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# Batch culture biodegradation of methylhydrazine contaminated NASA wastewater

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**Abstract**

The batch culture degradation of NASA wastewater containing mixtures of citric acid, methylhydrazine, and their reaction product was studied. The organic contaminants present in the NASA wastewater were degraded by *Achromobacter* sp., *Rhodococcus* B30 and *Rhodococcus* J10. While the *Achromobacter* sp. showed a preference for the degradation of the citric acid, the *Rhodococcus* species were most effective in reducing the methylhydrazine and the reaction product. Removals of more than 50% were observed for citric acid, methylhydrazine and the reaction product when the NASA wastewater was inoculated with the microbes in batch cultures. Simulation and chemical characterization of citric acid and hydrazine mixtures show that the interaction is partly of a chemical nature and leads to the formation of a conjugated UV/Visible absorbing compound. An 'azo' carbonyl derivative of the citric acid, consistent with the spectral data obtained from the investigation, has been proposed as the possible product.

## Introduction

Hydrazine, monomethylhydrazine (MMH) and some of their organic and inorganic derivatives are used as rocket propellants, fuels for aircraft emergency power supplies, in boiler water treatment, and in the manufacture of herbicides and medicines (Schmidt 1984). These uses have resulted in the accumulation of hydrazine wastes, such as scrubber water from the National Aeronautics and Space Administration (NASA) operations. The parent hydrazine as well as its salts have been found to be carcinogenic in mammals. Exposures to hydrazine and monomethylhydrazine liquids and/or their vapors can lead to systematic deleterious effects involving the central nervous system (IARC 1974).

In many industries and in particular in aerospace operations, the corrosiveness of waste hydrazine is being neutralized by the addition of calculated amounts of citric acid solution. At the NASA-Kennedy Space Center in Florida, USA, vent gaseous nitrogen ($GN_2$), contaminated with hydrazine and methylhydrazine vapors, are scrubbed with citric acid solution to absorb the hydrazines (hydrazine and methyl hydrazine) in scrubber liquor. While the cleansed $GN_2$ is vented to the atmosphere, environmentally hazardous citric acid/hydrazine mixtures are being accumulated, posing decontamination challenges.

Earlier studies have shown that hydrazine is amenable to microbial degradation in various environments. Microbial degradation was found to be responsible for 20% of hydrazine disappearance in Arredondo soil (Ou & Street 1987). *Achromobacter* sp. isolated from the soil was characterized to be responsible for the observed enhanced degradation. Methylhydrazine has also been shown to undergo degradation in various soil samples when inoculated with *Achromobacter* sp. (Ou & Street 1988; Ou 1988).

In this paper, we report the biodegradation of major organic constituents present in NASA scrubber wastewater by *Achromobacter* sp. and *Rhodococcus* sp. in batch cultures. A wide variety of organic compounds have been degraded by the *Rodococcus* sp. (Häggblom et al. 1988; Straube 1987). Its short ac-

climation period and relatively rapid growth rates provide great advantages in the bioremediation of industrial wastes.

## Materials and methods

### *Bacteria source and culture conditions*

*Achromobacter* sp. (ATCC 21910) was obtained from the American Type Culture Collection (ATTC) while *Rhodococcus* B30 and J10 strains were revived from previous isolates preserved by the Environmental Science Group of our University's Department of Agriculture. *Achromobacter* sp. was grown in ATCC Culture Medium 457 with the following constituents per liter: $K_2HPO_4$, 7.32 g; Ammonium tartarate, 4.6 g; $KH_2PO_4$, 1.09 g; $MgSO_4.7H_2O$, 0.04 g; $FeSO_4.7H_2O$, 0.04 g; and $CaCl_2.2H_2O$, 0.014 g (pH 7.5). The *Rhodococcus* sp. were grown in a modified Basal Salt Media with the following composition per liter: $K_2HPO_4$, 1.6 g; $NH_4NO_3$, 0.5 g, $KH_2PO_4$, 0.4 g; NaCl, 0.1 g, $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_20$, 0.025 g, $FeCl_3.6H_2O$, 0.003 g, glycerol, 0.1 ml (pH 7.0). All the chemicals, except ammonium tartarate, were from Fisher Scientific and were of Certified A.C.S. grade. Ammonium tartarate was obtained from Aldrich Chemicals. Both media and handling equipment were sterilized by autoclaving at 121 °C for 15 min.

