Biological Approaches for Treating Industrial Effluents Containing “Morpholine”

THESIS

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DOCTOR OF PHILOSOPHY

By

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Under the Supervision of

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Dedicated to two Mothers

(One gave the birth on earth and Other nurture the life)

A woman with strong will power and determination, who gave me a lesson

A mistake increases your experience & Experience decreases your mistakes. If you learn from your mistakes, then others will learn from your success

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CERTIFICATE

This is to certify that the thesis entitled **Biological Approaches for Treating Industrial Effluents Containing "Morpholine"** and submitted by Mr. Rupak Kumar, ID No: 2011PHXF0002H for the award of Ph. D. Degree of the Institute embodies original work done by him under my supervision.

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"We make a living by what we get, but we make a life by what we give"

Winston Churchill

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Date: **Rupak**

ABSTRACT

The heterocyclic xenobiotic compound morpholine (1-oxa-4-azacyclohexane) is of great importance for different industrial purposes. Morpholine is a colorless, oily, hygroscopic, volatile liquid with a characteristic amine smell and is completely miscible with water as well as with many organic solvents, but has limited solubility in alkaline aqueous solutions. Morpholine is an extremely versatile chemical. It is used as a chemical intermediate in the rubber industry, in corrosion control (plating industry), the synthesis of many drugs, crop protection agents, as a solvent in lyocell process (textile industry) and as optical brightener. Based on its chemical nature, being both cyclic ether and a secondary amine, it is also used as a solvent for a large variety of organic materials, including resins, dyes and waxes. Consequent to its wide range of applications and solubility, significant amount of this chemical is released via industrial effluents into the environment. In the natural environment, its secondary amine functionality leads to nitrosation to form N-nitroso morpholine, a well-characterized carcinogen as reported by many authors. The large-scale annual usage of morpholine (25,000 tons per year) and its potentially carcinogenic effects thus have environmental interest for biodegradation. Physical and chemical methods of morpholine removal like membrane polyelectrolyte filtration and ion exchange resins are expensive and hence uneconomical for use at commercial applications. Due to this the biodegradability of morpholine has attracted widespread interest. The principle objective is to allow industrial effluents to be disposed of without unacceptable damage to the natural environment by utilizing beneficial and effective microbes for a sustainable treatment. The goal is to reduce or remove organic compound morpholine in waste water. It is well known, that if favorable conditions are provided, the soluble organic matter like morpholine can be effectively removed through biological action by microorganisms. It can be achieved by providing nutritional requirement of microbes, optimized culture condition and establish a relationship between microbial growth and substrate utilization. Morpholine was earlier thought to be recalcitrant to biodegradation but later several microbes have been reported to be metabolically able to carry out biodegradation of morpholine. Most of the

reported studies showed that *Mycobacterium* sp. and *Pseudomonas* sp. are the two potential candidates for using morpholine as their sole carbon and nitrogen source. The present study aims at isolation, screening, adaptation and potentiality of naturally occurring microbes, or microbial consortium, for their capacity to degrade morpholine. Our pilot data, based on available morpholine in culture suspension, shows that certain bacterial strains are effective in biodegradation of this toxic, but industrially important commercial solvent. We have tested the isolated microbes adapted to gradual increase of morpholine in culture broth and have further identified the best isolate for effective removal of this toxic waste effluent candidate. In the consecutive study, different isolates from natural sources and industrial effluents showed complete removal of 0.10 to 0.28% concentration of morpholine. The operational % degradation was found to be over 99% in a 10-20 days old adapted culture supernatant of all potential isolates. The complete degradation of % morpholine of 0.28%, 0.21%, 0.20%, 0.20%, 0.20% and 0.15% by potential isolates follows the descending order of BAC 1 > SK 5/ BAC 2/ RK 1/ RK 2 > RK 11 isolate respectively. The result was well validated with gas chromatography analysis of bacterial culture supernatant with appropriate dilution of solvent in comparison with positive control and media blank. In this study, different approaches were used to elucidate the degradation pathway of morpholine either it follows the ethanolamine or diglycollic route of degradation. On the basis of chemical test of intermediate and MS analysis of culture filtrate reveal that certain potential isolate underdoes glycolic route of metabolic degradation pathway and supports the fact that in presence of morpholine, one of two branches of morpholine biodegradation pathway was induced while the other branch was inhibited. The illustrated degradative pathway might be starts with the first step of the cleavage of the C-N bond; this leads formation of an intermediary amino acid, which is further followed by deamination and oxidation of this amino acid to a diacid. Whatever the degradation pathway was exhibited by bacteria, the end product was ammonia which would be biochemically used by bacteria. Our studies confirm the presence of ammonia as end product and molar ratio of morpholine to ammonia was found to be 1:0.014. Due to low amount of ammonia produced, the pH of the media did not change throughout the experiment. However higher molar ratio of morpholine to

ammonia results the inhibitory effect on the growth of bacteria because it increases the pH of media to shift it toward alkalinity. The ratio of morpholine to ammonia was found different for different set of bacteria as reported by other authors. Further, effect of immobilization on degradation studies of morpholine was carried out and result showed that with respect to complete degradation of morpholine by acclimatized free RK-1 isolate, immobilized cells showed nearly 70% degradation when immobilized with 2% sodium alginate solution. One important finding was noticed that within 48 hours, beads itself decompose which might be due to fact that the either isolate itself produce alginase or other possibility is that the available amine in culture media itself reacts with alginate and undergoes reductive amination. Due to this limitation, we are looking for other suitable hydrophillic polymeric matrix to evaluate the degradation studies for industrial purpose, which is future scope of the present study. Thus, this biological approach uses the utilization of nature's own technology for pollution abatement through efficient and cost-effective methods.

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Abbreviation	
AIT: Auto ignition temperature	AP: Andhra Pradesh
APHA: American public health association	AUC: Area under curve
BOD: Biological oxygen demand	BP: Boiling point
C: Carbon	CFR: Code of federal regulations
cm: Centimeter	CP: Chemical pollution
COD: Chemical oxygen demand	*E.coli*: *Escherichia coli*
EB-1: Enable 1 column	ECD: Electron capture detector
EPA: Environmental protection agency	EV: Electron volt
F: Forward primer	Fig: Figure
FID: Flame ionization detector	FP: Freezing point
FPD: Flame photometric detector	FTD: Flame thermionic detector
GC: Gas Chromatography	Glu: Glucose
gm: Gram	H: Hydrogen
He: Helium	hrs.: Hour
HPLC: High pressure liquid chromatography	IARC: International agency for research on cancer
IC: Ion Chromatography	ICSC: International chemical safety cards
IDLH: Immediate danger limit for health	IPCS: International programme on chemical safety
ITP: Isotachophoresis	K: Potassium
Kg: Kilogram	KV: Kilo volt
lb: Pounds	LB: Luria bertani
LC: Lethal concentration	LCMS: Liquid chromatography mass spectroscopy
LD: Lethal Dose	lt: Litre
M: Molar, Morpholine	MAE: Man, and environment
MAID: Morpholine and its derivatives	mHZ: mega hertz
ml: Milii liter	mm: Millimeter
MP: Melting point	min: Minutes
MS: Mass spectroscopy	MM/NMM: N-methyl morpholine

MSS: Mineral salt solution	N: Normal, Nitrogen
Na: Sodium	ND: Not detectable
NMOR: N-Nitrosomorpholine	NH_3: Ammonia
NIOSH: National institute of occupational safety and health	NIT: 1-Naphthylisothiocyanate
NMR: Nuclear magnetic resonance	nm: Nanometer
NQ: 1, 2-Naphthoquinone	NPQ/NQS: 1, 2-naphthoquinone sulfonate
NSD: Nitrogen selective detector	OSHA: Occupational safety and health administration
PC: Positive control	PCR: Polymerase chain reaction
PEL: Permissible exposure limit	pH: Power of hydrogen
pK_a: Acid dissociation constant	ppm: Parts per million
psi: Pound square inch	r: Regression R: Reverse primer
REL: Recommended exposure limit	RI: Refractive index
rpm: Rotation per minute	rRNA: Ribosomal ribonucleic acid
RT: Retention time	S: Svedberg unit
SDA: Sabrauds dextrose agar	Sec: Second
SNPA: N-succinimidyl-P nitrophenyl acetate	sp: Species
TEA: Thermal energy analyser	TWA: Time weighted average
U.S.: United States	US FDA: United States food and drug administration
US EPA: United States environmental protection agency	UTI: Urinary tract infection
UVA: Ultra-violet A	VOC: Volatile organic chemical
VP: Vapour pressure	WHO: World health organization
Symbols	
A: angstron	
µ: mu/micron	ƛ: lambda
@: at	ε: epsilon
ºC: degree celcius	v: volume

CHAPTER 1

INTRODUCTION

1.0. Introduction

1.1. Chemical pollution: A manmade problem

An industry should accumulate wealth as a bee collects pollen from a flower. The bee harms neither the fragrance nor the beauty of the flower but gathers the pollen to turn it into sweet honey. Similarly, man is expected to make legitimate use of Nature to rise above Nature and realize his innate spiritual potential.

