



A new functional biofilm biocatalyst for the simultaneous removal of dibenzothiophene and quinoline using *Rhodococcus rhodochrous* and curli amyloid overproducer mutants derived from *Cobetia* sp. strain MM1IDA2H-1

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ABSTRACT

Biocatalyst systems based on biofilms were developed to remove nitrogen and sulfur-containing heterocyclic hydrocarbons using *Cobetia* sp. strain MM1IDA2H-1 and *Rhodococcus rhodochrous*. The curli overproducers mutants CM₁ and CM₄ were derived from *Cobetia* sp. strain and used to build monostrain biofilms to remove quinoline; and together with *R. rhodochrous* to simultaneously remove quinoline and dibenzothiophene using mixed biofilms. The quinoline removal using biofilms were 96% and 97% using CM₁ or CM₄ curli overproducers respectively, whereas bacterial suspensions assays yielded 19% and 24% with the same strains. At the other hand, the simultaneous removal of quinoline and dibenzothiophene using mixed biofilms were respectively 50% and 58% using strains *R. rhodochrous* with CM₁ and 75% and 50% using *R. rhodochrous* with CM₄. Results show that biofilms were more efficient than bacterial suspension assays and that in mixed biofilms the shared surface area by two or more bacteria could affect the final yield.

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1. Introduction

Among the risks that combustion of fossil fuels has for human health, the presence of organic molecules containing sulfur and nitrogen explain the emission of sulfur dioxide (SO₂) and nitrogen oxide (NO_x) contaminants [1]. Regrettably, this problem is increasing due to the need to exploit ever-deeper oilfields for petroleum production where abundance of sulfur and nitrogen-containing compounds is greater [2].

In the oil refining industry, the removal of sulfur and nitrogen has been performed by chemical catalysis [3]. This chemical process requires high-energy input uses high-cost noble metals and has limited duration and yields solid hazardous wastes. Therefore, conventional technology has several drawbacks that impact the environment and the entire process economy. For these

reasons alternative technologies are currently being explored to perform fuel refining.

Biocatalysis is a promising research area that uses living microorganisms or their enzymes to develop green applications for biorefining and depuration process [4]. A successful biocatalysis for oil refining process involves the use of specific microorganisms containing the proper metabolic pathways and the availability of the target molecules contained in the organic liquid phase. These factors must be linked in a rationally designed configuration of process and equipment. For these reasons, discovery and development of new microbial biocatalysts [5–7] and the optimizations of the contact between substrates and microbial cells using surfactants or immobilized bacteria, have been reported as novel strategies to improve the biocatalysis process [8,9].

Bacterial immobilization of *Rhodococcus rhodochrous* IGT58 (ATCC 53968) by adsorption using inorganic materials such as Silica (Si) and sepiolite (Sep) have been used in biodesulfurization (BDS) reactions because these materials increasing the bioavailability of sulfur substrates for bacterial cells, due the higher

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interaction between the cells and these supports [10]. On the other hand, bacteria such as *Cobetia* sp. strain MM1IDA2H-1 (CECT 7764) that grow using aromatic heterocycles hydrocarbons, produces a biosurfactant and generates biofilm, are interesting for the design of new biocatalytic process [11]. The annotated genome of this strain shows genetic clusters necessary for N-heterocycle metabolism (carbazole and quinoline) and for production of curli amyloid required for initiation of biofilm formation [24].

Biofilms are able to grow and develop on the surface of organic or inorganic particulate matter and structured by living microorganisms embedded into a self-produced polymeric matrix. As a living functional entity, the biofilm is a tight network of physical, chemical, genetics, biochemical, metabolic and eco-physiological relationships between microorganisms that share a self-generated micro-environment. For this reason biofilms have been proposed as a new generation of biocatalysts that can overcome the limitations of technology based on bacterial cells in suspensions [13].

