

## UNRAVELING THE NOVEL BACTERIAL ASSISTED BIODEGRADATION PATHWAY OF MORPHOLINE

RUPAK KUMAR<sup>\*1</sup>, SUMAN KAPUR<sup>2</sup>, AND SRINIVASA RAO VULICHI<sup>2,3</sup>

<sup>1</sup>Central Drugs Standard Control Organization, New Delhi, India

<sup>2</sup>Birla Institute of Technology and Science – Pilani, Hyderabad Campus, Hyderabad, India

<sup>3</sup>SVU College of Pharmaceutical Sciences, Sri Venkateswara University, Tirupati, India

Most xenobiotics are biodegradable, persistent or recalcitrant in nature. Morpholine, a typical xenobiotic, was initially regarded as recalcitrant, however, later proved to be biodegradable by bacterial species like *Mycobacterium* and *Pseudomonas* in particular. However, establishing the metabolic pathways involved for the successful biodegradation of morpholine is challenging because of its extreme level of water solubility that affects various analytical procedures. In addition, to date, no suitable analytical methods have been reported to directly estimate morpholine and its degradable products or intermediates. Nevertheless, methods, especially optical density, gas chromatography and mass spectrophotometric analysis, could indirectly estimate the degradation product(s) of morpholine formed as a result of its biotransformation. In the present study, the degradation pathway of morpholine was ascertained by selected bacterial isolates by measuring their capacity to degrade morpholine. Based on this analysis of culture filtrates, it was determined that the novel isolate is the genus *Halobacillus blutaparonensis* which follows the diglycolic acid route from the metabolic degradation pathway of morpholine to induce one of two branches of the morpholine biodegradation pathway. In the presence of concentration of morpholine, out of two branches of morpholine degradation one branch is induced, while the other branch is inhibited. Whatever the branches with regard to the degradation pathway of morpholine exhibited by bacteria are, ammonia is the final end product of degradation which might be biochemically utilized by the isolate.

**Keywords:** morpholine, xenobiotic, recalcitrant, glycolic acid route, ammonia

### 1. Introduction

Environmental pollution has become a global problem. Due to the indiscriminate and frequent release of xenobiotics as a result of different anthropogenic activities, each and every day our environment becomes increasingly devastated by the pollutants. Morpholine (1-Oxa-4-azacyclohexane) is one such heterocyclic xenobiotic organic chemical with different versatile applications in various processes in the rubber, paper, iron, textile, personal care, pharmaceutical and agricultural industries amongst others. As a consequence of its vast operational usage, a significant amount of this chemical is released into the environment through the differential process of discharging at both micro- and macro-concentrations. Therefore, it is necessary to mention that anthropogenic environmental pollutants, even at low concentrations, often produce deleterious effects on organisms, which are

difficult to predict because measurable effects are expressed only after prolonged exposure.

In the environment, the majority of exposure to morpholine originates from water and leads to the formation of the carcinogen N-nitrosomorpholine (NMOR) by the process of natural nitrosation [1] (Fig. 1). Furthermore, it is pertinent to mention that this process of nitrosation may occur in biological systems when directly consumed, ingested, inhaled and applied to the skin. In addition, NMOR is known as a mediator of various debilitating cancers associated with organs like the digestive tract, respiratory tract, kidneys and liver, which is eventually biomagnified through different trophic levels of biota by its application or the intake of polluted water leading to this carcinogen entering the food chain. In

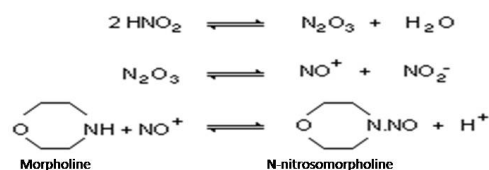


Figure 1: Formation of NMOR

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\*Correspondence: [rupakraman@gmail.com](mailto:rupakraman@gmail.com)

this regard, it would be best to provide a solution for its efficient discharge or effective removal by different physical and chemical processes. Recently, photocatalysis using catalysts irradiated by ultraviolet or visible light has been applied for the mineralization of toxic organic dyes in water and carbon dioxide [2, 3]. However, a cost-effective, environmentally-friendly biological tool powered by microbes has been widely used as an ancient core concept for the purpose of conserving the natural environment and resources to curb the negative impacts on biotic components. Therefore, a sustainable solution driven by microbes must be explored to elucidate the degradation pathway and measure how potent microbes are for the purposes of decontaminating a wide range of pollutants and their mitigation.

