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Phenol Biodegradation by *Corynebacterium glutamicum* Encapsulated in Electrospun Fibers

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ABSTRACT

In Northern Israel, olive mills discharge liquid waste causing contamination of subterranean aquifers with phenol, rendering them albeit temporarily, unfit for both drinking and irrigation. The impact of groundwater pollution due to phenol spillage can be extensive. We developed a model system for the biodegradation of phenol-contaminated wastewater by the bacterium *Corynebacterium glutamicum*. Experiments consisting of suspended cultures demonstrated the native ability of this organism to utilize phenol for its metabolic pathways enabling degradation, at levels of nearly 100 ppm within 24 hours. With the use of bioinformatic data, a complete degradation pathway was constructed. Quantitative Real Time PCR analysis of the first two enzymes in this pathway revealed very distinct expression patterns and two different regulation mechanisms were postulated. Additionally, an electrospinning core-shell system was used to assemble electrospun microtubes containing bacteria on porous metallic carriers. We used these carriers as a new immobilization technique and demonstrated their significant phenol degrading capacity in a batch bioreactor configuration. This system demonstrates the feasibility of constructing a water treatment system for the management of phenol-contaminated water.

Keywords: Phenol Biodegradation; Encapsulated Bacteria; Electrospinning; Microtubes; Phenol 2-monooxygenase; Catechol 1,2-dioxygenase; Olive Mill Waste Treatment; Olive Mill Waste; OMW

1. Introduction

During the winter of 2006, olive mill wastewater (OMW) was discharged into the local sewage system near the city of Maalot-Tarshicha in the Western Galilee, Israel. The OMW accumulated on the ground surface and quickly percolated into the subsurface nearby. A few days later, springs of three local rural villages (Kabri, Gaaton and Neve Ziv) suffered massive chemical and biological contamination that included a substantial increase in organic material (including phenol), metals and general bacterial count. As a result, 15 million cubic meters of drinking water were unfit for use for a period of ten months [1]. A joint survey conducted by The Geological Survey of Israel and The National Water Authority declared that the source of the phenol contamination (a toxic organic compound) was the olive mill wastewater described above [2].

Subsequently, the Ministry of Environmental Protection initiated a pilot project, wherein several Dissolved Air Flotation (DAF) tanks were installed to reduce the biological oxygen demand (BOD) and chemical oxygen demand (COD) from the OMW before discharging it into the public sewage system [3]. Being mechanical rather than biological, these devices cannot effectively reduce the phenol contamination (excluding tannins) in the OMW. The outcome was the need for a specific, targeted, biological process that can reduce phenol concentration to acceptable levels as regulated by legislative authorities.

Phenol (C_6H_5OH) is a relatively water miscible, slightly acidic and highly toxic compound [4]. According to the United States Environmental Protection Agency (US EPA), it is the 65th of 129 top water pollutants on the priority pollutant list within the US. It may cause harmful effects in humans after long-term exposure, when encountered in high concentrations [5]. In Israel, a significant local source of phenol contamination is the olive industry where sodium hydroxide is used to remove the bitter taste caused by polyphenols in ripe olives. Several washes with large amounts of water flush away this chemical. Additionally, OMW is generated in vast quantities during the production of olive oil with high concentrations of phenol. Therefore, the increase in anthropogenic industrial activities has made olive oil production environmentally unsustainable using the conventional methods for its production. An increasing source

of concern is the contamination of underground water aquifers by phenol, rendering the water unfit for drinking, irrigation or both. For example, phenol contamination near fisheries may taint the flavor of fish at concentrations as low as one ppm [6]. Three primary considerations drive industrial plants to treat their sewage independently before discharging it to the local treatment facility: practical financial reasons, increasing environmental awareness and legislative restrictions.