The culture flasks, each containing ∼100 ml growth cultures, were agitated on an orbital shaker at 200 rpm and maintained at their respective optimal temperatures in the laboratory's environmental chamber. The design ensured a high surface-to-volume ratio and allowed sufficient diffusion of oxygen to the bottom of the flasks. The bacterial stock cultures were maintained by serial transfers to fresh media every 3–5 days. Purity and phenotypic behaviors were ensured by continuous abbreviated characterization of each subculture. Microbial growth in the batch cultures was monitored by UV/Visible spectrophotometry, turbidimetry and total organic carbon measurements.

### *Harvesting and purification of cell suspension*

Concentrated cells for biodegradation experiments were harvested from the cultures by differential centrifugation. Cultures were harvested during their period of maximum stability and viability, characterized by the late logarithmic growth phase. Harvesting of the cells was done by filtering the cultures of each strain through Whatman No. 1 filter paper to remove any precipitate that may be present. The filtrate obtained was then centrifuged at 10,000 g for 10 min (Fischer-Marathon Model 22KR refrigerated superspeed centrifuge). The resulting cell pellet was washed twice by re-suspending in about 10 ml of sterile $KH_2PO_4/K_2HPO_4$ buffer (pH = 7.2), and centrifuged. The washings were repeated for three more times and the final re-suspended cells, estimated at $>300$ mg (wet weight) cells $ml^{-1}$, were used for the degradation experiments. The cell harvesting were all done aseptically.

### *Incubation of NASA wastewater with harvested microorganisms*

Harvested cells were used to inoculate sterilized NASA wastewater samples. 100 ml of diluted samples, each containing about 150 ppm total organic carbon, were inoculated with 10 ml of the resuspended cell concentrate. The carbon and nitrogen, from citric acid and hydrazine contaminants present in the inoculated wastewater, replaced the C and N source for the microbes usually derived from the ammonium tartarate, ammonium nitrate and glycerol in the original growth media. After pH adjustment to 7.5 for the *Achromobacter* sp. and 7.0 for the *Rhodococcus* sp., the inoculated samples were incubated in the laboratory's environmental chambers at ∼27 °C. All experiments were conducted in duplicates, accompanied by blank controls. Other quality control and quality assurance measures such as the daily calibration of the analytical equipment and repetitive experiments, for data validation, were also implemented. *Achromobacter* sp., *Rhodococcus* B30 and *Rhodococcus* J10 were investigated for their ability to degrade the residual citric acid, methylhydrazine and their possible reaction product present in the NASA wastewater.

Analytical studies were also conducted on surrogate samples simulated by neutralizing 15% citric acid solution with hydrazine until a pH of about 5. This was modeled after the industrial NASA wastewater citric acid treatment process. UV/Vis, HPLC and NMR analysis were done to establish possible chemical interactions between citric acid and hydrazine. The UV/Vis spectra of the aqueous reaction mixture were recorded *in-situ*. For the NMR characterization of the reaction mixture, $D_2O$ was used to prepare the initial citric acid solution (micro-scale) before the addition of hydrazine.