In general, most pollutants are organic and may be biodegradable (transformed by biological mechanisms which might lead to mineralization), persistent (fail to undergo bioremediation in the environment or under a specific set of experimental conditions) or recalcitrant (inherently resistant to biodegradation) in nature. Biogenic or naturally occurring compounds are biodegradable while man-made (anthropogenic) compounds may be biodegradable, persistent or recalcitrant. In terms of xenobiotics that are man-made, the microbial communities present in the environment may not have evolved suitable mechanisms for their degradation. Many possible mechanisms exist which differ from one xenobiotic to another. One common mechanism is the binding of enzymes analogous to their natural substrates which contain xenobiotic functional groups, assuming these do not greatly alter or change the active site which catalyzes a reaction with the xenobiotic. The success of this enzymatic reaction (as a biodegradation mechanism) also depends on other factors such as the ability of the xenobiotic as an inducer or inhibitor and the nature of the product/intermediate formed. Specific to morpholine, the metabolic degradation pathway has been very difficult to establish because of the aforementioned technical limitation.

### 1.1 Sustainable remediation of morpholine and its degradation pathway

Although morpholine was previously thought to be recalcitrant, several microbes have proven to metabolically degrade it. The majority of studies showed that the species *Mycobacterium* and *Pseudomonas* are the two potential bacterial isolates that utilize morpholine as their sole source of carbon and nitrogen, thereby undergoing degradation [4–7]. A few studies have been carried out to understand the biodegradation of morpholine and its regulation [8–10]. Later a hypothetical pathway was proposed for the complete mineralization of morpholine that could proceed via 2-(2-aminoethoxy)acetate to produce its diglycolate salt and/or ethanolamine [5, 11, 12]. These two different routes of degradation are called the ethanolamine/monoethanolamine pathway (Pathway 1)

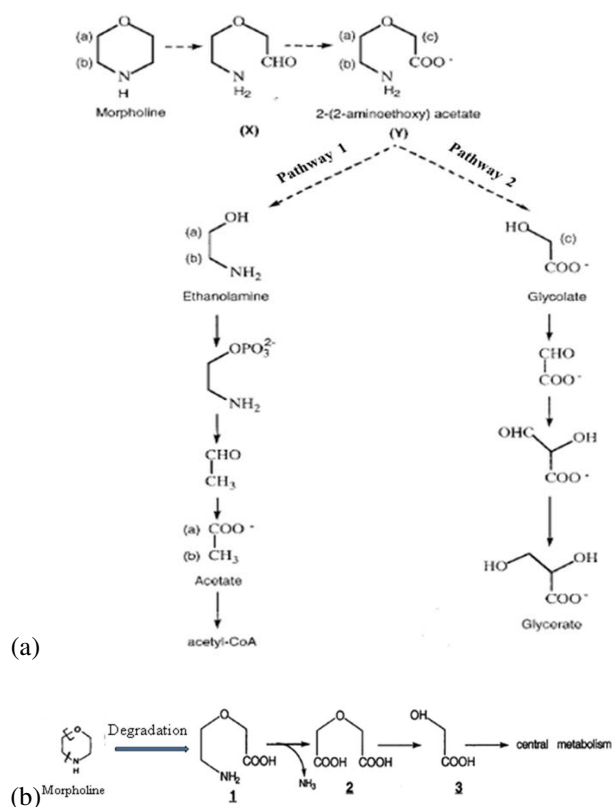


Figure 2: (a) Hypothetical pathway of morpholine degradation where X = 2-(2-aminoethoxy)acetaldehyde, Y = 2-(2-aminoethoxy)acetate and a, b, c indicate the position of carbon atoms in the ring. (b) Postulation of the morpholine degradation pathway after  $^1\text{H-NMR}$  and ion spectroscopic analyses where 1 = 2-(2-aminoethoxy)acetate, 2 = diglycolic acid and 3 = glycolic acid.

and diglycolic acid/glycolate pathway (Pathway 2), respectively (Fig. 2a). The illustrated degradation pathway might start with the cleavage of the C-N bond, leading to the formation of an intermediary amino acid which is followed by deamination and oxidation of this amino acid to form a diacid [11, 12].

The degradation of morpholine via the ethanolamine or glycolate pathways has been described in the presence of *Mycobacterium chelonae* and *M. aurum MO1* [8, 9] (Fig. 2a). The degradation of morpholine is likely to begin with the breakage of a bond between a heteroatom and an adjacent carbon atom by the enzyme *morpholine monoxygenase*, which is responsible for the ring cleavage. *Morpholine monoxygenase* is an important enzyme in the degradation of morpholine as it catalyzes the biotransformation of morpholine to form 2-(2-aminoethoxy)acetic acid and contains a catalytic subunit of cytochrome P<sub>450</sub> [1, 10]. Morpholine could serve as a substrate for flavin-containing monoxygenases or cytochromes P450 which is associated with oxygen consumption [13]. Further inhibitory effects of metyrapone on the degradation of the *Mycobacterium* strain RP1 have been attributed to the involvement of cytochromes P450

in the biodegradation of morpholine [5]. Depending on the concentration of morpholine in the culture medium, one pathway could be expressed while the other might be inhibited [11]. Recently, a new approach was applied in which the culture filtrate was analyzed by  $^1\text{H-NMR}$  spectroscopy and ion spectroscopy to identify the metabolic intermediates of morpholine degradation by *M. aurum* MOI [11, 12] (Fig. 2). Although many different species of *Mycobacterium* have been shown to degrade morpholine via this shared group of degradation reactions, little information is known about the enzymes involved (Fig. 2b). Furthermore, the byproducts of the microbial processes can be indicative of a successful bioremediation process. Consequently, since only hypothetical pathways have been proposed, limited interpretations of various experimental designs can be made to establish the degradation pathway that follows the route of degradation pathway that follows the route of Pathway 1 and /or Pathway 2 via the shared formation of 2-(2-aminoethoxy)acetate.

