Bacterial Strains for Morpholine Degradation: An adaptation and screening strategy

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***Abstract*—** Morpholine (C4H9ON) is a biologically active environmental xenobiotic often found as a pollutant in the natural environment. Morpholine and its derivatives are used for diverse industrial purposes and if present in effluents often pass through a biological treatment system unchanged. Further, under certain environmental conditions morpholine gets modified to N-nitrosamine which is a proven carcinogen. Till date several authors have described the ability of sludge-derived *Mycobacterium* or *Pseudomonas* strains to degrade morpholine. We here report a strategy for acclimatizing several fast growing gram negative and positive bacteria for near complete removal of morpholine from industrial effluents. In the present study a cohort of bacterial strains has been used for in vitro degradation of morpholine. The cultures were adapted in a step-wise manner in increasing concentration of morpholine. Over seventy different bacteria were screened and it was observed that nearly 10% of the exposed bacterial cohort was successfully able to use morpholine as the sole source of carbon and nitrogen and survive in morpholine concentrations ranging from 0.1 to 0.25%. These strains were able to degrade morpholine when grown individually and also as a consortium of bacterial mix. The decrease in morpholine concentration in the culture medium was estimated using gas chromatography.

***Keywords*—**Adaptation, gas chromatography, metabolic adaptation, morpholine biodegradation.

I. Introduction

BIOREMEDIATION is a waste management technique that involves the use of organisms to remove or neutralize pollutants. According to the Environmental Protection Agency, USA (EPA), bioremediation is a "treatment strategy that makes use of naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances". These can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site, while ex situ involves the removal of the contaminated
material to be treated elsewhere. In a non-polluted environment, bacteria, fungi, protists, and other micro-organisms are constantly at work breaking down organic matter. When exposed to an organic pollutant such as oil spill, most of the existing population would die, while a small fraction capable of degrading the organic pollution would survive. Bioremediation works by providing supporting nutrients to these small fractions along with other conditions that encourage their rapid growth. Hence bioremediation provides a technique for cleaning up pollutants by enhancing the naturally occurring biodegradation processes.

A Xenobiotic is a foreign chemical substance found in the environment or within an organism that is not normally naturally produced by or expected to be present within that organism. Morpholine is a biologically active environmental xenobiotic found as a pollutant in the natural environment. Morpholine is a common organic synthesis reactant used in many industries ranging from petroleum refining to nuclear power generation. It is also a common component of organic syntheses of many pharmaceuticals. It is a colorless, oily, hygroscopic, volatile liquid with a characteristic amine ("fishy") odor and is completely miscible with water, as well as with many organic solvents, but has limited solubility in alkaline aqueous solutions. Worldwide morpholine production is estimated at nearly 25,000 tons per year. While morpholine itself has shown little toxicity beyond slight mucous membrane irritation in rats [1,2], when in the natural environment its secondary amine functionality leads inevitably to nitrosation to form N-nitrosomorpholine, a well-characterized carcinogen [3]. The large-scale annual usage of morpholine and its potentially carcinogenic effects motivate investigation of the metabolic pathways involved in its biodegradation.

Morpholine biodegradation has commonly been done using *Mycobacterium* genus. Morpholine monooxygenase is an important enzyme in this pathway [3-5]. This enzyme contains a cytochrome P450 catalytic subunit and catalyzes the biotransformation of morpholine to 2-(2-aminoethoxy) acetic acid [4]. Many different species of *Mycobacterium* have been shown to degrade morpholine via shared group of degradation reactions for cyclic amines. The characterization of these reactions has been assayed by direct means but with little information about the enzymes involved in the reactions [5].

However, using *Mycobacterium* for this purpose has its own limitation like slower growth rate and pathogenicity. Therefore it becomes important to explore other microbes from diverse background for their biodegradation capability. This study was undertaken to screen and adapt bacteria to degrade morpholine when grown individually and also as a consortium of bacteria.

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II. Materials & Methods

A. Source of Samples & Reagents

Microorganisms used for biodegradation of morpholine were isolated from human body fluids, textile industry effluent, a local lake namely Husain Sagar in Hyderabad, India. Samples were collected in a clean, sterile plastic container, transferred to the laboratory. All chemicals were purchased from Sigma-Aldrich Corporation and were Analytical Grade.

B. Isolation of Microorganisms

For the initial isolation and cultivation of microorganisms Luria broth was used. 1ml of sample was taken in a test tube and isolation was done by standard serial dilution, pour plate and streak plate methods. Pure colonies isolated from the natural samples were characterized using morphological and biochemical tests.

C. Microbial Characterization

The biochemical tests performed in the present study were Gram staining and growth on selective medium (Hi-Chrome Media M1353) to identify the bacteria.

D. Acclimatization: Microbial Adaptation

Mineral Salt Solution (MSS) [6] was used as enrichment media for adaptation studies in presence of gradually increasing concentration of morpholine. Cultures were incubated at 37^0 C for different time points and absorbance at 600 nm was taken as a measure of growth.

E. Degradation Studies & Morpholine Estimation by GC

In the present study measurement of growth of bacteria and GC analysis for left over morpholine were chosen for confirming adaptation of bacteria. The method described by [7] was used for estimation of morpholine in the spent culture medium. The biological treatment studies included the microorganism/s capacity to degrade morpholine (from 0.1% onwards) and the remaining morpholine was estimated by GC. Gas Chromatograph Shimadzu GC-2010 Plus was used for morpholine quantification. Morpholine solution (0.05-2.5% v/v), in methanol, was used to obtain a standard curve. The culture supernatant was cleared by centrifugation and morpholine was estimated after 1:10 dilution with methanol. MSS was used as blank (1:10 dilution). The AUC obtained with unknown sample was extrapolated to the standard curve for calculating morpholine amount in the sample/spent medium (7).