The goal of this study is to use the bacteria *Cobetia* sp. strain MM1IDA2H-1 and *Rhodococcus rhodochrous* IGTS8 (ATCC 53968) to generate a mixed biofilm as a new biocatalyst for the simultaneous removal of nitrogen, and sulfur-containing compounds from the liquid phase.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains and their relevant genotype and phenotype are listed in Table 1. For sulfur-containing heterocycle metabolism, Medium A, supplemented with sodium succinate (30 mM) and citrate (0.1% w/v) as energy and carbon sources, respectively, or DBT (0.1 mM) (Merck) dissolved in isooctane (IOA) as the sulfur source, was used for growth [17,18]. For N-heterocycle metabolism with *Cobetia* sp. strain MM1IDA2H-1 (CECT 7764) quinoline (Merck, USA) 30 mM was used to supplement M9 minimal medium replacing NH_4Cl and citrate as the nitrogen or carbon source respectively, to evaluate the growth on this substrate under different conditions.

2.2. Obtaining curli overproducing mutants of *Cobetia* sp. strains MM1IDA2H-1

To ensure biofilms formation, curli amyloid overexpressing mutants of *Cobetia* sp. strain MM1IDA2H-1 (CECT 7764) [11] were obtained by chromosomal insertion of streptomycin cassettes using the puTminiTn5::Sm system [16] *Escherichia coli* containing puTminiTn5::Sm was used to transfer the transposon by

two-parental conjugations [19]. For mutants selection the exconjugants were plated on M9 minimal medium (DIFCO) supplemented with sodium citrate (0.2% w/v) and streptomycin ($100 \mu\text{g mL}^{-1}$). For selection of curli overproducers, Congo Red ($50 \mu\text{g mL}^{-1}$) and Brilliant Blue G250 ($1 \mu\text{g mL}^{-1}$) were added [20] and plates were incubated at 30°C for 48 h. Streptomycin resistant colonies showing curli overproducing phenotypes after 48 h were identified by the intensity of red coloration of the colonies, which were selected for further studies. The curli over producer phenotypes: white, dark, red, light red, pink and light pink were confirmed using the Congo Red agar method [21] Briefly, $50 \mu\text{L}$ of each mutant and wild type were added to the agar surface and incubated for 24–48 h at 30°C . After 24 h Congo Red agar plates were assessed for phenotypic classification and colonies of mutants with red phenotype were classified as curli. Mutants were evaluated for biofilm formation by crystal violet method in polyvinyl chloride plates [22] measuring absorbances at 540 nm and 600 nm in a microplate reader (Autobio Phomo Labtec Instruments) for adhesions and biomass respectively. The selected mutants were denoted CM_1 and CM_4 .

2.3. Generation of biofilms for biocatalyst of sulfured and nitrogenated hydrocarbons

For biofilms construction an initial step for bacteria cells adhesion it was followed by a second reaction step un a packed bed. For the first step, Silica (SiO_2) D11-10 BASF particles with a specific surface area of $80 \text{ m}^2 \text{ g}^{-1}$ were used as inorganic supports [23] for bacterial cells of *R. rhodochrous* and selected mutants of *Cobetia* sp. strain MM1IDA2H-1. For this, cells were collected from 24 h cultures of each strain by centrifugation at $4000 \times g$ for 5 min at 4°C . The pellet was suspended in 1000 mL of 0.85% w/v NaCl at pH 5.5 and circulated through the SiO_2 of packed bed column with a total volume of 94.2 mL (2 cm diam., 30 cm length) in a downward direction at 10 mL min^{-1} for 72 h [18]. The number of immobilized cells was measured by loss of turbidity of the initial cell suspension at 600 nm [18]. The estimated adsorbed cells ranged from 0.46×10^6 to 1.27×10^6 cells mL^{-1} .

2.4. DBT and QN removal using suspended and immobilized bacterial cells

2.4.1. Suspended bacterial cells assay

1 mL of QN solution in isooctane (IOA) (0.77 mM) was added to 10 mL of medium containing CM_4 or CM_1 suspended bacterial cells. The reaction was carried out at 30°C on a rotary shaker at 200 rpm for 24 h.