Sigalovadasutta

Pollution is the effect of inconvenient changes in our surrounding that impinge harmful effect on biota. This occurs when short-term economic gains are made at the cost of long term ecological sustainability. Global environment in 21st century provides us with infinite opportunities and challenges. One of the challenges is improving the quality of environment. Human beings in true sense are the product of their environment and exposed to extreme health risk. Man, and environment (MAE) relationship indicates that pollution has a social origin. The anticipated increase in human population by the turn of the century would naturally imply augmentation of our resources so that productivity is maintained for sustainable growth. Increased human activity has affected ecosystems in a variety of different ways. With the advent of heavy industrialization and manufacturing of different chemicals, introduction of potentially hazardous and toxic organic compounds in the environments cannot be prevented, and slowly the influx of such compounds in soil, air and water is increasing. Water is an important commodity which is rapidly becoming polluted with many inorganic (acid, metal salts etc.) and organic pollutants (oil, gasoline, plastic, pesticides, detergents and fertilizers, etc.). When they enter the water bodies, water system degrades due to bacterial activity which consume dissolved oxygen. Because of this, several living forms which are better suited to the polluted waters have growth burst in our aquatic environments causing ecological imbalance. Therefore, there is constant threat to the water bodies. While on one hand, there is an increasing problem of control of environmental pollution, on the other hand there is also a nagging problem of conservation of nature and natural resources. Hence chemical pollution (CP) is major economic and environmental issue of this era. Among all the wastes produced, industrial wastes demand the largest attention because they have been regarded as being of the greatest diversity and toxicity and are mostly recalcitrant. Hazardous wastes and toxic chemicals pose complex environmental problems by directly affecting the air, water, soil and sediments, while indirectly and unpredictably affecting living organisms that use these resources. Large area of the earth's

surface, the ocean and other water bodies are heavily contaminated with toxic chemicals. Many kinds of chemical substances are emitted from various sources into the environment. These chemicals transform by the processes such as transport, chemical reaction and removal to affect the environment in many ways (Fig. 1)

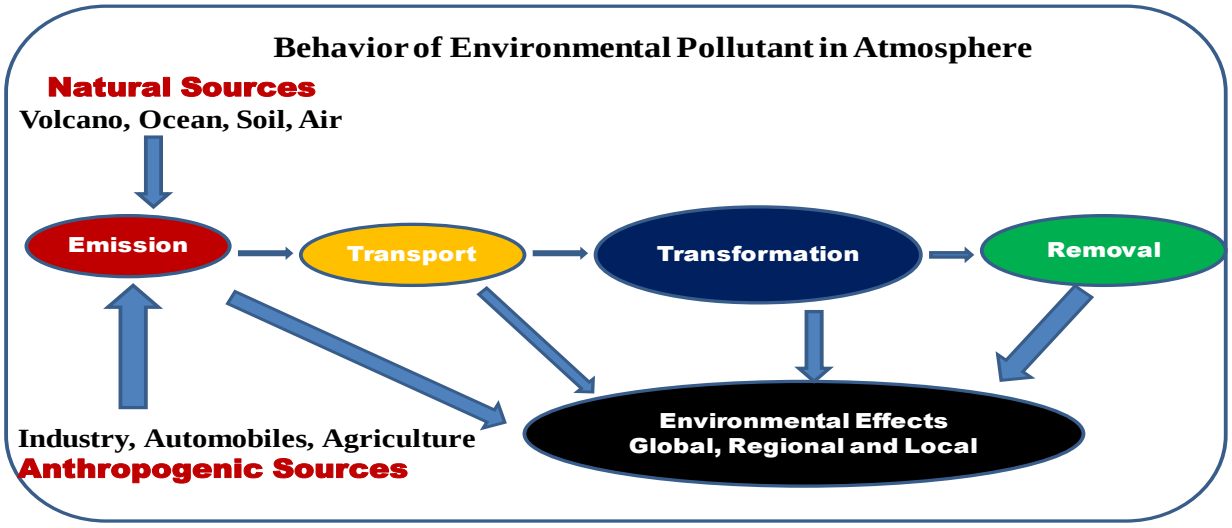


Fig. 1: Circulation mechanism of chemical pollutants in the atmosphere

Every second 310 Kg of toxic chemicals are released into our air, land, and water by industrial facilities around the world. This amounts approximately lead 10 million tons of toxic chemicals released into our environment by industries each year. Out of these 10 million tons, over 2 million tons per year (about 65 kg each second) are recognized carcinogens (http://www.worldometers.info/view/toxchem). As per compiled data by the tri-national North American Commission for Environmental Cooperation, the U.S. Toxics Release Inventory report and Canada's National Pollutant Release Inventory, chemical manufacturing ranked third highest among all industries for releasing pollutants in Canada and the U.S. in 2001 (Fig. 2). All industries together reported 1.3 million metric tons of pollutants releases in 2001. Electric utilities were the largest polluter, followed by metal-mining companies and chemical used industries. In chemical-based industries, paper, textile and leather industries are dominating with the highest BOD_s in their effluents. Morpholine and its derivatives (MAID) are used directly or indirectly in all reported industries except in electrical utilities.

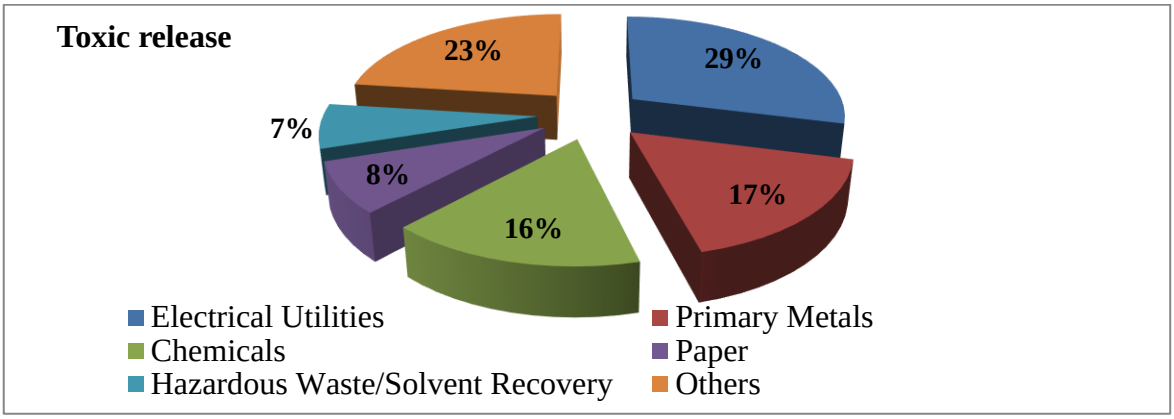


Fig. 2: Magnitude of toxic chemicals released by different industries

1.2. History of morpholine and its applications

Morpholine (C_4H_9NO or 1-oxa-4-azacyclohexane) is colorless, clear, synthetic, simple heterocyclic organic compound having characteristic functional group of amines and ether (Cromwell 1957) and it has a great industrial importance (Mijos 1978). It is an oily, volatile chemical with a perceptible amine-like odor and hygroscopic liquid of maximum water content of 0.3% (Global Trust Enterprises, product information of morpholine, 2011) with an annual production of ~ 25,000 ton (WHO 1995, http://eawagbbd.ethz.ch/mph/mph_map.html). A recent US EPA (United States Environmental Protection Agency) report data showed that 3.93 billion pounds of toxic chemicals were released into the environment. Morpholine is one of them and was listed in EPA master list for toxic substances control act (61 Fed. Reg. 65936, 13 Dec 1996, 57 Fed. Reg. 61240, 23 Dec 1992) and EPA registered pesticides (EPA inert ingredients list 03) and is designated as a volatile organic compound (VOC) (Morpholine Technical Advisory Panel Report-USDA United States Department of Agriculture). It was first prepared in 1898 (Budavari, 1996) and later in late 1970, its commercial production was started for different uses (Mannsvle Chemical Products Corp., 1981). Since onward, now a day's morpholine and its derivative are being used in various industries namely, rubber industry (vulcanization accelerator), iron industry (corrosion inhibitor), pharmaceuticals industry (catalysts, antioxidants, bactericides, analgesics, anesthetics, and other physiologically active agents- Lewis RJ Sr, 2007), solvent for resins, waxes, casein, and dyes (O'Neil MJ, 2006), personal care (hair conditioner, face wash, waxes and polishes), pesticides industry, tobacco industry (cigarette), refinery, power plants (additive in stream system to adjust the pH levels in water), paper industry (optical brightener),

food/vegetables coating, textile industry (to dissolve cellulose in a solvent spinning process to make lyocell fibres) and as a solvent in various other manufacturing processes or chemical reactions due to low cost and polarity (Fig. 3a). Typical use of morpholine in terms of percentage: rubber accelerator, 40%; corrosion inhibitors, 30%; waxes and polishes, 5%; optical brighteners, 5%; and miscellaneous, 20% (Mannsvile Chemical Products Corp., 1981) (Fig. 3b). The global market share of morpholine is represented in Fig. 3c.