In contrast to physical and chemical methods for phenol degradation, biological phenol treatment has several advantages such as: high specificity, low cost, potential of bacterial reuse by immobilization and limited negative environmental impact. Biological phenol treatment is beneficial because of its natural high substrate specificity as well as creation of minimal to no waste products. Although biological treatment can result in complete mineralization of the toxic compound thus eliminating the requirement for further treatment, it is beyond the scope of this work to test that [7].

We decided to utilize an aerobic phenol degradation pathway that has a higher efficiency and robustness in comparison to an anaerobic pathway. The aerobic phenol degradation pathway relies on a constant oxygen supply, which is crucial for both cell growth and phenol degradation reactions [8]. The products resulting in the aerobic process are fewer and less toxic compounds. We worked with a bacterial reference strain from an established culture collection, in order to have an annotated genome with the possibility for future genetic manipulation. To immobilize phenol-degrading bacteria, we used a core-shell electrospinning technique. This system facilitates the fabrication of microtubes in an electrostatic field. Two separate syringes continuously ejected solutions from a uniquely structured nozzle under high voltage. The internal part of this spinneret needle facilitated the flow of bacteria in an aqueous solution, while being enveloped by the external needle containing a non-biodegradable polymer solution. We collected the fabricated microtubes on a rotating disk collector (mandrel), without compromising their spatial structure.

We tested the phenol degrading capacity of the encapsulated bacteria in a batch bioreactor configuration, which can be scaled up to a semi-industrial setting.

2. Materials and Methods

Corynebacterium glutamicum a Gram-positive, rod-shaped bactirium was cultured from lyophilized sealed vials from the strain collection of the Department of Microbiology and Molecular Genetics (IMRIC), Hebrew University of Jerusalem. The identity of the bacterial strains was determined using 16S rRNA sequencing. Cells were grown in LB medium containing 10 g/l Bacto-tryptone (Becton Dickinson), 5 g/l yeast extract (Becton Dickinson), 10 g/l NaCl (Frutarom) and doubly distilled water (DDW) adjusted to pH 7.5 with NaOH. Incubation was at 30˚C and 150 RPM in baffled 250 ml Erlenmeyer flasks.

Phenol concentration was analyzed using a HP Agilent 1050 series RP-HPLC with a 1100 series chromatograph analyzer containing a C18 4 × 250 mm column and 5 µm oval particles at 110 ± 15 bar. The chromatograph detection wavelength was set at 270 nm and the pressure was stable throughout the run (< ±3%). Injection volume was 20 µl. Results were calculated according to a predetermined standard curve constructed with molecular grade anhydrous phenol. The mobile phase used was comprised of HPLC grade, A: 100% acetonitrile, B: 0.1% formic acid in DDW, using an isocratic flow of A: 50%, B: 50% at a constant rate of 1 ml/minute [9].

For quantitative real time PCR measurements, cells were grown as described above and cell pellets were treated with FastRNA Pro blue kit (MP Biomedicals). Isolated RNA was treated with RQ1 DNase (Promega) to eliminate DNA impurities. Rotor-Gene 3000A Real Time PCR machine and Rotor-Gene 6000 v1.7 software were used for the analysis of the transcription level of the following sequences (5' to 3'): Resuscitation promotion factor (*Rpf*) was chosen as the housekeeping gene using a 173 bp segment, forward primer: TAACGGCTACCACGGTGGTC, reverse primer: GGTTGGAGCGGAGTTCAGTC [10].

Two genes which are the two first consecutive enzymes in the phenol degradation pathway were examined. Phenol 2-monooxygenase, p2mo (EC 1.14.13.7), 127 bp segment, forward: GCCAATGCAGATGGCGTGCA, reverse: GGTTTTGCGGGTCCGGCTTC, and catechol 1, 2-dioxygenase, c1d (EC 1.13.11.1), 150 bp segment, forward: TTCGTTGAGGGCGATCCGCA, reverse: AAAACGTGTGCGTGCCCAGG. All three primer sets (Syntezza Bioscience) had an identical annealing temperature of 61.4˚C.

