DEVELOPMENT OF AN ENVIRONMENTALLY BENIGN MICROBIAL INHIBITOR TO CONTROL INTERNAL PIPELINE CORROSION

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Abstract

Title: Development of an Environmentally Benign Microbial Inhibitor to Control Internal Pipeline Corrosion

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Objective: The overall program objective is to develop and evaluate environmental benign agents or products that are effective in the prevention, inhibition, and mitigation of microbially influenced corrosion (MIC) in the internal surfaces of metallic natural gas pipelines. The goal is one or more environmental benign, a.k.a. "green" products that can be applied to maintain the structure and dependability of the natural gas infrastructure.

Approach: *Capsicum* sp. extracts and pure compounds were screened for their antimicrobial activity against MIC causing bacteria. Studies on the ability of these compounds to dissociate biofilm from the substratum were conducted using microtiter plate assays. Tests using laboratory-scale pipeline simulators continued.

Results: Preliminary results showed that the natural extracts possess strong antimicrobial activity being comparable to or even better than the pure compounds tested against strains of sulfate reducers. Their minimum inhibitory concentrations had been determined. It was also found that they possess bactericidal properties at minimal concentrations. Biofilm dissociation activity as assessed by microtiter plate assays demonstrated varying degrees of differences between the treated and untreated group with the superior performance of the extracts over pure compounds. Such is an indication of the possible benefits that could be obtained from these natural products. Confirmatory experiments are underway.

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EXECUTIVE SUMMARY

The main goal of this project is to develop an environmentally benign compound that could prevent and/or control microbially influenced corrosion (MIC) in the interior of metal gas pipelines. Presented herewith is a summary of research activities from April 2002 to June 2002.

Work for the first two objectives, that is, the cultivation of MIC-causing microorganisms and experimentation using a laboratory scale pipeline system, continued. In addition, this quarter's activities were mainly focused on the fourth and fifth objectives that is, evaluation of pepper components to inhibit biofilm formation and the identification of effective concentrations of the compounds tested. Pepper *(Capsicum sp.)* extracts and some pure pepper compounds were screened for their antimicrobial activity against a group of MIC- causing microorganisms, the sulfate reducers. Minimum inhibitory and bactericidal concentrations of these test agents were determined. It was interesting to note that preliminary data demonstrated the pepper extracts' strong antimicrobial activities that are comparable to and in most cases, even better than the pure compounds. More tests will be done in this connection using other organisms. The ability of these agents to dissociate biofilm from the substratum was evaluated using microtiter plate assays. Preliminary results showed varying degrees of difference between the treated and untreated group with the superior performance of the extracts over pure compounds in effectively detaching the attached biofilms. Confirmatory tests are underway and experiments on the inhibition of biofilm formation as well as some corrosion studies are still going on.

INTRODUCTION

The overall objective of this project is to develop, test, and apply environmentally benign agent(s) to control corrosion associated with internal surfaces of metal (iron or steel) pipes used in natural gas transmission. The overall hypothesis is that agents exist in nature that inhibit some or all of the steps executed by microorganisms in the formation of biofilm. As biofilm formation is an absolute prerequisite for the initiation and production of microbially influenced corrosion (MIC), blocking biofilm formation or propagation will block or mitigate MIC.

The general approach is to evaluate natural products isolated from plants, and possibly animals or microorganisms for their abilities to block the attachment, physiology, or reproduction of microbial agents that are responsible for microbiologically influenced corrosion. The first natural product to be tested is the oil that can be extracted from the seeds and pods of pepper plants. These plants are members of the Genus *Capsicum* and the first species evaluated were *Capsicum annuum* and *C. chinense*. The effective components or constituents (isolation and identification of these constituents in a previous and ongoing project funded by Gas Technology Institute) of this product will then be tested for its environmental impact and effects, effective concentrations, modes of application, and stability against isolated MIC microorganisms under simulated field conditions. A commercially viable agent that aids in MIC control and is environmentally friendly is the ultimate target with preliminary data to determine commercialization potential and cost-benefit analysis.

EXPERIMENTAL

Objective 4 – Evaluate Pepper Oil Components to Inhibit and Mitigate Biofilm Formation and MIC

Microtiter plate assay for the assessment of biofilm dissociation activity was done. Biofilm formation was initiated by inoculating ATCC # 1249 Modified Baar's Medium was supplemented with 20% Oxyrase for Broth with *Desulfovibrio* cell suspension of 10^8 cfu/ml and incubating anaerobically at 30^oC. Media was replaced with fresh one after 96 hrs and plates were re-incubated for another 48-72 hrs to ensure proper biofilm growth. Optical density was measured every 24 hrs using the MRX II Plate (Dynex Technologies) plate reader. After the final incubation period, media was removed and 100 ul of the test compounds were added to appropriate wells then incubated for 1 hr. The wells were then rinsed with distilled water, air-dried and stained with 150 ul 1% crystal violet for 45 mins. They were washed with distilled water and destained by 200 ul 95% ethanol. Quantitative analysis of the biofilm (Djordjevic, et al. 2002) present in the wells was done by transferring 100 ul of the destained biofilm into a new plate and measuring the optical density at 600 nm.