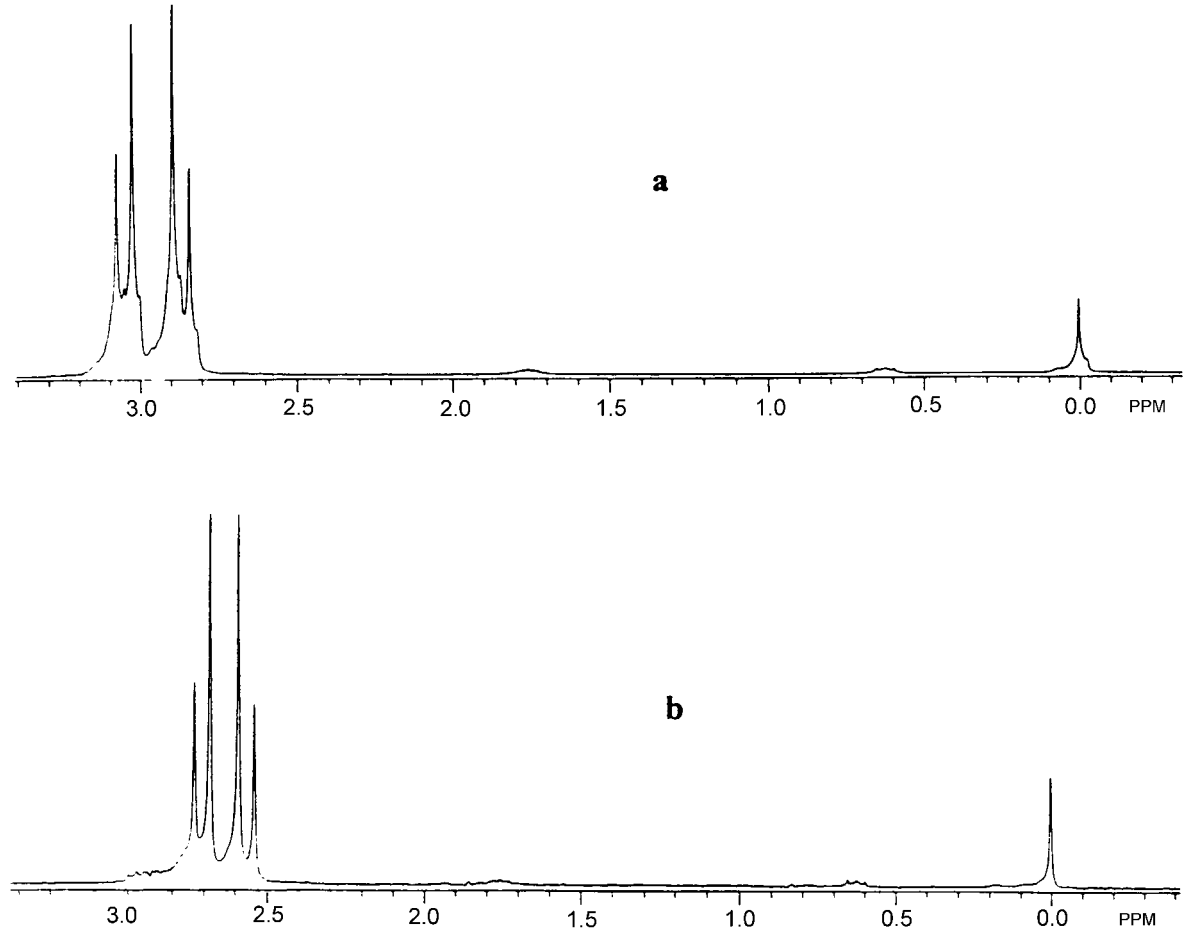


*Figure 1.* $^{1}$H NMR spectra of (a) pure citric acid and (b) citric acid and hydrazine mixture.

*Analytical methods*

Samples were aseptically collected from the inoculated flasks at regulated intervals. The samples were filtered, centrifuged and the supernatant collected. After appropriate dilution, the supernatants were subjected to both TOC and HPLC analysis. A Rosemount–Dohrmann DC-180 TOC analyzer utilizing the persulfate oxidation method was used for the total organic carbon analysis while a Hewlett Packard Model 1050 HPLC system with a variable wavelength UV detector was used for the HPLC analysis. Reverse-phase HPLC separation of the collected samples were accomplished on an ODS-Hypersil $C_{18}$ column (25 cm $\times$ 4.6 mm I.D). Programmed mixtures of 0.25 m $H_2SO_4$, methanol and acetonitrile (Fisher Scientific, HPLC Solvent Grades) served as the eluent with a flow-rate of 0.8 ml min$^{-1}$. The wavelength of detection was at 254 nm. Independent UV/Vis spectroscopic analysis was also undertaken for methylhydrazine after derivatization with *p*-dimethylaminobenzaldehyde.