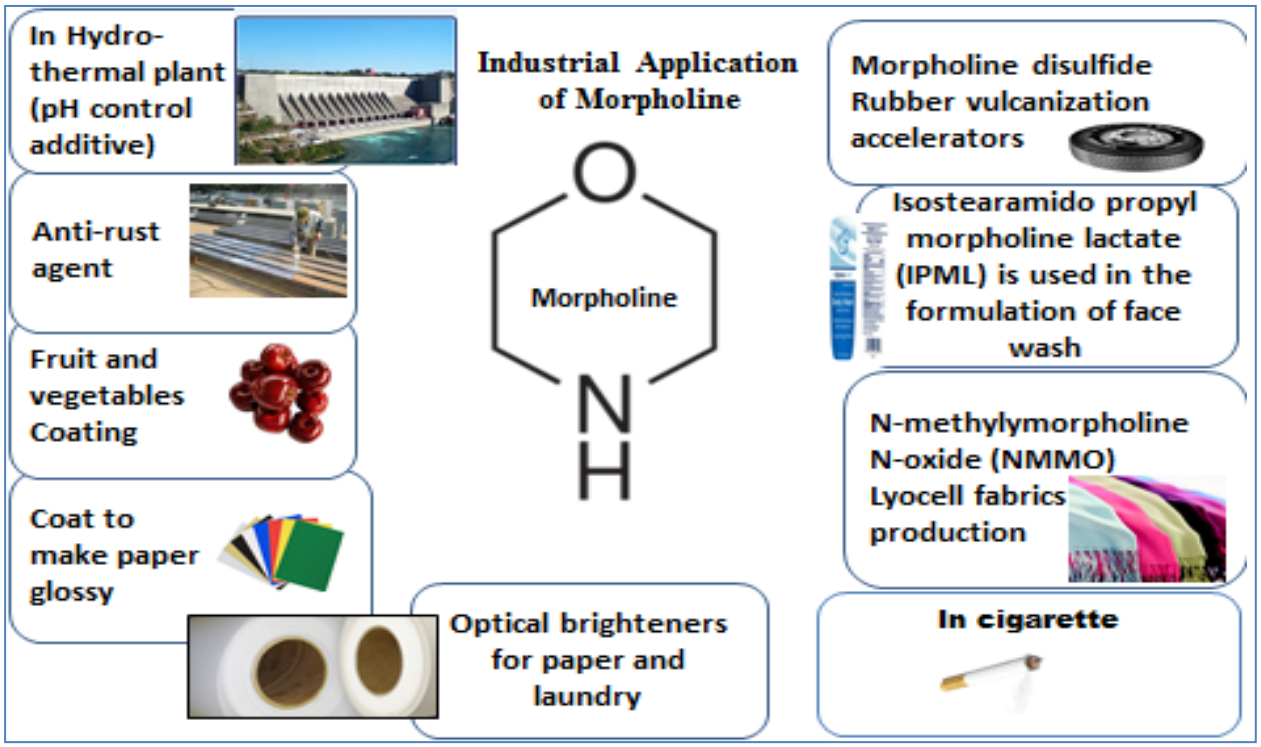


Fig. 3a: Different industrial applications of morpholine

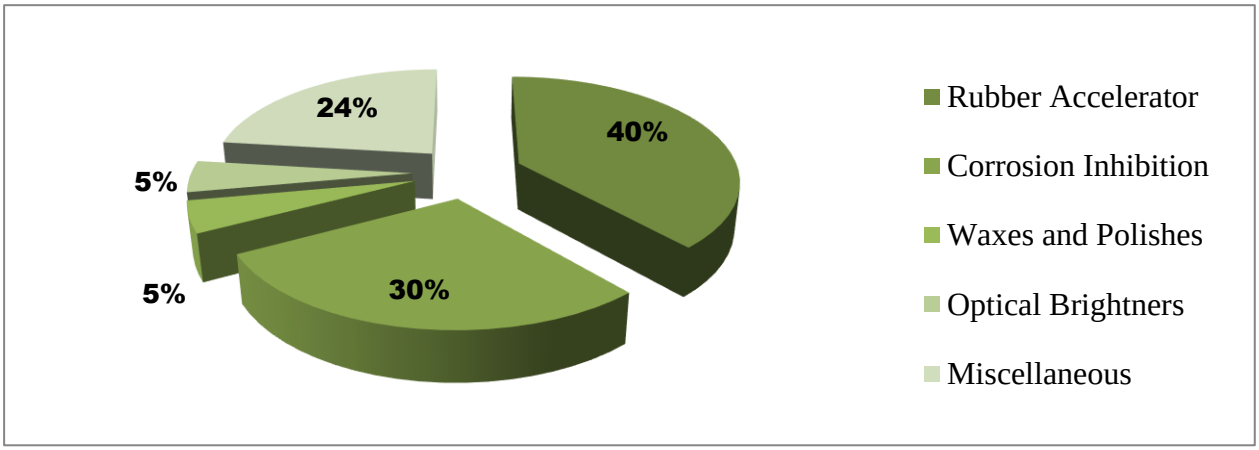


Fig. 3b: Morpholine applications (% contribution)
(Adapted from Mannsvile Chemical Products Corp., 1981)

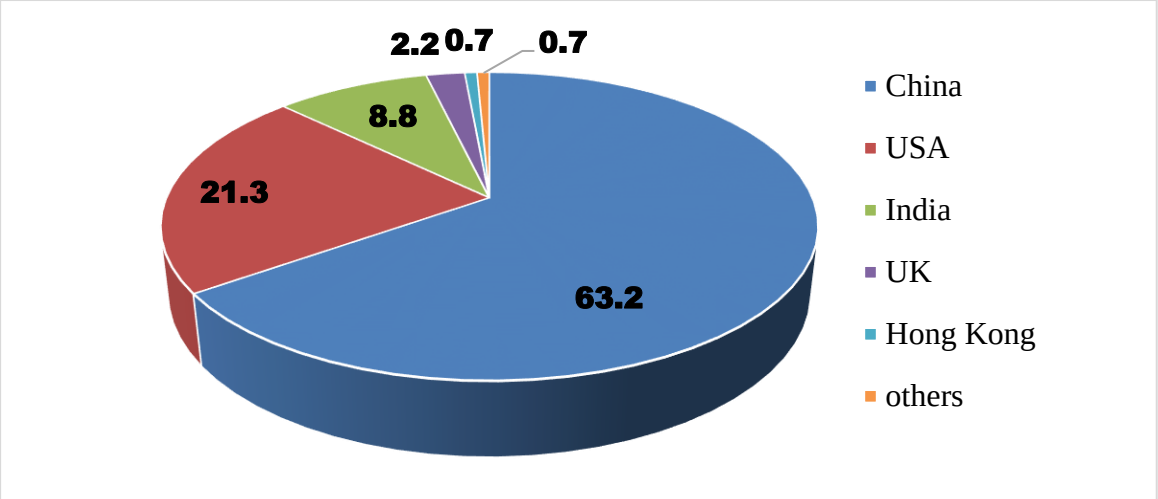


Fig. 3c: Global market share of morpholine (% contribution)
(http://www.guidechem.com/reference/dic-2241.html)

1.3. Morpholine: Why environmental interest?

Morpholine is one of the important chemical due to its versatile and diverse applications as discussed above. Morpholine occurs in the environment, is detected in foods (Mohri, 1987) and water and it is not surprising that morpholine appears in many industrial effluents with major contributors being rubber, metal and textile industries. Due of high solubility/complete miscibility in water (IPCS, Environmental Health Criteria 179, morpholine, 1996) and widespread applications, it is released via industrial effluents or other means into the environment where they can be transformed to N-nitroso compounds. These pro-mutagen chemicals are converted to the mutagenic hydroxy-N-nitroso compounds by metabolic activation or UVA irradiation (Fujiwara *et al.*, 1996). It then moves with soil moisture and running water but does not sediment (Lewis, 1995). In brief, in natural environmental condition, it undergoes chemical nitrosation to form potential carcinogenic product named N-nitrosomorpholine (NMOR) (Enzmann *et al.*, 1995; Sielaff *et al.*, 2001). NMOR is formed by reaction of aqueous solutions of nitrite with morpholine (Mirvish, 1975) or by reaction of gaseous nitrogen oxides, e.g., N_2O_3, N_2O_4, NO_x in aqueous solutions of morpholine, even under normal environmental conditions (Challis and Kyrtopoulos, 1979; Mirvish *et al.*, 1988; Schuster *et al.*, 1990). In aqueous solution, the chemical nitrosation reaction of morpholine was shown in Fig. 4. These reactions can also be catalyzed by various bacteria on secondary amines and nitrites or nitrates at neutral pH (Calmels *et al.*, 1988). Van *et al.*, 1995 reported the in vivo