## 2. Materials and methods

### 2.1 Environmental samples

The sample used in the present degradation study was collected from natural sources (soil) in and around Durgapur Steel Plant, West Bengal, India. The site is located in Durgapur at a latitude of  $51^{\circ}50'43.8''$  north and a longitude of  $8^{\circ}16'35.8''$  west in the state of West Bengal, India. Soil samples consisted of blackish fine-to-medium sub-angular gravel in the upper surface, including fine sand and a high content of iron flecks. Samples were collected in a clean, sterile plastic container before being transferred to the laboratory and stored at room temperature until used for further analysis.

### 2.2 Chemicals and reagents

All chemicals and reagents were of analytical grade and used as received without any further purification. Even though Milli-Q water (Elix Essential 3 Water Purification System with a conductance of 0.12 Siemens) was used to prepare an aqueous solution of reagents, autoclaved double distilled water was used because of the microbial cultures.

### 2.3 Screening, characterization and sequence accession of the morpholine-degrading isolate

For the initial isolation and cultivation of bacteria, ten-fold serial diluted samples were spread onto nutrient agar plates, which were prepared according to the manufacturer's instructions. The specific colonies obtained were subcultured further to isolate the pure bacterial strain. The selected pure bacterial isolate was identified based on morphological, biochemical and molecular characterization. Morphological characterization was achieved by

visually observing colonies in terms of their appearance, shape, color, arrangement, optical nature, margin, texture and elevation. However, the biochemical tests were performed as per standard methods [14]. Furthermore, the pure colony was then identified by 16S rRNA gene sequence analysis.

In order to verify the phylogenetic affiliation of the selected isolate, a single colony was collected for the purpose of DNA isolation (InstaGene<sup>TM</sup> Matrix Genomic DNA isolation kit (Bio-Rad Catalog # 732-6030) as per the kit instructions and procedures) and subjected to Polymeric Chain Reaction (PCR) analysis using primers targeting two 16S rRNA genes [27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'-)]. A PCR reaction (20  $\mu\text{L}$ ) was performed containing 8  $\mu\text{L}$  of Taq DNA Polymerase Master Mix, 1  $\mu\text{L}$  of both 10  $\mu\text{M}$  stock 27F/1492R primers, 9  $\mu\text{L}$  of double distilled water and 1  $\mu\text{L}$  of a DNA template. The PCR (MJ Research PTC-200 Peltier Thermal Cycler; Bio-Rad PTC-200) reaction was conducted using specified conditions from the literature [15]. DNA was denatured at  $94^{\circ}\text{C}$  for 5 mins, followed by 35 cycles of amplification, each consisting of the following components:  $94^{\circ}\text{C}$  for 45 secs (denaturation),  $55^{\circ}\text{C}$  for 60 secs (annealing),  $72^{\circ}\text{C}$  for 60 secs, (extension) followed by  $72^{\circ}\text{C}$  for 10 mins (final extension).

The PCR product was sequenced by Yaazh Xenomics, Chennai, Tamil Nadu, India. The 16S rRNA gene was sequenced using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). The phylogenetic analysis of the sequence using the closely related sequence of BLAST results was performed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple sequence alignments [16]. The resulting aligned sequences were filtered using the program Gblocks 0.91b, which eliminates poorly aligned positions and divergent regions, that is, removes alignment noise [17]. Finally, the program PhyML 3.0 aLRT was used for phylogenetic analysis and HKY85 as a substitution model.

The nucleotide sequence of the isolated bacterium was included in NCBI's GenBank and assigned an accession number consisting of 2 letters and 6 numbers [18].

### 2.4 Cultivation and acclimatization of the isolate: Microbial adaptation against morpholine

Bacterial inocula were prepared by aseptically transferring the selected identified pure colonies to 10 mL of an enriched media called Knapp Buffer. Alternatively, a mineral salt solution (MSS) comprised of 100 mg of  $\text{KH}_2\text{PO}_4$ , 100 mg of  $\text{K}_2\text{HPO}_4$ , 4 mg of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.2 mg of  $\text{FeCl}_3$  was used as previously described by the author supplemented with 0.1% v/v morpholine as previously described by the author [19]. Cultures were incubated at  $37^{\circ}\text{C}$  as well as 150 rpm for 1–2 weeks and