For the electrospinning, the core and shell solutions were forced through the spinneret needle by a stable flow from two separate syringes at the rate of 0.5 and 5 ml/hour, respectively. The needle was fixed at a distance of 10 cm from the tip of a 30 cm diameter flat bed rotating disk collector in an electrostatic field of 900 V/cm. Temperature was maintained at 23.5˚C ± 3˚C and relative humidity was 36% ± 16% throughout the process. The bacteria for the process were grown in LB medium for 12 hours, collected by centrifugation and washed 3 times in H_2O before the procedure. The electrospun fibers were collected on the external surface of high-density mesh metallic carriers and photographed using a confocal flourescent microscope after suspension in live/dead dye (Invitrogen). The composition of the microtube shell solution was 13% w/w PVDF-HFP of average M_w of 400,000 g/mol, 1% w/w PEG of average M_w of 6000

g/mol, 56% w/w THF and 30% w/w DMF. The inner core solution contained 13% w/w PVP of average M_w of 1,300,000 g/mol, 76% v/v of H_2O and 11% v/v of *Corynebacterium glutamicum* bacteria at approximately 10^{11} CFU/ml. The process is detailed by Zussman *et al*. [11-13].

Sodium alginate beads were created by slowly dripping 10 ml of a 4% sodium alginate suspension containing bacteria into a gently stirred 0.2 M $CaCl_2$ solution. The beads were suspended in the solution for 15 minutes and then rinsed twice in PBS before use.

3. Results

3.1. Transcription of the Two First Genes of the Phenol Degradation Pathway

The data in **Figure 1** show that before the addition of phenol, the cells which were in a logarithmic growth phase, exhibited a very low transcription level of both genes p2mo and c1d, of 4.24 and 2.66 relative concentration units, respectively. Following the addition of phenol, the transcription level of p2mo quickly rose and reached a peak of 1375 and 7.1 at 5 minutes, then fell to a steady state of 208.3 and 4.15 at 12 minutes, respectively.

3.2. Electrospinning of *Corynebacterium glutamicum*

Figure 2(a) shows *Corynebacterium glutamicum* bacteria encapsulated in the electrospun microtubes from a top view. The image shows a single layer of fibers collected on a glass microscope slide in which the bacteria are visible as small round red (electronic version) dots in along the tubes. Image b shows a horizontal cross-section picture of microtubes, showing that the bacteria are located in the center of their height, meaning that they are encapsulated within the tubes and demonstrating the successful encapsulation process.

We tested the phenol degradation capacity of different immobilization methods, compared to a control. We used four 250 ml Erlenmeyer flasks at 30˚C and 150 RPM for a batch bioreactor experiment and used 10^9 bacteria for each method. Each flask contained three carriers or an equivalent number of suspended cells. The results are shown in **Table 1**.

4. Discussion

The two very different and distinctive patterns of gene transcription shown in **Figure 1** support the assumption that these two genes have very different regulatory methods. The transcription level of phenol 2-monooxygenase is induced considerably in the presence of phenol, whereas catechol 1,2-dioxygenase which is a constitutive gene, is not greatly affected by the addition of phenol.