Upon completion of the degradation experiment, the samples were adjusted to pH $<$ 3 and extracted with dichloromethane (DCM) for possible acidic metabolites. Further extraction with DCM were conducted after adjusting the pH to $>$ 11 for possible base/neutral fractions. The DCM extracts were concentrated on a rotary evaporator. The polar (acidic) fractions were treated with diazomethane to yield possible methyl ester derivatives before the GC/MSD analysis (Schwartz & Bright 1974).

The GC analysis were conducted with a HP 5890 gas-chromatograph coupled to an HP 5970 Mass Selective Detector (MSD). The injection and detector temperatures where set at 250 °C while the oven was temperature programmed (100 °C held for 2 min, then ramped to 210 °C at 7 °C min$^{-1}$). A HP-5MS column

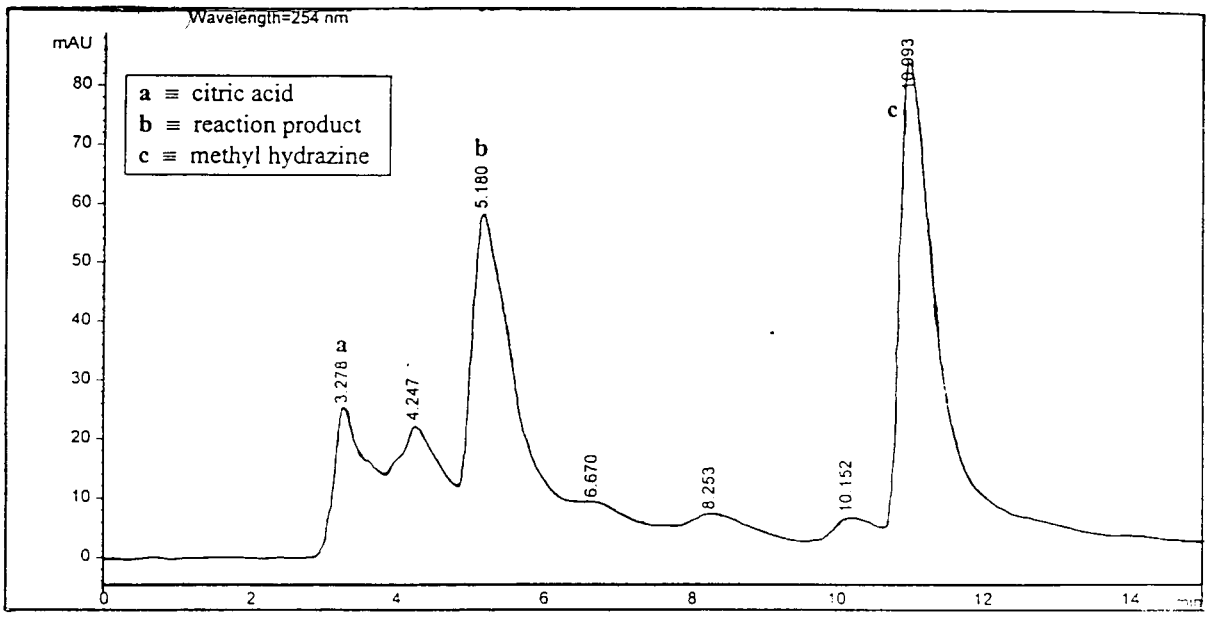


*Figure 2.* HPLC chromatogram of NASA industrial fuel wastewater.

(low breed 5%-diphenyl–95%-dimethylsiloxane copolymer), 0.25 mm ID., 0.25 $\mu$m (film thickness) and 30 meters long was used for the analysis.