Table 1: GC parameters for the estimation of the monoethanolamine concentration

Parameters	Specificity
Column and its configuration	Rtx-35 30 mm × 0.32 mm × 1 μm
Oven/Column temperature	Initial temp.: 60 °C Hold: 1 min Ramp rate: 30 °C/min Final temp.: 240 °C maintained for 3 mins Linear velocity: 37.6 cm/sec (for nitrogen)
Injection port	Temp.: 200 °C Split Ratio: 30:1 Injection Volume: 1 μL
Carrier gases (mobile phase)	Column gas flow rate: 2 mL/min Purge gas flow rate: 1 mL/min Hydrogen gas flow rate: 40 mL/min Zero air flow rate: 400 mL/min Nitrogen gas flow rate: 15 mL/min
Stationary phase	60% Dimethylpolysiloxane and 35% Diphenyl polysiloxane
Detector	Flame ionization detector at 300 °C
Analysis time	10 mins
Software	GC Solution
Workstation	Windows 8

their absorbance at 600 nm was taken regularly as a measure of growth. Based on their growth, when an optical density of 0.5 was reached (data not shown), the culture was diluted to 1 : 100 before being further spread onto MSS-agar plates (treated with 2% agar + 0.1% morpholine) to confirm the acclimatization of the isolate against morpholine stress. Furthermore, the growing culture was centrifuged at 6500 rpm for 10 mins and the pellet was re-suspended in the MSS medium while gradually increasing the concentration of morpholine to 0.2% which was referred to as a seeded acclimatized bacterial inoculum. For each increased acclimatization study, the tested bacteria were grown in an MSS broth supplemented with an increased concentration of morpholine and a respective MSS-agar plate with the same concentration of morpholine to confirm the said acclimatization.

The acclimatized inoculum was later grown in the presence of an intermediate degradation product of morpholine to explore whether this particular isolate follows Pathway 1 or 2. This was further validated by performing in-vitro chemical and analytical assay(s) with the availability of intermediate product of morpholine degradation in the culture filtrate. Lastly, estimation of the ammoniacal nitrogen (measure of the amount of ammonia) in the culture filtrate revealed the complete degradation of morpholine by this isolate following the concerned pathway.

### 2.5 Growth on different hypothetical degradation intermediate compounds

The growth of the isolate on various substrates (degradation intermediate compounds) was investigated by adding the corresponding compounds (0.15%) to the MSS. The pH of the media was adjusted to 7 and growth carried

out at 37°C as well as 150 rpm for 48 hours. At regular time intervals, the absorbance was measured in terms of optical density to establish whether these degradation products might have been formed to facilitate the growth of the isolated bacteria.

### 2.6 Chemical tests of intermediate(s) in the degradation pathway

Chemical tests on degradation products, mainly monoethanolamine (primary amine) and morpholine (secondary amine), were carried out by the standard Simon test - 1 (Rimini test) and Simon test - 2 (modified Rimini test) on the culture filtrate to determine the presence of primary and secondary amines [20]. The amine undergoes a nucleophilic addition reaction with nitroprusside ions in the presence of acetaldehyde or a ketone to yield the characteristic color of primary amines (blue) or secondary amines (violet).

### 2.7 Gas Chromatography (GC) studies of degradation intermediate(s)

A GC system (Shimadzu GC-2010) equipped with a standard oven for temperature ramping, split condition, injection ports, a flame ionization detector and a Rtx-35 amine column (30 mm × 0.32 mm × 1 μm film thickness) in the presence of nitrogen as a carrier gas by the direct injection method was used for the analysis of monoethanolamine (MEA). The analytical parameters for the analysis of MEA are summarized in Table 1, as per the method (by modifying the column and its parameters) reported in the literature [21].

Table 2: MS operating parameters for intermediate(s)

Parameter	Specificity
Ionization	electrospray ionization Needle voltage = 4.5 kV
Interface temperature	350 °C
Temperature of heating block	200 °C
Sheath/Drying gas flow rate	15 L/min
Nebulizer gas flow rate	1.5 L/min
Acquisition time	2 mins
Acquisition mode	Positive/Negative Scan m/z 50 – 200 Scan speed = 52 units/sec Sampling acquisition time = 1.56 Hz (640 msec)
Detector	Electron multiplier
Software	Lab Solutions
Workstation	Windows 7

A standard solution of 0.125 to 0.5% v/v MEA (corresponding to ppm and prepared in methanol) was injected along with the processed culture supernatant (1:10, filtrate volume of 1 and 9 volumes of methanol), as per the method described above. GC of the test samples was run against blank media using positive controls to quantify or estimate the presence of MEA in the culture filtrate by analyzing the Area Under the Curve (AUC) calculated by the machine.

### 2.8 Mass spectrometry studies of degradation intermediate(s)

The mass spectrometry (MS) system of an integrated Liquid Chromatography-Mass Spectrometry instrument (Shimadzu LCMS-2020) equipped with an inlet interface, ion source, mass analyzer and detector was used to analyze the degradation products of morpholine. The analytical parameters for ascertain the morpholine degradation products are summarized in Table 2.

The sample for injection was prepared without using a solvent, as per the method reported in the literature [12]. The culture sample (5 mL) was centrifuged at 10,000 rpm for 10 mins before the supernatant was filtered through a nylon filter with a pore size of 0.22 µm (Axiva Sichem Biotech, India) to remove any bacterial cells. 1 mL of neat filtrate was injected directly into the MS instrument.