Table 1
Strains and relevant genotype and phenotype

Strain or plasmid	Description	Phenotype relevant	Reference or source
<i>R. rhodochrous</i> IGTS8 (ATCC 53968)	dszABC genes for non-destructive Sulphur removals from DBT	Biodesulfurization selective of DBT	[14]
<i>Cobetia</i> sp. strain MM1IDA2H-1	-csgA, csgB genes for major curlin precursors	Growth in quinoline as the carbon source.	[12], This work.
Wild type (CECT 7764)	-IQOb; IQOba for growth in the medium with heterocycles/ quinoline/ carbazole		
CM_1	csgA, csgB; genes for major curlin precursors; -IQOb; IQOba for growth in the medium with heterocycles/ quinoline/ carbazole; Sm ^r	Curli overproducer, biofilm formation	This work
CM_4	csgA, csgB; genes for major curlin precursors; -IQOb; IQOba for growth in the medium with heterocycles/ quinoline/ carbazole; Sm ^r	Curli overproducer, biofilm formation	This work
<i>Escherichia coli</i> CC118 (λpir)	CC118 lysogenized with λpir phage		[15]
puT-mini-Tn5Sm	Ap ^r Sm ^r ; mini-Tn5 suicide donor		[16]

2.4.2. Packed bed column assay with SiO₂ immobilized bacterial cells

For the reaction step, packed bed columns with respective biofilms were used as bioreactors for removal of QN and the simultaneous removal of DBT and QN. Each glass column was filled with SiO₂ particles with a bed height of 15 cm and particle sizes in the range 3.35–5.6 mm [18]. For reactions QN solution (0.77 mM) with nitrogen-free Medium 9 or a mixture of DBT (6.23 mM) and QN solution (0.77 mM) with a mixture of nitrogen-free Medium 9 and sulphur-free Medium A were fed continuously into the bioreactor in a downward direction at 0.9 mL min⁻¹ using a peristaltic pump over two hours. Samples were then collected at 20 min intervals for 72 h. As a control, QN and DBT were fed into a packed bed containing no immobilized bacteria.

2.5. Analytical methods

DBT and QN were extracted with 20 ml of ethyl acetate and analyzed by gas chromatography using a Shimadzu GC-2010 equipped with an SPB-5 capillary column (L 30 m, ID 0.25 mm, film 0.25 μm) and an FID detector [18]. Each experiment was repeated at least three times. The degradation percentages of DBT (% DBT) and QN (% QN) were expressed as % DBT = [(DBT₀ - DBT)/DBT₀] × 100 and % QN = [(QN₀ - QN)/QN₀] × 100, respectively.

3. Results and discussion

3.1. Obtaining curli overproducer mutants of *Cobetia sp* strain MM1IDA2H-1 for the development of biocatalysts for quinoline removals

In this study we propose the marine bacterium *Cobetia sp.* strain MM1IDA2H-1 for the development of new biocatalysts destined to the removal of N(S)-heterocycles hydrocarbons. For this purpose, was tested the wild type *Cobetia sp.* strain MM1IDA2H-1 to use quinoline as the sole nitrogen source and the sole carbon source using modified minimal M9 medium. The results presented in Table 2, show that this bacterium used quinoline as the nitrogen source and as the carbon source when it was grown in M9 medium with quinoline replacing NH₄Cl (nitrogen source) or succinate (the carbon source). These results are explained by the detected cluster for N-heterocyclic aromatics degradation in the *Cobetia sp.* strain MM1IDA2H-1 genome [12]. The cluster included genes encoding for: Isoquinoline 1-oxidoreductase beta subunit (IQOb), Isoquinoline 1-oxidoreductase alpha subunit (IQOba), and an aminopeptidase for degradation of N-heterocycle aromatics such as carbazole.

Mutants derived from the wild type *Cobetia sp.* strain MM1IDA2H-1 with curli overproduction phenotype were isolated using mutagenesis by transposon and selection in Congo Red agar (Fig. 1). Selected mutants named CM₁ and CM₄ generated curly phenotype in less than 24 h of incubation at 30 °C and according to results obtained with the crystal violet method, mutants formed more biofilm than wild type strain (unpublished data). This can be explained because curli is the main protein component of the extracellular matrix playing a key role for the initial phase of cellular adhesion [24]. Recently genetically modified bacteria overexpressing curli has been used to design new biocatalytic

biofilms where an efficient bacterial adhesion is associated with catalytic functionalities for contaminants removal [25,26].