The $^1$H NMR analysis of the surrogate mixture and that of pure citric acid were conducted using a General Electric Model QE-300 nuclear magnetic resonance spectrometer. The spectra were acquired in $D_2O$ at a rate of 16 scans per second. Tetramethylsilane [$(CH_3)_4Si$] was used as the internal reference standard. A Hewlett Packard Model 8453 single beam UV spectrometer was used for the scanning UV/Vis measurements while a portable HACH DR/2000 UV spectrometer was used for the single wavelength determination of the methylhydrazine after derivatization.

## Results

### *Surrogate mixtures of citric acid and hydrazine*

Instrumental analysis of surrogate mixtures of citric acid and hydrazine at pH of about 5 gave distinct results from those observed for pure hydrazine and citric acid. In the UV/Vis spectra, while neither hydrazine nor citric acid absorbs significantly above 250 nm, the mixture exhibited a second maxima at about 470 nm. The $^1$H NMR also exhibited a high field shift of the citric acid methylene protons from $\delta$ 2.9 and 3.0 for the pure citric acid to $\delta \sim 2.6$ and $\sim$2.7 respectively for the reaction mixture (Figure 1).

Furthermore, isocratic HPLC chromatogram of the citric acid/hydrazine mixture gave three peaks. By analyzing pure samples of hydrazine and citric acid under the same conditions, two of the peaks, with retention times 2.4 and 2.8 minutes, were identified as hydrazine and citric acid residues respectively. The third peak with a retention time of 3.1 minute was assigned to a product of the reaction between hydrazine and citric acid. A model mixture simulated from citric acid and methylhydrazine and analyzed under gradient elution with a different solvent system, however eluted in the order; citric acid, possible reaction product and methylhydrazine.

### *Degradation of NASA wastewater*

The HPLC analysis of the industrial NASA wastewater is shown in Figure 2. The wastewater in use for this work were accumulated when methylhydrazine was the predominant propellant in use at the NASA Kennedy Space Center and analysis of pure samples of citric acid and methylhydrazine led to the assignment of the bands as shown in Figure 2. In addition

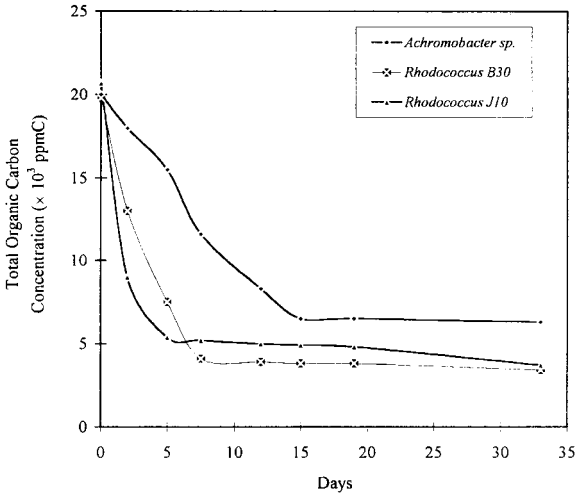


*Figure 3.* Changes in total organic carbon concentration during the degradation of NASA fuel wastewater by various microbes.

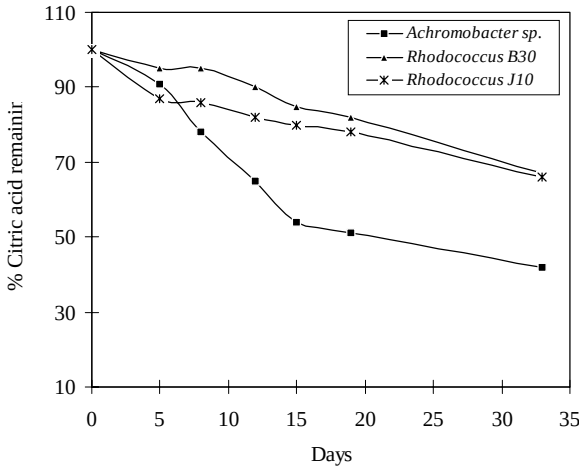


*Figure 4.* Changes in citric acid concentration following batch inoculation of NASA fuel wastewater with various microbes. The initial citric acid content of undiluted NASA wastewater is about 5 mg $ml^{-1}$.