nitrosation reaction in mice by the action of NO_2 on morpholine. Many studies assessing the carcinogenic activities of nitroso compounds have shown that this compound could enhance, even at low doses, the development of early stages of hepato-carcinogenesis in rats (Enzmann *et al.,* 1995). As morpholine is a precursor of carcinogenic nitrosamine, so, its removal from industrial wastewaters is in the interest of protecting the environment and demands the development of biological approaches of remediation treatment because morpholine was earlier thought to be recalcitrant to biodegradation. Calamari *et al.,* 1980 and Tölgyessy *et al.,* 1986 both reported the resistance of morpholine to biodegradation. However, later it has been reported to be biodegradable (Rothkopf and Bartha, 1984; Poupin *et al.,* 1999). Many aerobic bacteria capable of degrading secondary amines have been isolated and characterized (Knapp *et al.,* 1982; Swain *et al.,* 1991; Emtiazi and Knapp, 1994; Combourieu *et al.,* 2000; Schreader *et al.,* 2000). Except for two bacteria belonging to the genus *Arthrobacter* (Dmitrenko *et al.,* 1985), all morpholine-degrading bacteria are *mycobacteria* (Brown *et al.,* 1990, Cech *et al.,* 1988) and each capable of growth in a simple mineral salt solution (MSS) with morpholine as the sole source of carbon, nitrogen and energy.

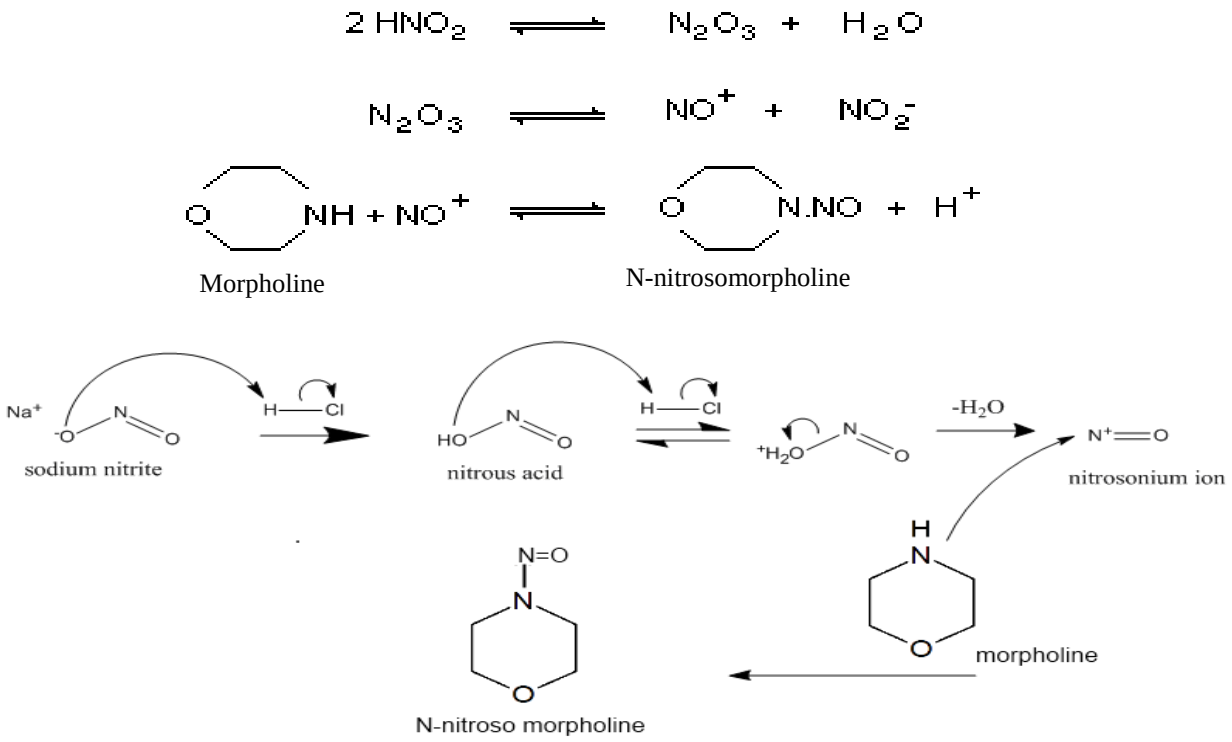


Fig. 4: Reaction mechanisms of the formation of NMOR
(Mirvish, 1975, 1988, Challis and Kyrtopoulos, 1979)

1.4. Gap in existing research

With the advances in chemistry and its applications, vast arrays of synthetic chemicals have been introduced over the past century. Morpholine is among one of them. While properties like polarity, basicity and inertness which make this synthetic chemical attractive to industry and so, causes greater environmental hazards too. This brings focus towards biological treatment of morpholine containing industrial effluents. Among the candidates of many microbes, sludge derived *mycobacterium* or *Arthrobacter* strain based biodegradation of morpholine is described by several authors and considered the most suitable. 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 morpholine biodegradation capability. This study was undertaken to screen and adapt bacteria to degrade morpholine when grown individually and as a consortium of bacterial mix. The metabolic pathway involved in biodegradation of morpholine has been very difficult to establish. As this chemical does not possess any chromosphere and is highly soluble in water, which does not amenable for easy extraction. Consequently, it poses a challenge for direct measurement of morpholine and only indirect strategies have been used till date. These strategies include COD, optical density, NH_3 measurements and NMR/GC/MS monitoring to identify the intermediates or degradation products/efficacy, etc. Thus, there is scope for optimizing an individual or a consortium of microbe which would effectively remove morpholine and its derivatives (MAID) from waste water and may be used for the effluent treatment by the industry.

1.5. Hypothesis

I. Develop a strategy to screen and adapt several fast-growing bacteria isolated from different natural or industrial sites.
II. These acclimatized microbes would degrade morpholine when grown individually and as a consortium of bacterial mix.
III. It would create near complete or partial removal of morpholine from industrial effluents and may provide sustainable environmental solution for the industry.

1.6. Morpholine used in the current study

Morpholine, which is 99% pure and commercially available was used in this study, without any further purification. The same was also used for its suitability in adaptation studies to select strains suitable for biological treatment of industrial effluents.

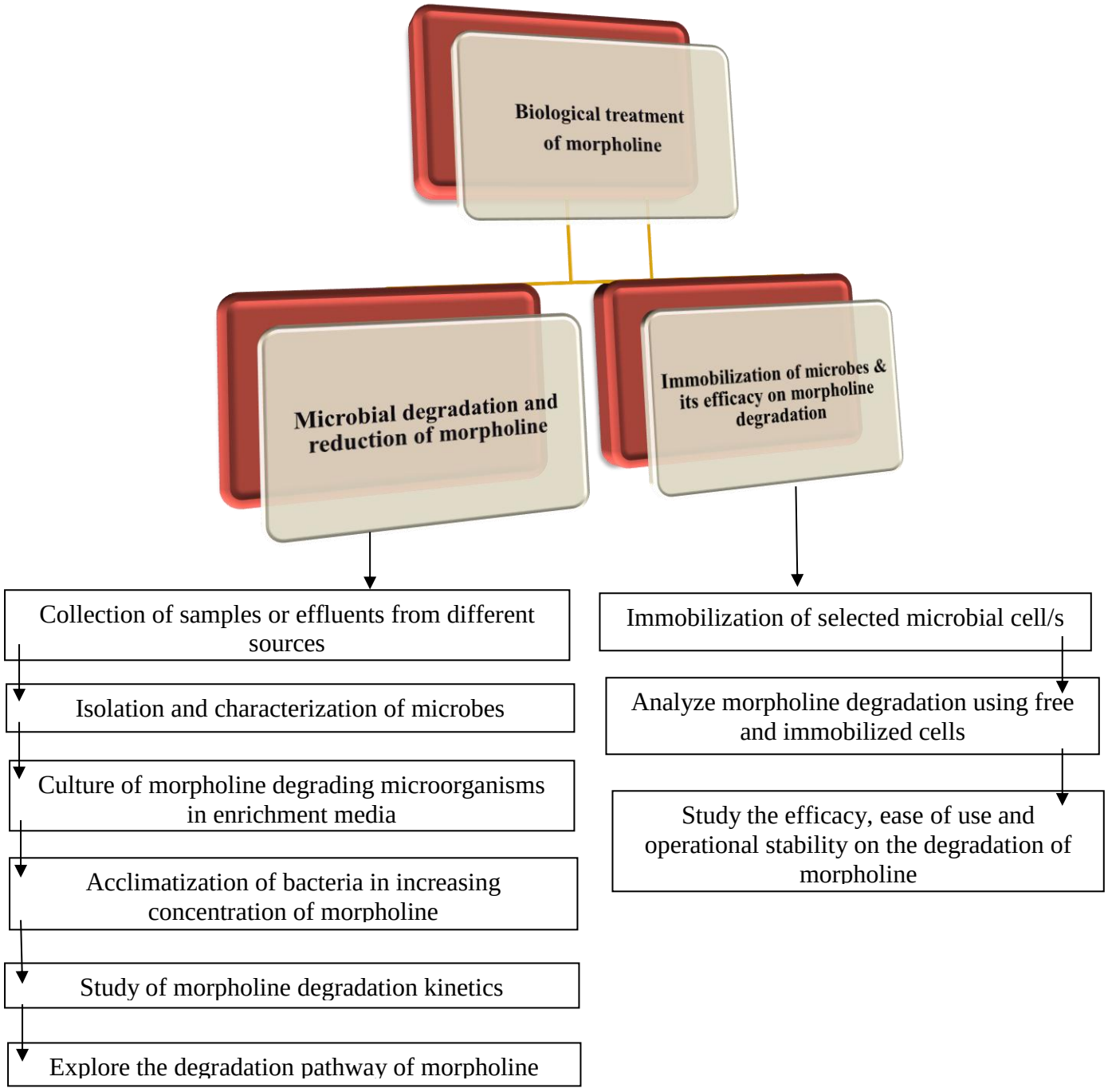
1.7. Objectives and work flow of the study