Regarding to quinoline (QN) degradations activity using bacteria in suspension it is possible to appreciate that QN removal was 19% and 24% in mutants CM₁ and CM₄ respectively, showing that these bacterial strains are metabolically active for quinoline degradations when are used in assays using bacteria in suspensions (Table 3).

3.2. QN removal in biofilms formed by curli overproducers strains of *Cobetia sp.* MM1IDA2H-1

To test quinoline (QN) removals, biofilms were constructed to be used as biocatalyst using separately the selected mutants CM₁ and CM₄. Kinetics of QN removals using biofilms with curly overproducers strains are shown in Fig. 2, meanwhile Table 3 summarized the final percentages of quinoline removal obtained with respective biocatalytic biofilms of selected mutants CM₁ and CM₄. Results show that in the first 5 h QN removal increase until 50% and 60% for CM₄ and CM₁ strains, respectively. In the next stage, the process after 72 h slowly reached 97% and 96% of QN removal with CM₄ and CM₁ respectively.

The kinetic pattern observed using both mutants must be the result of changes on metabolic and physiological status of bacteria caused by a decrease in substrate concentration and the occurrence of metabolic intermediaries during QN biotransformation. According to abiotics controls, the process is biologically catalyzed since QN removal was negligible in controls assay without biofilms. Literature reports show that similar QN removal values have been observed using bacteria isolated from contaminated soil or activated sludge. Qiao [27] studied the biodegradation of QN using *P. putida* isolated from a municipal wastewater treatment plant, observing that QN at a concentration of 500 mg L⁻¹ could be completely removed within 3 h. Similarly, Bao-Hua [5] demonstrated that *Bacillus sp* isolated from a refinery wastewater treatment plant, could completely remove QN from a 500 mg L⁻¹ solution within 30 h.

In relation to QN removals, the performance obtained using biofilms of *Cobetia sp.* was noticeably higher than obtained using bacteria in suspensions that did not exceed 24% (Table 3). Advantages of biofilms performing in packed systems, can be related to the substrates bioavailability and the tight interaction cell-substrate occurring in the biofilm matrix playing relevant roles in pollutants removal [18]. Additionally, silica offers an important opportunity for the development of functional biofilms owing to its mesoporous structure, which provides an environment conducive to cell encapsulation [28].

3.3. Simultaneous removal of QN and DBT using a biofilm as a biocatalyst

Kinetics and final values for simultaneous removal of QN and DBT using mixed biofilms formed by curli overproducers strains of *Cobetia sp.* (CM₄ and CM₁) and *R. rhodochrous* are shown in Fig. 3 and Table 3. For *R. rhodochrous* and CM₄ biofilm (RCM₄) is possible to observe that both substrates were actively removed in the first

Table 2
Dibenzothiophene and quinoline uses by strains of this work.

	DBT	QN
Carbon source	<i>Cobetia sp.</i> strain MM1IDA2H-1, CM1 and CM4	<i>Cobetia sp.</i> strain MM1IDA2H-1, CM1 and CM4
Sulphur source by nondestructive 4 S pathway	<i>R. rhodochrous</i>	Not apply
Nitrogen source	Not apply	<i>Cobetia sp.</i> strain MM1IDA2H-1, CM1 and CM4