III. Observation & Results

The isolates were identified using HiCrome selective medium. Figure I shows the bacterial diversity of the cohort used for further morpholine adaptation.

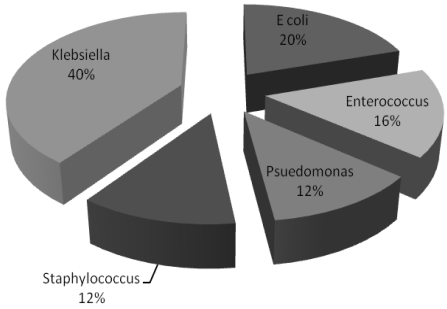

Fig. 1 Bacterial diversity observed in the cohort

Acclimatization to increasing morpholine concentration:

Overnight cultures of different bacteria were inoculated at 1:100 dilutions in liquid MSS media starting with 0.1% morpholine and kept for incubation at 37^0C and bacterial growth was monitored with absorbance at 600nm and when an OD of 0.5 was reached a subsequent 1:100 dilution was prepared and spread on MSS Agar plates with 0.1% morpholine. A total of 25 bacteria out of 70 isolates grew on 0.1% morpholine and were used for further acclimatization The growing culture was centrifuged and bacterial pellet was re-suspended in MSS media with gradually increasing concentrations of morpholine up to a maximum of 0.25%. For each step in the acclimatization, culture was also plated on a MSS agar plate with matching morpholine concentration and preserved as glycerol stocks for further characterization.

Out of 25 isolates, majority of the cultures i.e. 48% (n=12) showed successful growth till 0.16%; whereas 20% (n=5) stopped growing at 0.14% and 8% (n=2) of the isolates were unable to metabolize more than 0.1% morpholine (Figure II). Staphylococcus sp. (CIG 146) was the only strain in successful degrading morpholine up to 0.25% followed by *Klebsiella* where all isolates successfully adapted to 0.16% morpholine. *E.Coli* and *Pseudomonas* sp. were found to be least adaptable as these isolates survived till 0.1-0.14% morpholine only (Figure III). As can be seen from Figure IV the number of surviving bacterial cells decreased on each subsequent day after addition of morpholine to the culture medium. There was a change observed in the morphology of the bacterial colonies growing on the plate.

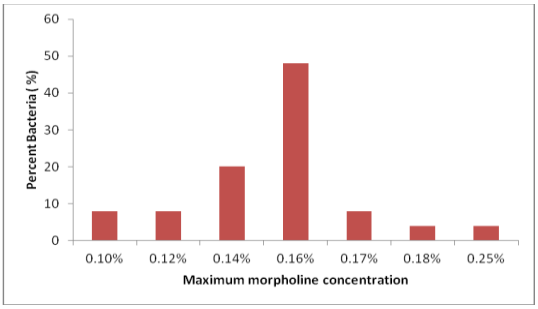

Fig. 2 Percentage of successfully adapted isolates

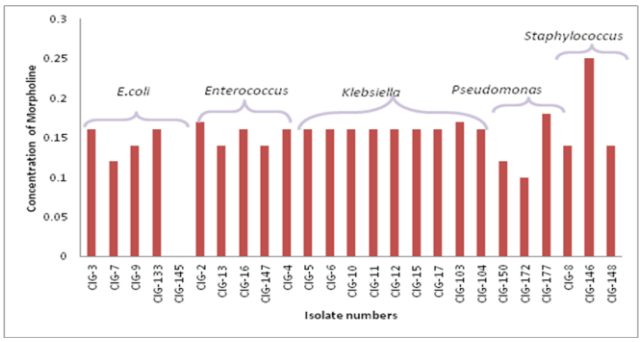

Fig. 3 Distribution of bacterial strains and percentage of morpholine metabolized

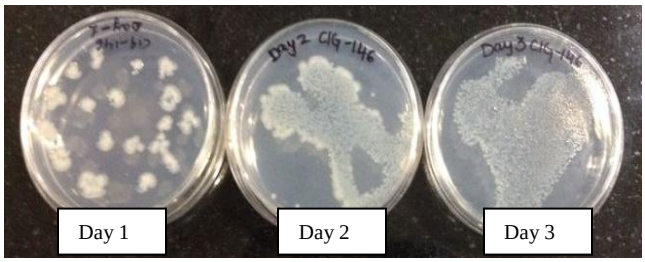

Fig. 4 Growth profile of CIG 146 on 0.25% morpholine

Estimation of Morpholine in Culture Supernatant:

The quantification method used was percentage reduction in AUC. AUC obtained for positive control, viz., 0.2% morpholine was 1648380.9 and for the spent culture supernatant was found to 77.8 only indicating that the bacterial metabolism led to 99.9% degradation of morpholine.

IV. DISCUSSION

When the bacteria were grown under the presence of increasing concentration of morpholine a differential adaptation rate was observed. At low concentration up to 0.14%, all the isolates were able to grow. It was observed that isolates belonging to *Enterococcus sp.* and *Escherichia sp* were unable to survive in 0.16% morpholine. A single isolate *Staphylococcus* sp. was found to degrade 0.25% morpholine and grow in the absence of any other source for carbon and nitrogen.

In an attempt to understand the underlying mechanism for morpholine degradation the sensitivity to β-Lactam class of antibiotics was determined in the isolates before and after morpholine adaptation. It was found that at each stage of adaptation the zone of inhibition observed in classical Kirby-bauer disc-diffusion method [8] was found to increase indicating a probable role of β -lactamases in the degradation of morpholine (data not presented). In conclusion it can be said that using natural microorganisms can be a promising approach to treat morpholine containing industrial waste water so as to reduce its hazardous impact in the environment.

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