Objectives of the proposed research are following;

I. Characterization and acclimatization of microbes from various sources which show ability to degrade morpholine.

II. Standardizing methods for immobilizing microbes for ease of use, operational stability and desired level of degradation

The work flow of the study has been represented in a flow chart.



CHAPTER 2

REVIEW OF LITERATURE

2.0. Literature Review

There was no to many studies reported for the biological degradation of morpholine. So many current and old literature were cited and studied to find the research gap and develop the method of analysis of morpholine.

2.1. Microorganisms in pollution control: Biological treatment of waste water

Waste water includes all liquid effluent from household, commercial establishments, institutions, hospitals, agricultural runoff, industries and other so on. It must be treated before it is discharged back to the environment. The major aim of wastewater treatment is to removal of suspended solid so it to make less toxic and eco-friendlier and biodegradable**.** Treating wastewater generally involve five major stages namely called segregation, preliminary, primary, secondary and tertiary treatment (Fig. 5).

- Segregation: It is carried out at source of generation point based on physical and chemical nature of the effluents.
- Preliminary treatment: It involves many unit processes to eliminate undesirable characteristics of wastewater. Processes include use of screen, grit chambers for removal of sand and large particles, communities for grinding of coarse solids, pre-aeration for odor control and removal of oil and grease.
- Primary treatment: It involves removal of settable solids prior to biological treatment. The general treatment units include: Equalization (Each unit volume of waste is mixed thoroughly with other unit volumes of other wastes to produce homogeneous and equalized effluent, Neutralization (Acidic effluents may be neutralized by treatment with lime or lime slurry or caustic soda), Sedimentation (Separation of suspended solid particles by gravitational settling in the primary clarifier). Primary treatment removes about 60 % of suspended solids from wastewater (https://water.usgs.gov/edu/wuww.html)
- Secondary/Biological treatment: It involves purification of wastewater primarily with dissolved organic matter by microbial action. It includes mainly anaerobic and /or aerobic treatment methods Aerobic treatment uses activated sludge, aerated lagoons, trickling

filters/bio filters, membrane bioreactor and others. However anaerobic treatment employed for the effluents with high organic load of >2g/l (Technical EIA guidance manual for common effluent treatment plant, The Ministry of Environment and Forests, Govt. of India, 2010). It includes anaerobic contact reactor and fixed-bed reactor. Secondary treatment removes more than 90 % of suspended solids. (https://water.usgs.gov/edu/wuww.html)

- Tertiary treatment: This mainly includes physical and chemical treatment processes that comprises the use of carbon filters, microfiltration, ultrafiltration and reverse osmosis. it can be applied to secondary effluents to meet the water quality.

Although these treatment processes are routine practice for conventional waste water purification but secondary (biological) treatment considered the main process where it removes dissolved and suspended solid/ biological matter and is typically performed by indigenous microorganisms in a managed habitat (Tilley DF 2011). In that process, bacteria consume hazardous waste, particularly contaminant soluble organic chemicals through the route of environmentally friendly, cost effective process of bioremediation (El Bestawy *et al.*, 2014).

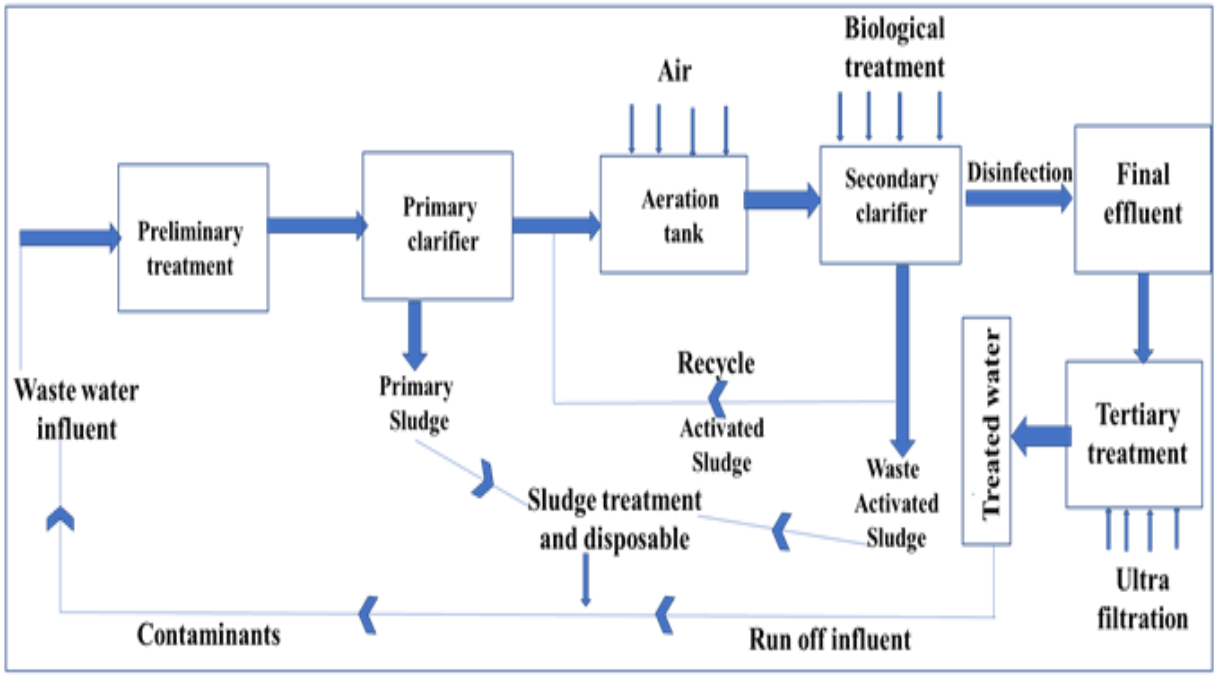


Fig. 5: Illustration of waste water treatment

2.1.1. Role of Microbes: Biological transformation of xenobiotics

More than ten million organic compounds are generated by biosynthetic pathways in animals, plants, and microorganisms either by natural processes, or by industrial synthesis (Fetzner S, 2002). Whilst the organic structures found in nature are created by many organisms and biological processes, microorganisms also perform most of the biodegradation of both natural products and industrial chemicals. These chemically synthesized chemicals (xenobiotic) are foreign to the biosphere and have unnatural structural features to which microorganisms have not been exposed to during evolution and so are difficult to biodegrade. The possible fate of xenobiotic compounds is summarized in Fig.6.