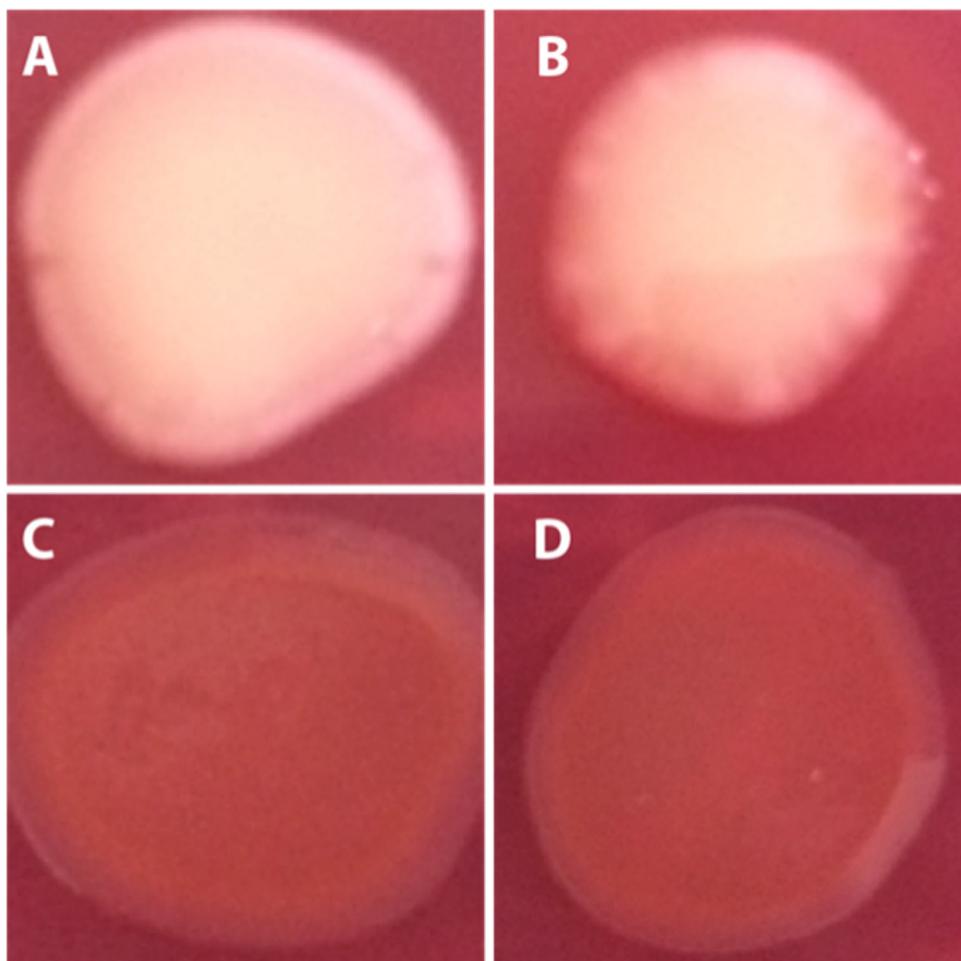


Fig. 1. Phenotypes for curli over-production in mutants derived from *Cobetia* sp. strains MM1IDA2H-1. Image shows colonies after 24 h of incubations growing in agar Congo Red with pink light phenotype of wild type (a and b) and the red phenotype described for curli production (c and d). The selected curli over producer mutants were named: CM₁ and CM₄.

Table 3
Maximum DBT and QN removal values for different cell systems and curli overproduction of two mutant strains.

Bacterial Cell Systems			DBT Removal (%)	QN Removal (%)
Bacterial suspensions		<i>R. rhodochrous</i>	33	-
		CM ₁	-	19
		CM ₄	-	24
Packed Biofilm	Mutant of <i>Cobetia</i> sp <i>R. rhodochrous</i> and <i>Cobetia</i> sp Biofilm	CM ₁	-	96
		CM ₄	-	97
		RCM ₁	58	50
		RCM ₄	50	75

7 h, followed by a phase of 70 h where a slight and constant removal activity was achieved with a maximal of 75% for QN and 50% for DBT degradation. In *R. rhodochrous* and CM₁ biofilm (RCM₁) a similar kinetic in time for simultaneous removal of QN and DBT was obtained (Fig. 3), with maximum removal yield of 50% for QN and 58% for DBT.

Comparing results of mixed biofilm (*R. rhodochrous* and curli overproducers strains of *Cobetia* sp.) with biofilm formed only by *Cobetia* sp., is evident a significant reductions in quinoline removals. This finding can be explained by physical and biological factors involved in surface interactions and metabolism. It is proposed that in comparisons to the biofilm formed by only one strain, in a mixed biofilm *Cobetia* sp. and *R. rhodochrous* cells must

share the available area in where each substrate is metabolized. This means that, although the catalytic activity per cell unit for each substrate can be constant, the specific metabolic activity per unit of area will be lower when using mixed biofilms.