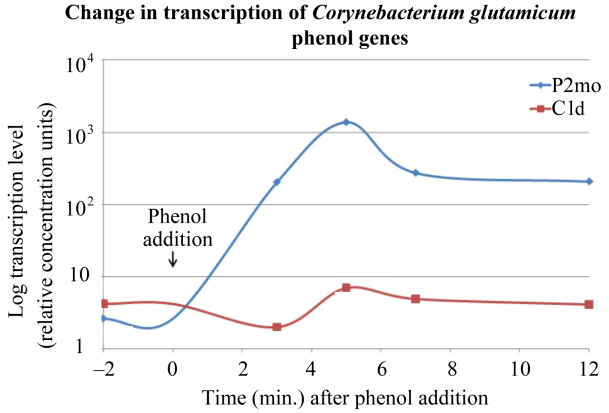
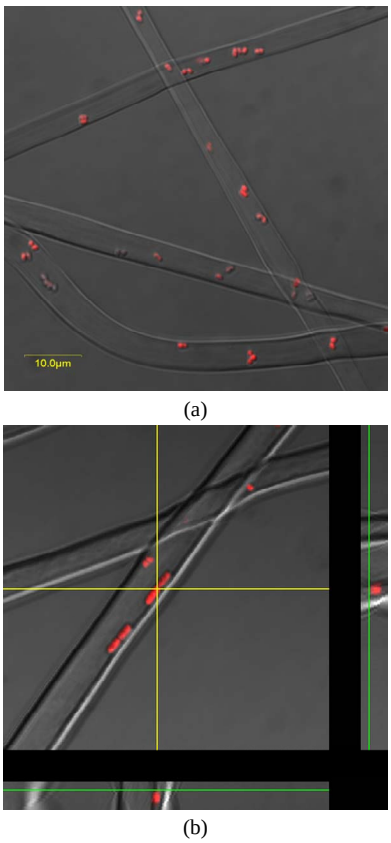


Figure 1. Change in bacterial gene transcription using relative quantitative analysis. Genes tested: p2mo = phenol 2-monooxygenase, c1d = catechol 1,2-dioxygenase.



(a)

(b)

Figure 2. Bacteria encapsulated in electrospun microtubes; image from top view (a) and side view (b).

Table 1. Phenol degradation by immobilized bacteria, units are in ppm.

Type/time (day)	0	1	2
Control (no bacteria)	751	760	730
Suspended cells	751	<75	<75
Alginate beads	751	480	<75
Metallic Carriers	751	580	570

These are novel findings. The large difference in gene transcription can be explained by a substantially higher catalytic rate of catechol 1,2-dioxygenase, compared to phenol 2-monooxygenase.

The bacteria were situated in the hollow micro-tube cavity as is shown in **Figure 2(a)** and remained in that location even after several washes to remove excess dye. This conclusion is also supported by the fact that when examining the individual cross-section images using a confocal microscope, the fluorescence appeared in the center of the tubes (**Figure 2(b)**).

To test the application of this new system, we examined the phenol degradation capacity of the encapsulated bacteria versus suspended cells in growth medium. We configured a batch bioreactor experiment and used the same suspension and therefore approximately the same number of bacteria for each method. The identity of the bacteria was confirmed by sequencing their 16S rRNA as previously noted. The results clearly show that the phenol degradation was most efficient in suspended cells and lowest in bacteria encapsulated in carriers. This can be explained by the microenvironment of the fabricated tubes. Encapsulated bacteria are under constraints of physical pressure, molecular diffusion and equilibrium of metabolism products, which may adversely affect the rate of phenol degradation. It is also possible that the viable bacterial yield following the encapsulation process is low, requiring several days for cell recovery and proliferation. This immobilization procedure leave the capacity of the bacterium to degrade phenol over time, even though the degradation rate is lower compared to suspended cells. In contrast, these limitations have little effect on suspended cells, which were exposed directly to the aqueous environment.

In contrast to existing immobilization techniques, the method described here encapsulates one or a few cells at a time. Although other immobilization procedures were not examined, it is clear that this technique can be manipulated in a number of ways, especially with the use of non-biodegradable polymers. The deleterious effects of this immobilization method, compared to alginate beads should be examined more extensively.

5. Conclusion

In this work, we described two distinct patterns of gene transcription involved in phenol degradation in *Corynebacterium glutamicum*. The first gene in this pathway was found to be inductive whereas the second was constitutive. We assembled electrospun microtubes on carriers and demonstrated their significant phenol degrading capacity in a batch bioreactor configuration. This system demonstrates the feasibility of construction and use of filters fabricated in a similar fashion for the treatment of phenol-polluted water. This system can be especially beneficial in treating liquid olive mill waste. Future plans include finding parameters that will allow for keeping the encapsulated bacteria within the microtubes and study phenol degradation capability over extended time periods.

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