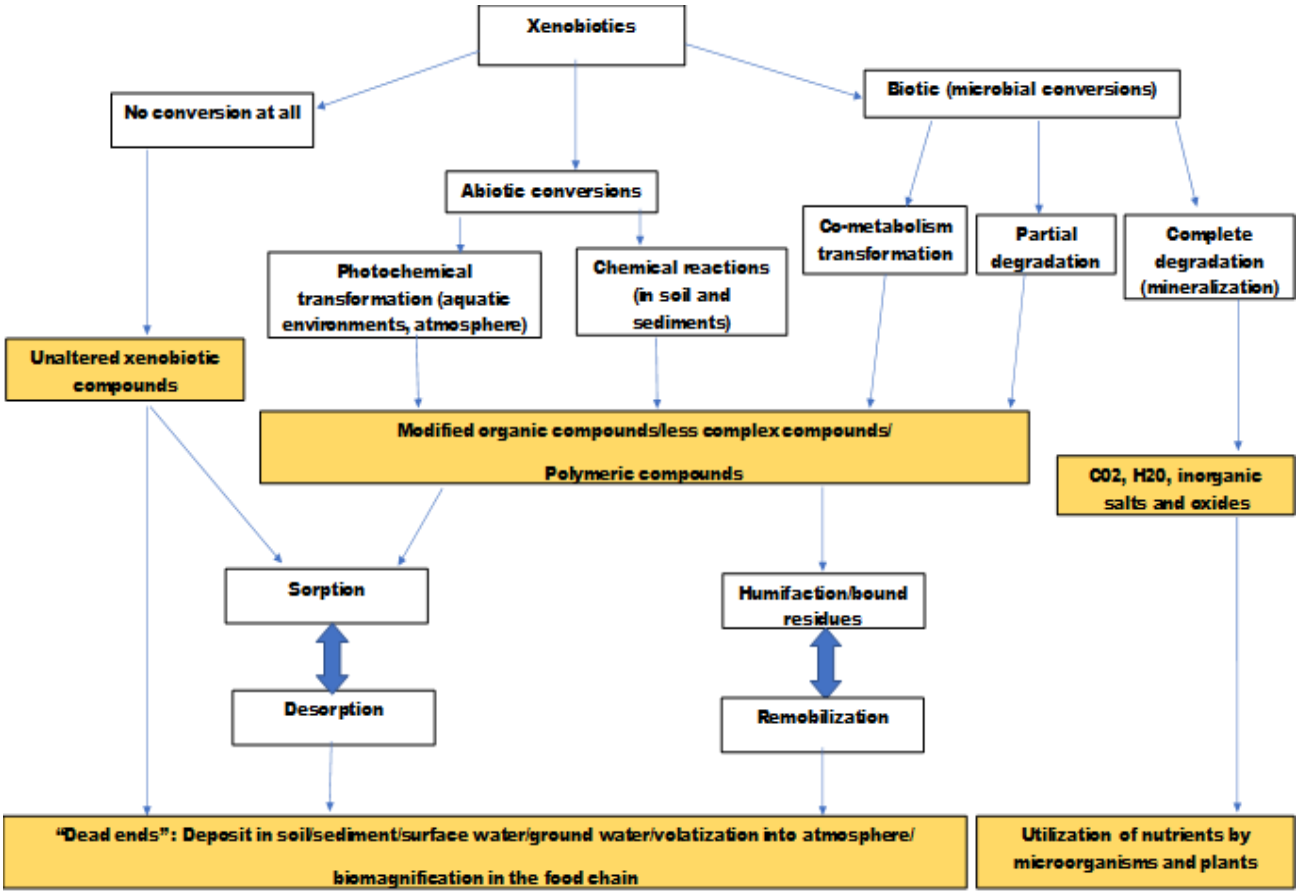


Fig. 6: Fate of xenobiotics in environment (as described by Fetzner S, 2002)

Collectively, microorganisms play a key role in the biogeochemical cycles of the earth. Since the beginning of recorded history, microorganisms have been used for the benefits of human beings

in many ways and one of them is the purification of sewage and the elimination of an important proportion of the organic matter contained in residual effluents by aerobic and anaerobic microorganisms by the process of bioremediation. So, around the world bioremediation technologies are categorized as innovative technologies. Hence, bioremediation in an environment requires the presence of suitable microorganisms. This may involve the presence of complex microbial community. The environment must also be suitable both for the growth of these organisms and for any chemical transformation reaction to proceed at a significant rate. Important factors include the concentration of the toxic chemicals (which most probably will be toxic to the microorganisms carrying out the transformation), the presence of other substrates and nutrients, temperature, pH, oxygen concentration, etc. The unique properties of microorganisms, not seen in plants and animals, are fast growing, small cell size and diversified metabolic pathways. The first two properties allow microbial applications to operate in a fast mode while the third characteristics enable microorganisms to degrade or transform large number of compounds that are harmful to our ecosystem. Microbes are potential candidates for biological transformation of xenobiotics compounds that are introduced into the ecosystem. A microbial population in natural environments exists in a dynamic equilibrium that can be altered by modifying the environmental conditions such as nutrient availability. The metabolic effect of microorganisms on pollutants can take many forms and not always to the environmental advantage of the ecosystems (Table 1).

Table 1: The important characteristics of microbes in biological treatment of chemical pollutants/xenobiotics

Category	Chemical Change	Features
Degradation	Complex compound transformed into simple products, sometimes mineralization	Their ability to grow rapidly on easily available cheap raw materials
		Their ability to maintain a physiological constancy
Conjugation	Formation of complex or addition reactions to more complex compounds	Their ability to bring about biochemical transformations under simple culture conditions
Detoxification	Conversion to non-toxic compounds	High ratio of surface area to volume which facilitates rapid uptake of nutrients required to support high rates of metabolism, biosynthesis and conversions
Activation	Compound converted into more or less toxic compound	

2.1.2. Microbes in bioremediation of pollutants/xenobiotics

The workhorses of bioremediation are microbes, more precisely bacteria and today they seem to provide many solutions to manmade problems. Microorganisms are the oldest inhabitants of earth. They are masters in versatility and adaptability to the changing environment. They will prove to be most cost-effective partners in our efforts for sustainable development. So, limitations faced by physical and chemicals methods of pollution control will be overcome if we take help of these environmental masters (microorganisms). The uniqueness of microorganisms and their often-unpredictable nature and biosynthetic capabilities, given a specific set of environmental and culture conditions (available water, oxygen, pH and temperature), has made them potential candidates for solving pollution problems. Microorganisms especially bacteria, are capable of mediating biodegradation of a wide variety of simple to complex and recalcitrant organic compound owing to their ability to adaptation and genetic manipulation under the prevailing environmental conditions.

Many microbial strains can be isolated to control various forms of chemical pollution, for instance decompose biocides, xenobiotics, detergents, plastic materials or hydrocarbons as shown in Table 2 (Brown, C.M, Campbell, I, Priest, F.G. Introduction to Biotechnology, 1987 Oxford, Blackwell Scientific Publications) which lists some of microorganisms which have been reported to be metabolically active to carry out biodegradation of some known compounds. The substances transformed or degraded by microorganisms are used as a source of energy, carbon, nitrogen, or other nutrient, or as final electron acceptor of a respiratory process. Thus, biodegradation involves the breakdown of organic compounds, usually by microorganisms, into biomass and less complex compounds, and ultimately to water, carbon dioxide and the oxides or mineral salts of other elements present in the environment.

Table 2: List of toxic chemicals degrading microorganisms

Microbes	Chemicals/Xenobiotics
Pseudomonas sp.	4-alkylbenzoates, Alkylammonium, Alkylaminoxides, Anthracene, Benzene, Hydrocarbons, Malathion Naphthalene, Methyl naphthalenes, Organophosphates, PCBs, P-xylene, P-cymene, Parathion, Phenanthrene, Phenoxyacetates, Phenylureas, Polycyclic Aromatics, Rubber, Secondary Alkylbenzenes, Toluene, Phenolics,

	Oleaginous materials, Pulp byproducts
Alcaligenes sp.	Halogenated hydrocarbons, Linear alkyl benzene, Sulphonates, Polycyclic aromatic PCBs
Arthrobacter sp.	Benzene, Hydrocarbons, Pentachlorophenol, Phenoxyacetates, Polycyclic aromatics.
Bacillus sp.	Aromatics, Long chain alkanes, Phenylureas.
Corynebacterium sp.	Halogenated hydrocarbons, Phenoxyacetates.
Mycobacterium sp.	Aromatics, Branched hydrocarbons, Benzene, Cycloparaffins.
Nocardia sp.	Hydrocarbons, Alkylbenzenes, Naphthalene, Phenoxyacetates, Polycyclic aromatics
Streptomyces sp.	Diazinon, Phenoxyacetates, Halogenated-hydrocarbons
Xanthomonas sp.	Hydrocarbons, Polycyclic hydrocarbons
Candida tropicalis	PCBs
Fusariumsolani	Propanil
Cunninghamellae legans	PCBs, Polycyclic aromatics

(Adapted from Brown, C.M, Campbell, I, Priest, F.G. Introduction to Biotechnology, 1987 Oxford, Blackwell Scientific Publications)

2.2. Xenobiotic morpholine: Availability and properties

Morpholine (C_4H_9NO also called diethylene imidoxide) is an extremely versatile synthetic organic compound. Chemically it is six-membered heterocyclic compound that features cyclic amine II and ether functionalities (Fig. 7). It is designated as a volatile organic compound (VOC) and commonly used in various organic synthesis. The major physical, chemical and biological properties of morpholine are summarized in Table 3.