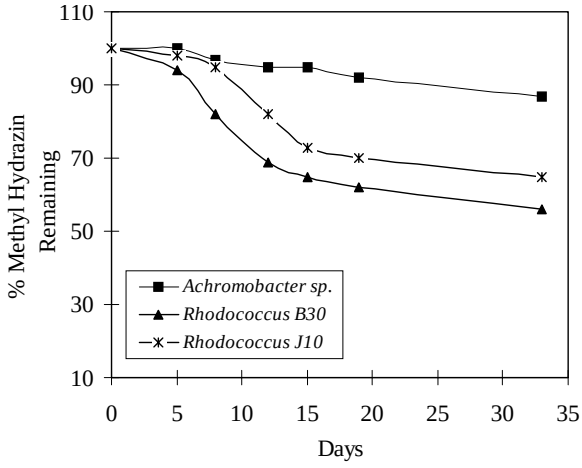


*Figure 5.* Changes in methyl hydrazine concentration following batch inoculation of NASA fuel wastewater with various microbes. The initial methyl hydrazine content of undiluted NASA wastewater is about 11 mg $ml^{-1}$.

to the three major organic substances, the industrial wastewater contains traces of other species that are evident in the HPLC chromatogram.

Figure 3 shows the variations in total organic carbon present in NASA wastewater during degradation in batch cultures by various microbes. The organic carbon content of the wastewater should originate mainly from the three major components.

Figures 4–6 show the degradation curves for citric acid, methylhydrazine and the reaction product, respectively, after inoculation by various microbes in batch cultures. Standard samples of citric acid and methylhydrazine were used to quantify their respective amounts during the degradation while those of the ascribed product were evaluated from carbon balancing based on the postulated structure and the TOC results. Methylhydrazine concentrations were further confirmed from the independent UV/Vis measurements done after derivatization.

## Discussion

### *Reaction between citric acid and hydrazines*

Chemical and instrumental analysis of surrogate NASA wastewater mixtures simulated by reacting the hydrazines with citric acid indicated that the interaction of both compounds leads to a chemical reaction that results in the formation of a chemical product. The three bands observed in the HPLC chromatogram of the mixture unambiguously confirms the presence of a third compound, a reaction product. Though this reaction was observed to be exothermic with temperatures rising up to 40 °C from room conditions, the reaction proceeded very slowly yielding a fully-developed surrogate mixture after ∼48 h, that is characterized by a deep-brown coloration resembling that of the industrial NASA wastewater supplied.

The absorption at ∼470 nm in the UV/Vis spectrum suggests the presence of a conjugated chromophore while the NMR shifts to lower delta values indicate a more shielded environment for the citric acid methylene protons in the mixture relative to the

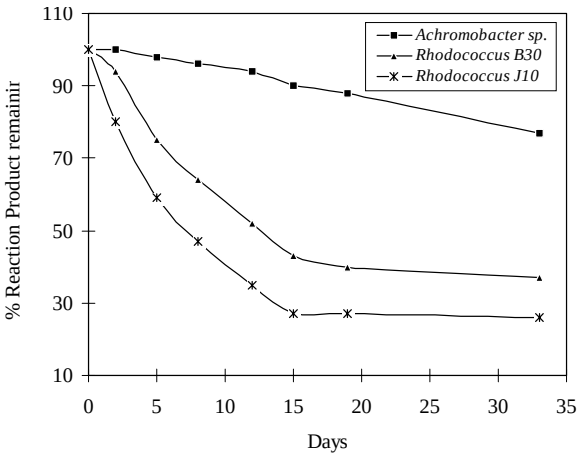


*Figure 6.* Changes in the concentration of the reaction product between methyl hydrazine and citric acid following batch inoculation of NASA fuel wastewater with various microbes.

pure citric acid. Though the identity of this product is yet to be fully confirmed, we postulate the reaction pathway (Figure 7) which leads to an 'azo' compound as the most probable reaction product.