### 2.9 Estimation of the ammonia concentration

The presence of ammonia in the culture supernatant was estimated by the standard Nessler's method [22], which involves coupling of ammonium to the Nessler's reagent

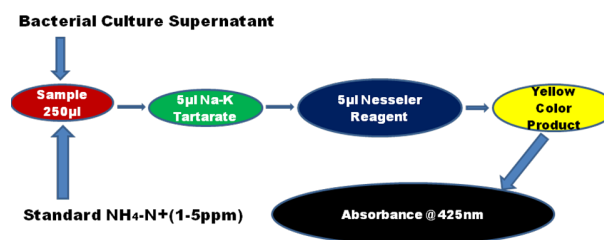


Figure 3: Estimation of the ammoniacal nitrogen concentration by Nessler's method

to produce a yellow color under strongly alkaline conditions (Fig. 3). The resulting yellow color was formed in proportion to the ammonium ( $\text{NH}_4^+$ ) concentration and was measured at a wavelength of 405 nm using an Elisa reader (ELx50/8MS BioTek India) against a reagent blank. The ammonia level in terms of ammoniacal nitrogen was expressed in mg/L (ppm). A standard solution of 10 ppm of  $\text{NH}_4^+ -\text{N}$  was prepared by dissolving 4.773 mg of ammonium chloride in 125 ml of double-distilled water and further diluted to make solutions of 1 – 5 ppm  $\text{NH}_4^+ -\text{N}$ . A calibration curve was plotted and is presented in the results section.

## 3. Results and discussion

### 3.1 Morphological, biochemical and molecular identification

Morphologically, the isolate was found to be white in color with a dull opaque appearance, rod-shaped, have a smooth texture and grow as a convex elevation colony. Standard staining reported it to be a Gram-negative bacterium with high motility which also showed signs of growth on a selective medium, namely HiCrome UTI Agar M1353.

The primary sequence of the 16S rRNA from the present bacterial isolate was determined. The program PhyML 3.0 aLRT for phylogenetic analysis and HKY85 as a substitution model on the 16S rRNA gene sequences determined the phylogenetic position of said isolate to be a species closely related to the genus *Halobacillus blutaparonensis* with a sequence representative of *E. coli* (Fig. 4).

Nucleotide sequence accession was assigned by GenBank, NCBI and an accession number of KC345029 was

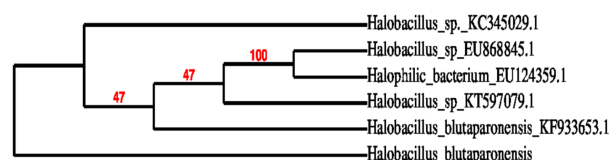


Figure 4: Molecular phylogeny of the 16S rRNA gene sequence and sequences from identified bacteria in the database. The sequence of *E. coli* served as the outgroup for rooting the tree.

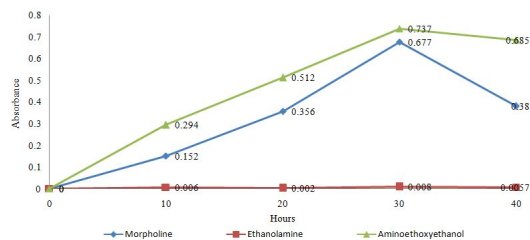


Figure 5: Growth of the isolate in the presence of intermediates of morpholine degradation

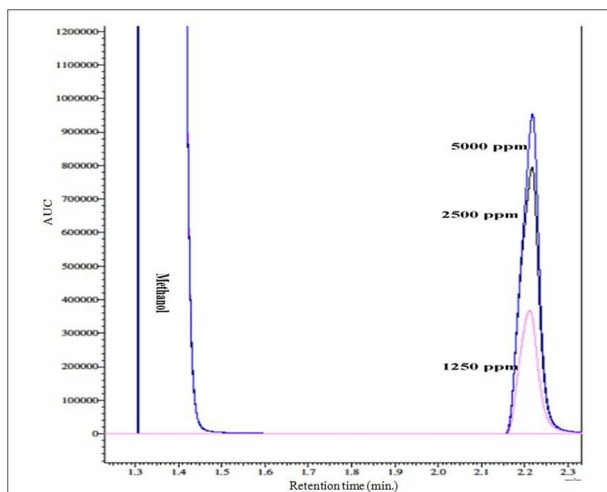


Figure 6: GC (Rtx-35)- flame ionization detector chromatogram of MEA

assigned to this bacterial isolate of genus *Halobacillus blutaparonensis*.

### 3.2 Growth on intermediates

The isolate grew in the presence of morpholine and the intermediate, namely aminoethoxy ethanol (reduced product of aminoethoxy acetate) by consuming it as a source of carbon and nitrogen. However, no growth was recorded in the presence of ethanolamine in the culture media shown in Fig. 5. The count of bacterial cells was adjusted to  $1 \times 10^8$  cells/mL (1 unit of absorbance =  $5 \times 10^8$  cells) by varying the incubation periods up to 48 hours.