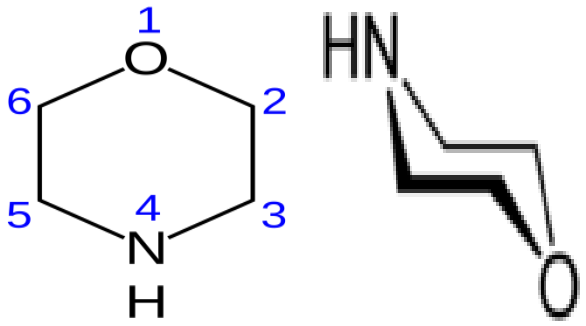


Fig. 7: Molecular structure of morpholine
Where 1,2,3,4, 5 and 6 shows the position of carbon atom in the aromatic ring

Table 3: Physical, chemical and biological properties of morpholine

Morpholine	Features	References
Physical	Liquid, Colorless, Oily	Lewis, R.J. Sr. 2007
	Odor: Amine like	
	Flammable, Corrosive	NIOSH 1999
	Hygroscopic, Volatile, Sensitive to moisture	Texaco Chemical Co. 1986
	Density: 1.007g/cm^3	
	RI: 1.454 @ 20°C	Weast, 1985
	MP: -5ºC	
	BP: 129ºC	
	VP: 6mmHg, VD: 3 g/cm^3	Lewis, R.J. Sr. 2004
	FP: 31ºC	
	Specific gravity: 0.994-0.997 @ 20ºC	
	AIT: 275ºC	
	Purity: 99-100% (contaminated with N-ethylmorpholine and ethylenediamine)	Gerhartz, W. 1985
	Storage: in cool dark place, Stability: Room temp and should be protected from atmospheric moisture and carbon dioxide	WHO 1995 ITII. 1988
Chemical	Chemical formula: C_4H_9NO, Molecular wt.: 87.122g/mol	https://pubchem.ncbi.nlm.nih.gov/compound/morpholine
	Synonyms: 1-oxa-4-azacyclohexane, Diethylene oxamide, Diethyleneimide oxide	
	Solubility: Miscible in water and other solvent ethanol, benzene, and acetone	Lide DR 2003
	pH: 9.4 (0.01% w/w) Acidity (pK_a): 8.36 (of conjugate acid) Strong Base, Polar and 2º Amine in nature	Perrin DD 1965
	Reactivity: Moderate but violently with oxidizing agent	Lewis, R.J. Sr. 2004
	Serious health concern if exposure	National fire protection association 2010
	LD $_{50}$ (Oral): 525 ppm in mouse LC_{50}: 1.32 ppm in mouse	Lewis, R.J. Sr 2004, Wishnok JS and Tannenbaum SR1976, Traut *et al*., 2014, European Chemical Bureau 2000
	PEL-TWA: 20 ppm	OSHA 29 CFR 1910.1000
	IDLH: 1400 ppm	NIOSH 2005
	Human olfactory threshold: 0.036 mg/m^3.	

Biological	REL-TWA: 20 ppm	NIOSH 2005
	Antibacterial and antimycotic properties 0.5% and 10% morpholine inhibits the growth of a variety of bacteria and fungus respectively.	WHO 1995 WHO 1996
	Blue vision, grey vision or haloes, "glaucopsia". Corneal oedema with "hazy vision" and halo phenomena around lights	Mastromatteo, E.1965 Jones, WT., Kipling, MD. 1972, Grant, WM. 1986
	It has toxic properties which affects digestive, respiratory, and cutaneous systems. Harmful if swallowed. Toxic in contact with skin, irritating to eyes, nose and throat and causes severe skin burns and eye damage and necrosis. Toxic if inhaled and causes nausea, headache, or difficult breathing, suspected of causing genetic defects, causes damage to organs through prolonged or repeated use	Brunnemann et al 1982 BASF. Morpholine: 1976 Shea TE Jr. 1939
	Harmful to aquatic life. Damage to living resources. BOD: 0.9%, 5 days; 5.1%, 20 days	https://pubchem.ncbi.nlm.nih.gov/compound/morpholine

Where: MP: melting point, BP: boiling point, VP: vapor pressure, VD: vapor density, FP: flash point, RI: Refractive Index, AIT: auto ignition temperature, LD: lethal dose, LC: lethal concentration, PEL: permissible exposure limit, REL: Recommended exposure limit, TWA: time weighted average, IDLH: immediate danger limit for health, BOD: biological oxygen demand, OSHA: occupational safety and health administration, NIOSH: national institute of occupational safety and health.

Morpholine derivatives and salts are used in the metal industry, as an anti-corrosion and rust-proof factor in the protection of various metals and as a wax and polishing material. It is a strong alkaline that rapidly absorbs humidity and evaporates into the air (Mercer EI. 1991). Morpholine is used in cosmetics as an additive to hair dye, conditioners, and shampoos (Lupp *et al* 2006), and as a disinfectant of water (Rekka *et al*., 1990). To preserve fruits for a longer time and to process food, morpholine is used in waxes, wrapping coatings (NIOSH 1999, Traut *et al* 2014). Morpholine is also a good solvent for organic materials such as paints and resins in the paint industry (Budavari 1996, Summerton 1999). Morpholine is a cheap and readily available alkaline catalyst (Mohammadi Ziarani *et al* 2013). It is used in agriculture as a pesticide, bactericide, herbicide, and fungicide, such as nitrophenyl derivatives which are composed of sulfur and morpholine and are used against powdery mildew on plants (Fleck *et al.,* 2003). Because of its

solubility in water, significant amounts of this chemical compound are released via industrial effluents into the environment where it undergoes chemical or microbiologically-mediated nitrosation leading to formation of the carcinogenic compound *N*-nitrosomorpholine (NMOR) (discussed earlier in introduction). It was also reported that morpholine is found in baked ham (0.20 ppm- Hamano *et al.*, 1981), fishes (<0.6 ppm in canned tuna- Singer *et al.*, 1976a), Fruits/Vegetables coating (Orange pulp 5-71.1mg/kg, orange peel 0.3 mg/kg- Ohnishi et al., 1983), cigarettes and cigarette smoke condensate/snuff (0.3 mg/kg- Singer *et al.*, 1976b, Brunnemann *et al.*, 1982) and paper and paper board food packages (0.018 mg/kg- Hotchkis and Vecchio, 1983) and can enter our body through absorption through the skin, ingestion and other exposure routes, when improperly handled or consumed directly. The type and severity of symptoms of morpholine toxicity vary depending on the amount of morpholine and the nature of exposure. The link between industrial discharge containing morpholine and direct consumption of coated fruits/vegetables is shown in Fig. 8.

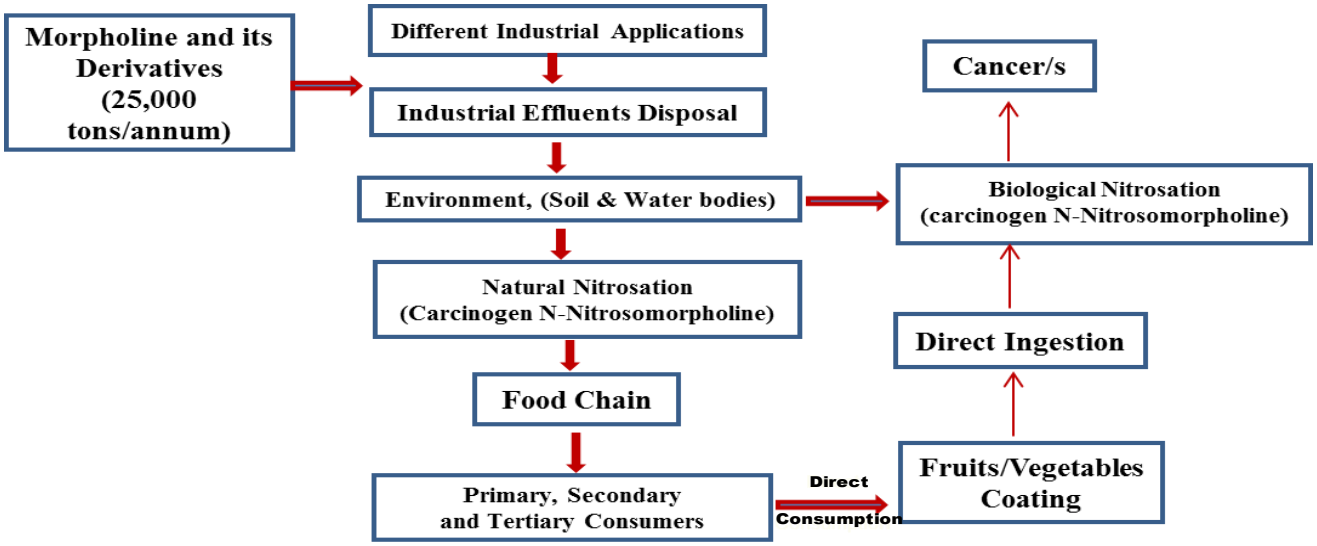


Fig. 8: Link of morpholine in industrial effluents and fruits/vegetables coating

2.3. Occurrences of morpholine and its nitrosation: A case study

Morpholine and its salts have been used as components of protective coatings applied on fruits and vegetables (Ohnishi *et al.*, 1984, US FDA 1988). There are many reports about the presence of morpholine in different fruits and vegetables. Ohnishi *et al.*, 1983 found morpholine at concentrations of < 71.1 mg/kg in the peel of retail citrus fruits (orange, lemon and mandarin) in

Japan while that in the pulp (flesh) of the fruits was much lower, being < 0.7 mg/kg (Table 4). Recently, Rupak *et al.,* 2016 also quantified the presence of morpholine in some fruits and vegetables purchased from super market in Hyderabad, India (Table 4). It was found that if the fruits were washed in washing liquid, morpholine concentrations were reduced, but only by about 25%. Even when the fruit was boiled for 20 minutes, a third to a quarter of the morpholine remained. Morpholine leached by these processes was detectable in washing and boiling water.