Objective 2 – Identify and Test Effective Concentrations of Pepper Oil Components

Microdilution assay method (Eloff, 1998) was used to determine the minimum inhibitory concentration of the pepper components. The pepper extracts and pure compounds were serially diluted 50% with sterile distilled water in 96-well microtiter plate. For the inoculum, an overnight bacterial culture was centrifuged at 4,000 rpm for 5 mins, washed with 50mM potassium phosphate buffer, pH 7.0 and the optical density was adjusted at 600 nm to obtain a final cell concentration of 10^6 cfu/ml per well. ATCC # 1249 Modified Baar's Medium was supplemented with 20% Oxyrase for Broth to ensure confluent cell growth in the microtiter plate. The plates were then placed inside a modular anaerobic chamber, purged with nitrogen gas and incubated at

30^{o}C for *D. vulgaris* and 37^{o}C for *D. desulfuricans*. Optical density at 600 nm was measured using the MRX II (Dynex Technologies) plate reader from 0 up to 168 hrs.

RESULTS AND DISCUSSION

The extracts tested include C. *annuum* ether extract (S1E), C. *annuum* methylene chloride (S1M), C. *chinense* ether (H1E), C. *chinense* (H1M). The pure compounds include capsaicin, dihydrocapsaicin, caffeic acid, chlorogenic acid and sitosterol. Ampicillin and methanol were used as controls. Preliminary data showed that among the ten compounds tested, C. *annuum* ether extract (S1E) at 156 and 312 ug/ml exhibited potency in detaching D. *vulgaris* biofilm from the substratum with 18.90% and 18.70%, respectively, difference from the untreated biofilm. C. *chinense* ether extract (H1E) at 625 ug/ml showed 18.49% difference from the untreated one (Figure 1). The overall performance of all extracts was better than that of the pure compounds tested except for S1M at 156 ug/ml where biofilm formation was enhanced. Concentration range used for each compound varied depending on their Minimum Inhibitory Concentration (MiC) values from the antimicrobial screening as shown in Table 1. Per cent difference from the untreated group implies direct proportionality to detached biofilm (Figure 2).

For the antimicrobial screening, the minimum inhibitory concentration (MiC) of H1E against D. *vulgaris* was found at 312 ug/ml while the rest of the extracts was at 156ug/ml (Table 1). When tested against D. *desulfuricans*, the MiC of all extracts was found to be 78 ug/ml (Table 1) suggesting that the second organism was more sensitive than the first one. Corresponding minimum bactericidal concentrations (MbC) were determined as well (Table 1). These data are results of initial screening and confirmatory studies are underway.

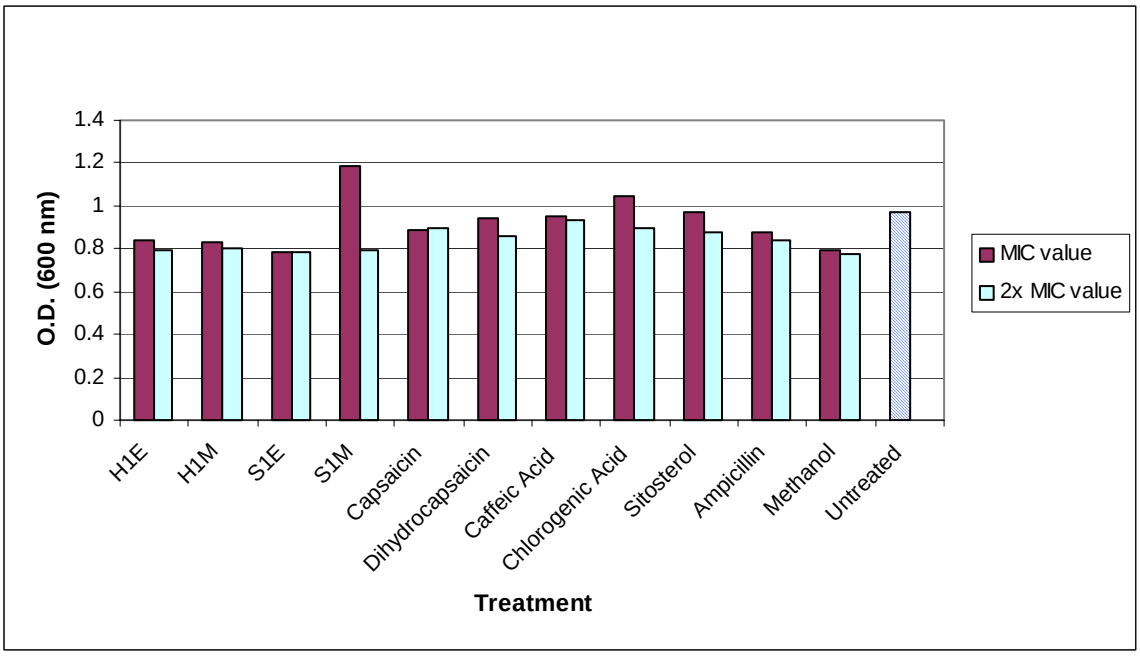

Figure 1. D. *vulgaris* biofilm after treatment with *Capsicum* sp. compounds. OD_{600} shows the quantity of destained biofilm after treatment.

