rapidly (24h) using CM with similar concentration[26] while in Guan's report he isolated 25 bacterial isolates using 1% CM indicating that this method is selective and accurate [6].

Ten isolates could grow on 0.2% CM while only one bacterial isolate (SZ1) could grow on 0.5%. However, Kunc reported that 31 bacterial strains grew on mineral medium with 0.5% coumarin as the sole carbon source [4].

The bacterial isolate SZ1 was identified using traditional bacteriological media and several biochemical tests as a *Pseudomonas fluorescens* strain. *Pseudomonas fluorescens spp.* Occur generally in hydrocarbon-contaminated soils and have been reported to degrade several heterocyclic hydrocarbons such as benzene, toluene, naphthalene, pyridine, quinoline, benzothiophene and benzofuran [30] and coumarin is also a heterocyclic compound.

The bacterial isolate SZ1 revealed microscopically Gram negative short rods and yellowish green slimy convex colonies with smooth circular margins on nutrient agar. Majority of genus *Pseudomonas* produce pyocyanin (blue-green pigment), while the nonpathogenic saprophyte *Pseudomonas fluorescens* produces a greenish fluorescent pigment. Bacterial strain SZ1 displayed bright greenish fluorescence on King B media under UV light (365nm).The production of a fluorescent pigment on King's medium B was characteristic of most isolates of *P. putida* and *P. fluorescens* [31]. Similar morphological results were obtained with Jayashree et al. 2000, who isolated pseudomonads from the rhizospheres of black gram, carrot, banana, pepper, rice and forest trees and then confirmed the fluorescent colonies by viewing under UV-light [3].

SZ1 Isolate was further identified using several biochemical tests as oxidase test, Catalase test, Starch hydrolysis, Triple sugar iron agar (TSI), citrate test, gelatin hydrolysis , nitrate reduction test, Indole production, Hydrogen sulphide production, Methyl red test (MR test) and Voges-Proskauer test (VP Test). As reported in our study, positive, motility, catalase and oxidase

test, confirmed the strain to be *Pseudomonas spp.* While positive nitrate reduction, glucose fermentation and gelatine hydrolysis indicated that the *Pseudomonas* isolate SZ1 have similar

characteristics with that of P. fluorescens, according to Bergey's Manual of Determinative Bacteriology (1974)[27]. Also Angayarkanni et al. (2005) reported that *P. fluorescens* can dissolve solid gelatine into a liquid form in room temperature[33]. *Pseudomonas fluorescens* can utilize the carbohydrates and glucose utilization was displayed by isolate SZ1.

Negative Indole production, TSI, Methyl red test (MR test) and Voges-Proskauer test (VP Test) was also reported. Similarly eight *P. fluorescens* isolated from rhizosphere of different vegetable crops showed the same biochemical characteristics[34]. However contradicting results was displayed by 30 *Pseudomonas fluorescens* isolates from rice rhizosphere of the Rangareddy district [10].

Based on the following morphological and biochemical characteristics isolate SZ1 was identified as *Pseudomonas fluorescens* and assumed to be a potential strong coumarin degrading strain.

## 6. Conclusion

This is first report to specifically mention a *Pseudomonas fluorescens* as a coumarin degrader which could be used later on in industrial and medical applications. The strain was identified using several morphological and biochemical tests.

## 7. References


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**Table 1:** Growth of soil isolates on variable Coumarin Media

| | Sample ID | source | Coumarin concentrations in media | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | (0.01 %) | (0.05 %) | (0.1%) | (0.2%) | (0.3%) | (0.5 %) |
| **1** | SZ1 | soil | + | + | + | + | + | + |
| **2** | SZ2 | soil | + | + | + | + | + | - |
| **3** | S18 | soil | + | + | + | + | + | - |
| **4** | SA21 | soil | + | + | +/- | +/- | - | - |
| **5** | SA 22 | soil | + | + | + | + | + | - |
| **6** | SB | soil | + | + | - | - | - | - |
| **7** | S 1 | soil | + | + | +/- | - | - | - |
| **8** | S 3 | soil | + | + | + | - | - | - |
| **9** | S 5 | soil | + | + | + | - | - | - |
| **10** | S 7 | soil | + | + | + | - | - | - |
| **11** | S 8 f | soil | + | + | + | + | + | - |
| **12** | S 9 | soil | + | - | - | - | - | - |
| **13** | S 8c | soil | + | + | + | + | + | - |
| **14** | S 11 | soil | + | + | + | - | - | - |
| **15** | S 12 | soil | + | - | - | - | - | - |
| **16** | S 17 | soil | + | + | + | - | - | - |
| **17** | S 18 | soil | + | + | + | - | - | - |
| **18** | S 20 | soil | + | - | - | - | - | - |
| **19** | S 23 | soil | + | - | - | - | - | - |
| **20** | S 26 | soil | + | + | + | + | + | - |
| **21** | S 27 | soil | + | - | - | - | - | - |
| **22** | S 29 | soil | + | + | + | + | + | - |
| **23** | S 32 | soil | + | + | + | + | + | - |

+= indicates growth in media -= indicates no growth in media +/-=indicates slight or weak growth in media

**Table 2:** Morphological and biochemical identification of *Pseudomonas fluorescence* isolate SZ1

| Method | Result | Method | Result | Method | Result |
|---|---|---|---|---|---|
| Gram stain | Gram negative, short rods | Starch hydrolysis test | Positive | Citrate test | positive |
| 3 % KOH test | Viscous strings | Oxidase test | Positive | Indole test | Negative |
| Motility test | positive | Catalase test | positive | Denitrification test | positive |
| MacConkey agar | Pale yellow (NLF) colonies | Methyl red test | Negative | Gelatin hydrolysis test | positive |
| Pseudomonas base agar | Yellow green circular slimcolonies | Voges-proskauer test | Negative | Glucose fermentation test | positive |
| Fluorescent pigmentation test | green fluorescent pigment | Triple sugar iron agar test | Negative | | |