The initial reaction is the condensation of the hydrazine group with one of the end acidic groups and the elimination of water. This is followed by the elimination of a hydrogen molecule and the formation of an N=N double bond in conjugation with the carbonyl group. This conjugation would be responsible for the orange-brownish coloration of the wastewater and the broad absorption in the UV/Vis spectrum around 470 nm. The two doublets observed in the $^1$H-NMR spectra are assigned to the methylene groups from the citric acid skeleton. Citric acid is a typical example of molecules with prochiral centers in which two H-atoms attached to a methylene group are not chemically equivalent (Figure 8). The two hydrogen atoms are not interchangeable and are therefore not chemical shift equivalent (Silverstein et al. 1991). Thus one set of identical protons causes a splitting of the other set of equivalent protons, and vice versa, giving rise to the observed double doublet of the methylene protons in the $^1$H NMR spectrum.

The presence of residual amounts of both the citric acid and the hydrazine is suggestive of a reversible reaction. Also the probability exists for the reaction to occur at any one or more of the citric acid functional groups, though no evidence has been obtained in our work to that effect. However, elucidation of the unambiguous structure of this compound is continuing through the use of citric acid and methyl hydrazine containing isotopically-labeled atoms.

### *Microbial degradation of NASA wastewater containing citric acid-methyl hydrazine mixtures*

The organic suites present in NASA wastewater were found to be susceptible to biodegradation by both the *Achromobacter* sp. and *Rhodococcus* B30 and J10 strains. An acclimation period of less than 2 days was followed by rapid degradation of the organic substances. In relative terms, the consumption and degradation of the organic carbon sources present in NASA wastewater was more rapid with both the B30 and J10 strains of the *Rhodococcus* sp than with the *Achromobacter* sp. Almost 80% of the organic carbon had been consumed by the 7th day in experiments with both strains while only about 50% depletion of the carbon was observed for *Achromobacter* sp. within the same period.

The J10 strain of the *Rhodococcus* sp. exhibited a special capability of being able to degrade the organic carbons under a variety of conditions. No significant difference was obtained in terms of utilization of the carbon in the NASA wastewater by this microorganism with and without added ammonia nitrate, one of the major natural inorganic nutrients for the bacteria. The microbe was also the only one found to degrade appreciable amounts of the organic carbon contained in the wastewater in the absence of any additional glycerol which is the natural carbon source for the B30 and J10 strains of the *Rhodococcus*. For both species however, the supply of calculated amounts of glycerol, the microorganisms' natural carbon source was found to catalyze the biodegradation of the organic substances present in the wastewater.

The HPLC analysis yielded information on the extent of microbial degradation of the specific organic compounds present in the wastewater. The initial target for degradation by the *Achromobacter* sp. was the residual citric acid present in the wastewater. For this microbe, more than 50% of the citric acid components of the wastewater were degraded by the 15th day after inoculation. Evidence of significant degradation of both the major product and the residual methyl hydrazine were however observed from the 3rd week after significant reduction in the citric acid content.

The *Rhodococcus* B30 was found to be most effective in the degradation of methylhydrazine in batch cultures among the three microbes screened. About 40% of the hydrazine residues present in the waste-