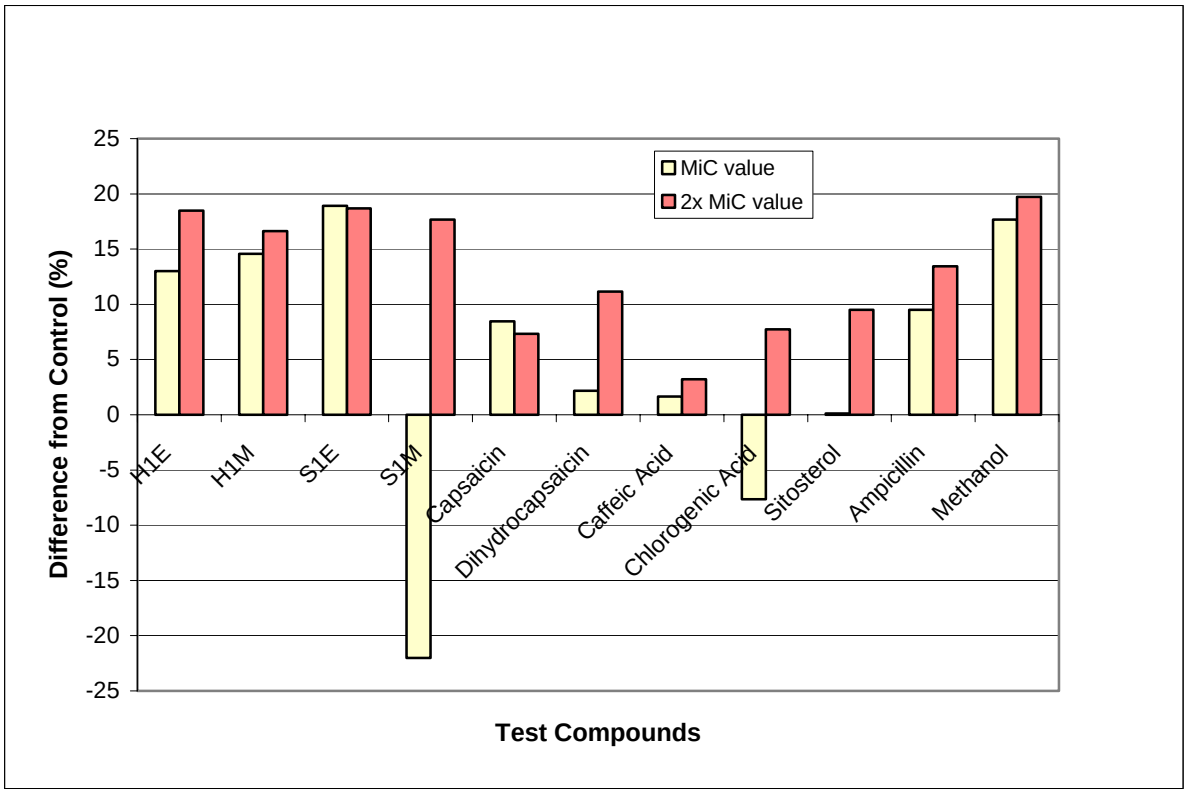

Figure 2. Effect of *Capsicum* sp. Compounds on D. *vulgaris* biofilm. Per cent difference from the control coincides with the detached biofilm.

Table 1. Preliminary data on the Antimicrobial Acitivity of *Capsicum* sp. Compounds against Sulfate-Reducing Bacteria

Agents	*D. vulgaris*		*D. desulfuricans*	
	MiC	**MbC**	**MiC**	**MbC**
H1E	312.5	625.0	78.0	312.5
H1M	156.0	312.5	78.0	312.5
S1E	156.0	312.5	78.0	156.0
S1M	156.0	156.0	78.0	156.0
Capsaicin	156.0	312.5	312.5	1,250.0
Dihydrocapsaicin	240.0	480.0	240.0	960.0
Caffeic Acid	302.5	302.5	302.5	302.5
Cholorogenic Acid	312.5	312.5	312.5	625.0
Sitosterol	4.11	16.43	65.75	65.75
Kanamycin	>200.0	ND	>200.0	ND
Ampicillin	6.25	6.25	6.25	25.0
Methanol	0.625%	1.25%	1.25%	2.5%

Note: ND – Not Determined

Concentrations were all in ug/ml unless otherwise stated.

MiC – Minimum Inhibitory Concentration

MbC – Minimum Bactericidal Concentration

CITATIONS

Eloff, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*. **64**: 711-713.

Djordjevic, D., M. Wiedmann and L.A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol*. **68**: 2950-298-58.