### 3.3 Chemical assay of intermediate(s)

Based on Simon tests - 1 and 2 [20], the presence of MEA and morpholine in the culture filtrate is shown in Table 3.

### 3.4 GC studies of MEA in the culture supernatant

GC of the culture supernatant was run at different concentrations (ppm) of a standard MEA solution. Table 4 and Fig. 6 indicate a retention time of MEA equal to 2.2

mins which was absent in the diluted culture supernatant. GC analysis revealed that no MEA was present in the culture supernatant suggesting that bacteria might prefer the diglycolic route (Pathway 2) of morpholine degradation which was later confirmed by MS analysis.

### 3.5 MS studies of the culture filtrate

MS was run directly with a neat culture filtrate. Each sample was analyzed separately in both the positive and negative ion modes (Table 5 and Fig. 7).

It was observed that the m/z peak of the neat culture filtrate (Fig. 7) indicates the presence of 2-(2-aminoethoxy)acetate ( $C_4H_9NO_3$ , molecular weight = 119.119 and m/z = 120 as  $[M+H]^+$ ) and an anion of diglycolic acid ( $C_4H_6O_5$ , molecular weight = 134.09 and m/z = 133 as  $[M-H]^-$ ) which supports the fact that this particular isolate prefers the degradation pathway of diglycolic acid (Pathway 2), similar to a strain of *mycobacterium* reported earlier by conducting electrospray ionization mass spectrometry on the culture filtrate [12].

Further MS analysis supports the GC findings that MEA is not present in the culture filtrate because it might have an inhibitory effect on the bacteria. Therefore, the said bacterial isolate prefers the diglycolic acid route of the metabolic pathway given the fact that in the presence of morpholine, one of the two branches of morpholine biodegradation was induced while the other was inhibited. The illustrated degradation pathway might start with the cleavage of C-N bond, leading to the formation of an intermediary amino acid followed by deamination and oxidation of this amino acid to form a diacid as is shown in Fig. 2b.

### 3.6 Ammonia release: As the end product of morpholine degradation

Morpholine can be degraded by bacteria which releases ammonia. Whichever degradation pathway of morpholine is followed, ammonia is produced as an end product.

The concentration of ammoniacal nitrogen produced by the isolate was calculated (Table 6 and Fig. 8) by the regression equation of a standard curve ( $y = 0.137x$  with  $r^2 = 0.98$ ) and found to be present at a concentration of 5.2 ppm based on Nessler's quantification. The initial morpholine concentration in the culture supernatant (before degradation) was reported to be 2000 ppm. The molar ratio with regard to the conversion of morpholine into ammonia was found to be 1 : 0.014. Furthermore, it was shown that the final pH of the media throughout the experiment did not change, supporting the fact that a low concentration of ammonia was released as an end product of morpholine degradation.

Table 3: Simon tests for the presence of the primary amine MEA and secondary amine morpholine in the culture supernatant






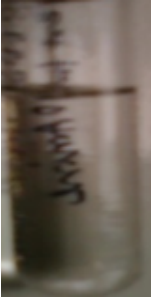
Sample	Test	Feature	Remark	Result
Morpholine	Simon 1	Characteristic blue color of the secondary amine		Morpholine positive
MEA	Simon 2	Characteristic violet color of the primary amine		MEA positive
Culture media	Simon 1	No characteristic blue color		Morpholine negative
	Simon 2	No characteristic violet color		MEA negative
Culture Supernatant (Filtrate)	Simon 1	No characteristic blue color		Morpholine negative
	Simon 2	No characteristic violet color		MEA negative

Table 4: GC analysis of the diluted culture filtrate

Vial	Retention time (mins)	AUC	Interpretation (Compound)
Methanol	1.331	378534920.9	Methanol
5000 ppm MEA	1.333	366649701.7	Methanol
	2.218	2748948.5	MEA
2500 ppm MEA	1.331	374551161.2	Methanol
	2.216	2397300.9	MEA
1250 ppm MEA	1.331	378803557.4	Methanol
	2.211	1149593.1	MEA
Culture Supernatant (1:10)	1.334	310947764.4	Methanol
	2.331	92353.6	No/Negligible MEA

Table 5: Expected intermediate according to the MS analysis of the culture filtrate.