Relative to biological factors, it should be noted that in this mixed biofilm respective metabolism of DBT and QN in *R. rhodochrous* and mutants are carry on by different pathways were occurrence of metabolic intermediates and culture media could affect the biocatalyst efficiency. DBT in *R. rhodochrous* is used as the sulfur source (but not as the carbon source) by the non-destructive "4S pathway" that selectively removes the sulfur generating 2-hydroxybiphenyl as toxic end product affecting bacteria with lower growth rate [29,30]. Nevertheless, according

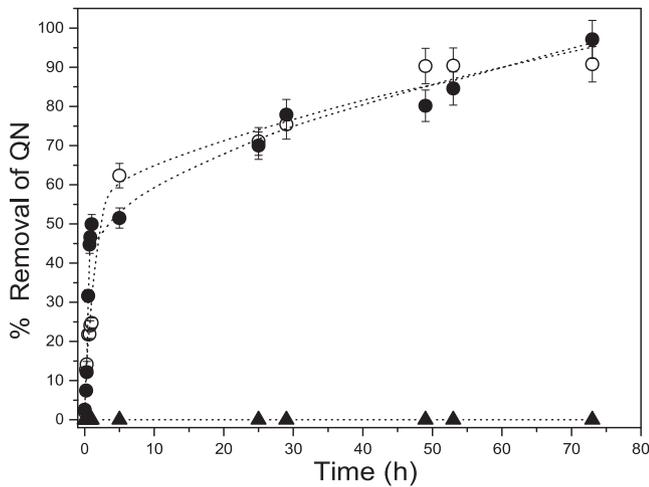


Fig. 2. Removal of QN in packed bed column using biofilms of genetically modified *Cobetia* sp. strains MM1IDA2H-1 adsorbed on the surface of SiO₂ particles, CM₄ (●) and CM₁ (○). The control was the QN removal on a packed bed column of silica without biofilm (▲). Data show mean values for three independent assays. Error bars correspond to the standard deviation.

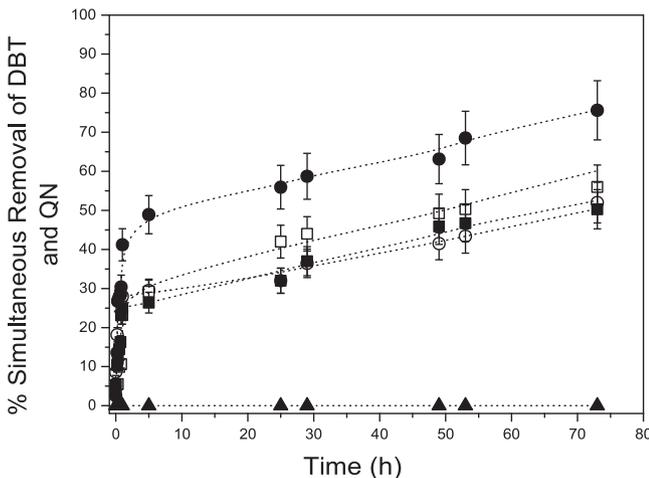


Fig. 3. Simultaneous removal of DBT and QN in a packed bed column using a functional biofilm formed by *R. rhodochrous* (■) and CM₄ (●) adsorbed on SiO₂ (RCM₄), and a functional biofilm formed by *R. rhodochrous* (□) and CM₁ (○) adsorbed on SiO₂ (RCM₁). The control (▲) was removal of DBT and QN on a packed bed column of silica without biofilm. Data are mean values for three independent assays. Error bars correspond to the standard deviation.

to genomic and microbiological evidence *Cobetia* sp. strain MM1IDA2H-1 is able to use 2-hydroxybiphenyl, so toxicity associated with the accumulation of this metabolic intermediate can be ruled out. For the exposed, in addition to physical factor, the observed DBT and QN removal in the mixed biofilm can be explained by the metabolic and ecophysiological status of both strains sharing a common space in a complex matrix, with changes in turnover and availability of nutrients.

4. Conclusions

Biofilms formed by the biodesulfurizing *Rhodococcus rhodochrous* and two quinoline degrading, curli overproducer mutants derived from *Cobetia* sp. strain MM1IDA2H-1 were functional as biocatalysts for simultaneous removal of DBT and QN. On the other hand biofilms formed by two quinoline degrading, curli overproducer mutants derived from *Cobetia* sp. strain MM1IDA2H-1

were more efficient than assays performed using bacteria in suspensions.

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