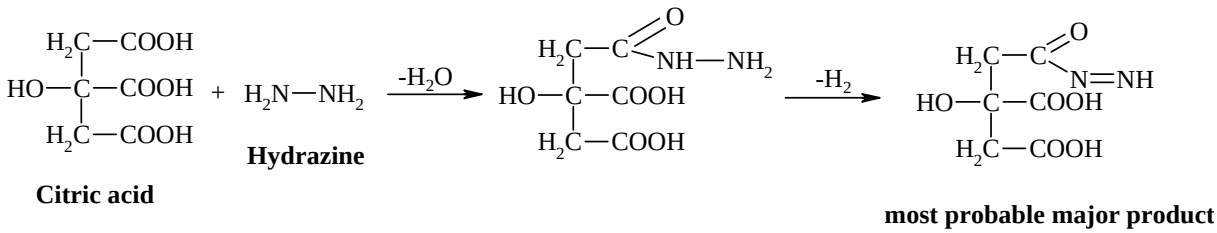


*Figure 7.* Postulated reaction of citric acid and hydrazine

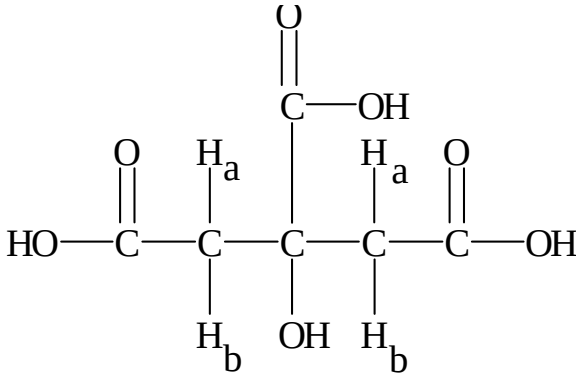

*Figure 8.* Citric acid showing the apparently identical methylene protons in different chemical environment. In $^{1}$H NMR, 'a' ~2.5 ppm while 'b' ~2.7 ppm.

water matrix were degraded by this microbe within the first 15 days of microbial incubation. The *Rhodococcus* J10 exhibited similar behavior though degradation was at a slower rate than that observed for the B30 strain. All three microbes showed longer lag period for methylhydrazine degradation in comparison to citric acid.

The postulated reaction product was also a target of rapid degradation by both strains of the *Rhodococcus* sp. By the 15th day of microbial incubation, more than 55 and 70% of the reaction product had been degraded by *Rhodococcus* B30 and J10 respectively. The utilization of one major component of the NASA wastewater as a substrate by each of the microbes makes mixed culture biodegradation of the industrial waste particularly viable.

Undiluted NASA wastewater contains about 5 mg ml$^{-1}$ of citric acid and 11 mg ml$^{-1}$ of methylhydrazine. Microbial degradation was still observed, at about the same rates, when the microbes were inoculated to the undiluted wastewater as with the diluted feed stocks. This implied that these levels of contaminants in the full-strength wastewater do not inhibit microbial activities, making the method potentially cost-effective for the degradation of the industrial wastewater.

No new bands appeared on the HPLC chromatograms obtained during the microbial degradation. A broad number of organic and inorganic compounds as well as ions absorb UV radiation at 254 nm and so can be conveniently and directly detected at this wavelength. In addition the GC/MSD chromatograms of the processed and derivatized samples did not reveal any new bands attributable to metabolites. The absence of any metabolic bands on both the HPLC and the GC chromatograms after biodegradation suggests strongly that microbial action on the NASA scrubber wastewater leads mainly to the mineralization of the organic constituents.

## Conclusion

The results of this study show that NASA wastewater containing methylhydrazine propellant residues and citric acid as well as their reaction product are amenable to microbial degradation. Both the citric acid and the methylhydrazine residues present in the wastewater in addition to their reaction product were all degraded significantly by *Achromobacter* sp. and *Rhodococcus* B30 and J10. While the *Achromobacter* sp. mainly targeted the residual citric acid in the industrial NASA wastewater matrix, the *Rhodococcus* B30 and J10 utilized mostly the methylhydrazine and the reaction product, respectively, as substrates for their metabolic energy.

These promising degradative abilities exhibited by these microbes, has now led to their further investigation on continuous biodegradation studies being carried out in flow-through experiments using solid support media (biofilms). These column reactor experiments are designed to yield increased biomass concentrations and enable the investigation of the effects of various experimental conditions on process effi-

ciency during microbial degradation of the wastewater in the reactors.

## Acknowledgments

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