Sample	m/z Positive mode	m/z Negative mode	Remark
Neat Culture filtrate	120		[M+H] <sup>+</sup> 2,2 Aminoethoxy acetate
		133	[M-H] <sup>-</sup> Anion of diglycolic acid

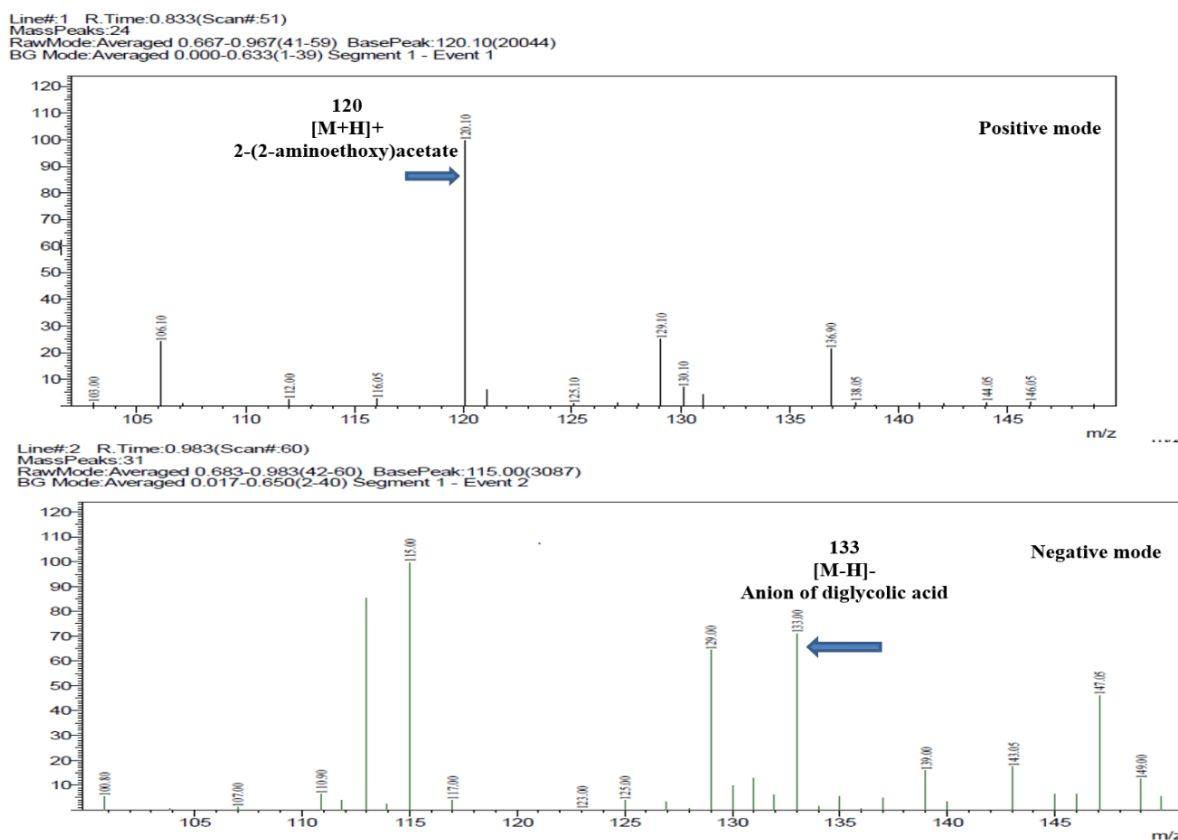


Figure 7: Electrospray ionization - MS spectra recorded under positive and negative ionization of the neat culture filtrate.



Table 6: Estimation of ammoniacal nitrogen concentration by Nessler's reagent

Well	10 ppm Stock NH <sub>4</sub> -N+ (μL)	Milli-Q water (μL)	Culture media (μL)	50% Na-K Tartrate (μL)	Nessler's reagent (μL)	Net absorbance at 405 nm
1 ppm	25	225	—	5	5	0.091
2 ppm	50	200	—	5	5	0.284
3 ppm	75	175	—	5	5	0.353
4 ppm	100	150	—	5	5	0.552
5 ppm	125	125	—	5	5	0.725
Culture supernatant	250	—	—	5	5	0.725

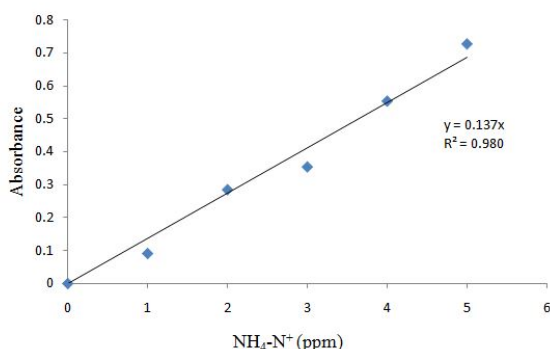


Figure 8: Standard curve of ammoniacal nitrogen concentration by Nessler's reagent

#### 4. Discussion

Based on the results summarized, it has been reported that the isolate prefers to undergo the diglycolic acid route of degradation instead of the ethanolamine pathway, which might be an inhibitory effect on bacterial growth. The illustrated degradation pathway starts with cleavage of the C-N bond, leading to the formation of an intermediary amino acid which is followed by deamination and oxidation to form the diacid (Fig. 9). This diacid, namely diglycolate, later participates in intermediate metabolism and is converted indirectly into TCA by the Krebs cycle, which is beyond the scope of the present article.

Moreover, the presence of degradation intermediate compounds in culture filtrate also favors this finding with the conclusion that the diglycolic acid route of biodegradation might be a common degradation mechanism, which is also shown by other strains of bacteria, proceeding via 2-(2-aminoethoxy)acetate. The said investigation to reveal the degradation pathway of morpholine is supported by similar findings published by other authors [5, 10–12].

Furthermore, whatever the degradation pathway exhibited by the bacterial isolate, the end product, that is, ammonia, will be biochemically produced and used. Our studies confirm the presence of ammonia as an end product in a molar conversion ratio of morpholine to ammonia of 1 : 0.014. Due to the low concentration of ammonia produced, the pH of the culture medium did not change throughout the experiment. However, a higher molar ra-

tio of morpholine to ammonia brought about an inhibitory effect on the growth of bacteria by increasing the pH of the medium and making it more alkaline. The molar ratio of morpholine to ammonia was found to be different for different strains of bacteria as viz., namely 1 : 0.5 for *Mycobacterium sp. HE5* [6], 1 : 0.89 for *Mycobacterium sp.* [7] and 1 : 0.82 for *Mycobacterium sp. MO1* [9].

#### 5. Conclusions

The large scale industrial applications of morpholine and its known carcinogenic effect thus have an environmental interest for its biodegradation and exploring the degradative pathway so that unrevertable damage to the natural environment and biota can be minimized. Along with the *Mycobacterium* and *Pseudomonas sp.* another potential isolate namely *Halobacillus blutaparonensis* has been investigated for its ability to removal of morpholine by adopting the diglycolate degradation pathway. Hence, sustainable remediation practice by utilizing effective microbes should be applied to bring the environmental cleanup or facilitate the existing system of effluent treatment mechanism incorporation with biological approaches to minimize the impact of xenobiotic pollutants in the anthropocentric epoch.

#### Conflicts of interest

The authors confirm no conflicts of interest with regard to the results derived from this study on the sustainable remediation of morpholine and its micro-scale degradation pathway.

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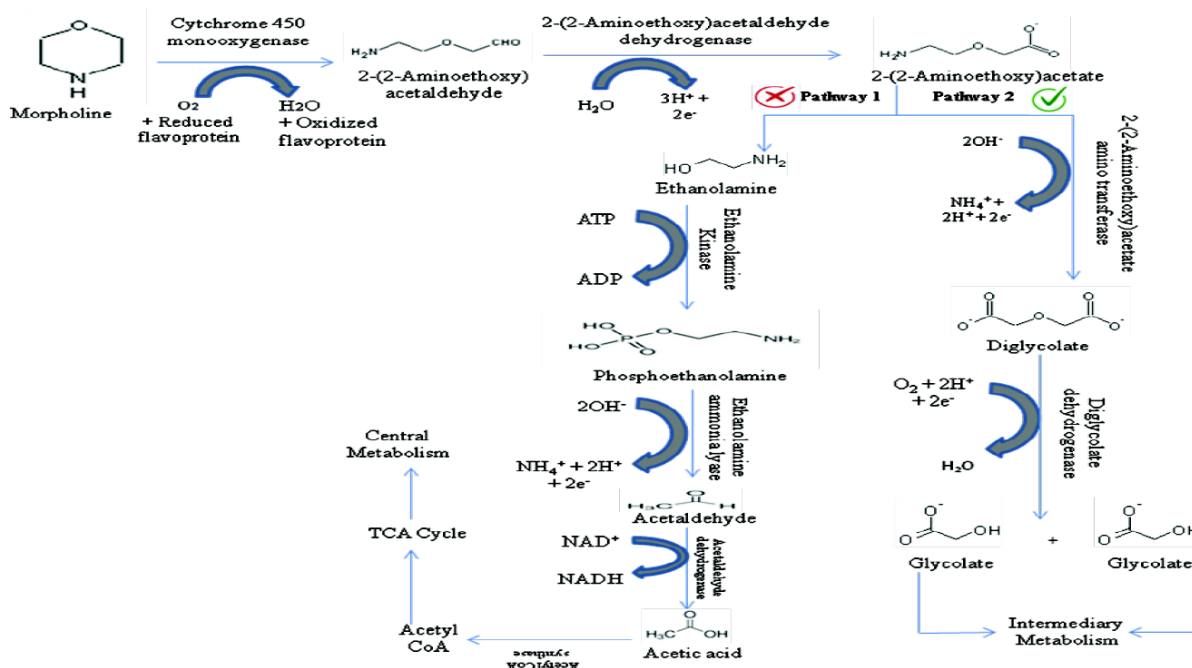


Figure 9: The complete illustration of a possible degradation pathway of morpholine. The isolate, namely *Halobacillus blutaparonensis*, prefers Pathway 2 for the successful removal of morpholine. Abbreviations used - TCA: Tricarboxylic acid; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; H<sup>+</sup>: Hydrogen atom; e<sup>-</sup>: Free electron; O<sub>2</sub>: Oxygen molecule; NH<sub>4</sub><sup>+</sup>: Ammonium ion

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