Table 4: Morpholine content in fruits and vegetables

Sample	Part	No. of sample	Morpholine concentration (mg/kg)	Reference
Orange (Variety a)	Peel Pulp	12 03	ND-57 0.2-0.7	Ohnishi *et al.,* 1983
Orange (Variety b)	Peel Pulp	06 01	5.0-71.1 0.3	
Mandarin	Peel Pulp	02 01	16.1-18.0 ND	
Lemon	Peel Pulp	02 01	ND-5.2 ND	
Grape	Peel Pulp	02 01	2.8-7.0 ND	
Tomato Carrot Capsicum	Peel Peel Peel	02 02 02	0.17 1.8 0.34	Rupak *et al.,* 2016

Where a and b variety of Orange, N.D. = not detectable with detection level of 0.2 mg/kg

Sen and Baddoo, 1989 reported the morpholine and NMOR content of waxed and un-waxed apples of Canadian origin obtained either direct from the packers or from retail sources. Apple homogenates and coated liquid waxes were analyzed for morpholine contents and are shown in Table 5. Although the concentration of morpholine found in waxed apples was high, NMOR could not be found in any of the waxed or un-waxed samples. Low levels of morpholine in the un-waxed apples could be due to contamination during packing or transport. Morpholine concentration like to those found in orange pulp (Ohnishi *et al.,* 1983), have been found in cigarette tobacco, at a concentration of 0.3 mg/kg, and in snuff and chewing tobacco at concentrations up to 4.0 mg/kg respectively indicating that both smokers and nonsmokers are at an equivalent risk for developing cancer by NMOR. Brunnemann *et al.,* 1982 analyzed the 10 popular snuff brands from USA and Sweden for morpholine and NMOR (Table 6). In five USA

brands, morpholine and NMOR concentrations was between 1.5-4.0 mg/kg and ~0.7 mg/kg respectively and in Swedish products, these concentrations were between 0.2 and 2.5 mg/kg and 0.044 mg/kg respectively. NMOR formed by nitrosation from morpholine was found in 5/5 of USA and 2/5 of Swedish snuff samples.

Table 5: Concentration of morpholine and NMOR in liquid waxes, waxed and un-waxed apples

Sample	Number of sample	Morpholine Concentration (mg/kg)	NMOR concentration (mg/Kg)	Reference
Liquid wax	300	27	0.286	Sen NP and Baddoo PA 1989
	500	31	0.668	
	400	24	0.138	
	500	38	0.277	
	500	22	0.152	
	300	33	0.585	
Un-waxed apples	300	ND	ND	
	500	0.118	ND	
	400	0.016	ND	
	500	0.041	ND	
	500	ND	ND	
	300	0.018	ND	
Waxed apples	300	4.3	ND	
	500	4.9	ND	
	400	6.3	ND	
	500	7.1	ND	
	500	4.0	ND	
	300	7.7	ND	

Where N.D. = not detected with detection limit is 0.005 mg/kg for morpholine and 0.0005 mg/kg for NMOR

Table 6: Morpholine and NMOR concentration in snuff tobacco/cigarette

Concentration (mg/kg)	Snuff tobacco									Reference
	Snuff brand (USA)					Snuff brand (Sweden)				
	I	II	III	IV	V	I	II	III	IV	V
Morpholine	2.8	1.5	4.0	3.2	2.2	0.82	0.2	0.78	0.94	2.5	Brunnemann KD *et al*., 1982
NMOR	0.024	0.7	0.7	0.63	0.03	0.044	ND	ND	0.01	ND	

Where N.D. = not detectable with detection limit is 0.002 mg/kg

2.4. Health effects of morpholine

Morpholine is a toxic substance used in industry and agriculture and can be absorbed into the body through ingestion, inhalation, and the skin. There is ample evidence that this substance can be nitrosated to the carcinogenic N-nitrosomorpholine (NMOR) by reactions within the human body. In mice, rats and hamsters, NMOR may be formed following concomitant administration of morpholine and nitrite or nitrous oxide under physiological conditions (WHO 1995, IARC 1978, Van Stee et al 1983). In vitro nitrosation of morpholine to NMOR was observed when morpholine was added to human saliva (Tannenbaum *et al.*, 1978). Additionally, a new type of metabolite, N-cyanomorpholine, was identified when morpholine was incubated in vitro with whole human saliva (Wishnok and Tannenbaum, 1976, WHO 1996). In a study, Wishnok and Tannenbaum, 1976 found the carcinogenic properties of NMOR in rats, mice, hamsters, and various types of fish after oral administration of this chemical and resulting liver and lung cancer in mice, liver and kidney cancer in rats, and liver cancer in hamsters. Many authors reported that where rats were exposed to morpholine and nitrite, they showed fore-stomach tumors and other carcinogenic properties, e.g. Mirvish SS *et al.,* 1983 reported the liver and fore-stomach tumors and other fore-stomach lesions in rats when treated with morpholine and sodium nitrite. Kitano *et al* 1997 also reported the carcinogenicity of methylurea or morpholine in combination with sodium nitrite in rat multi-organ carcinogenesis bioassay. After oral and parenteral administration or after inhalation, morpholine is well absorbed and distributed in all tissues and body fluids. Morpholine is eliminated mainly in a non-metabolised form in the urine of the rat, mouse, hamster and rabbit (Griffiths, 1968; Tanaka *et al*., 1978; Van Stee *et al*., 1981; Sohn *et al*., 1982) but metabolized only to a limited extent, appearing unchanged in the urine of humans (American Conference of Governmental Industrial Hygienists, Inc. Documentation of the Threshold Limit Values and Biological Exposure Indices. 6th ed. Volumes I, II, III. Cincinnati, OH: ACGIH, 1991. p. 1059). However, Sohn *et al.* 1982 reported that morpholine is metabolized by N-methylation followed by N-oxidation in the guinea-pig.

Animals exposed to morpholine showed liver and kidney damage (http://www.ams.usda.gov/sites/default/files/media/Morph%20Technical%20Advisory%20Panel%20Report.pdf). There is direct relationship between morpholine consumption to

development of liver or kidney cancer, impaired liver and kidney function in animal models because of the biotransformation and detoxification of foreign substances and hence increased susceptibility to effects of morpholine. Many animal studies have been reported to exposure of morpholine and its effects through various routes as shown in Table 7a.

Table 7a: Adverse health effects of morpholine administration to animal models

S. No.	Animal Model	Route	Morpholine dose	Effects observed	Reference
1	Guinea-pigs and rabbit	Dermal application	0.9 g/kg bw	Necrosis of liver and tubules of kidney	Available from https://monographs.iarc.fr/ENG/Monographs/vol47/mono47-11.pdf
2	Guinea-pigs and rats	Single oral administration	0.1-10 g/kg bw	Hemorrhages in stomach and small intestine	
3	Guinea pigs	Oral, 30 days	0.5 g/kg bw	Necrosis of liver and renal tubules	
4	Rats	Inhalation up to 42 hr.	1200 or 1800 ppm (42720 or 6400 mg/m^3)	Liver and kidney necrosis	Shea, TE, Jr. 1939
5	Rats	Oral, 30 days	0.16-0.8 g/kg bw	Severe damage of secreting tubules of the kidney, fatty degeneration of liver and necrosis of stomach epithelium	
	Guinea-pigs		0.09-0.45 g/kg bw		
6	NMRI male albino mice (5)	Intraperiton-eal injection for 15 days	300 mg/kg bw	Changes in blood parameters, increased filtration, decreased reabsorption, absorption, inflammation, hyperemia, urinary tract reconstruction, polyuria	Zare *et al* 2014
7	Rat, Hamster, and Guinea pig	Intraperiton-eal injection	125 mg/kg ^{14}C-morpholine /animal	Half-lives of blood plasma become 115, 120, and 300 min in the rat, hamster, and guinea pig respectively	Sohn OS *et al.,* 1982
8	Guinea pigs	Intraperiton-eal injection	135 mg/kg ^{14}C morpholine	20% of the dose as N-methylmorpholine-N-oxide was found in the urine.	European Commission, ESIS; IUCLID Data set, Morpholine-2000

9	Rats	In diet	1 g/kg	Fatty degeneration of the liver after 270 days	Sander J and Bürkle G. 1969
	Female Sprague-Dawley Rats (7)	In diet for 12 weeks	5g morpholine together with 5g nitrite/kg diet	All develops hepato-cellular adenomas after 39 weeks	
		In diet for 12 weeks	5g morpholine or 5g nitrite/kg alone	No tumors in any tissue after 39 weeks	
10	20 males and 20 female rats	Inhalation	25 ppm 6 hours/day, 5 days/week 13 weeks	No significant differences in body weight, hematology, or organ weight; no nasal lesions	Conaway *et al.*,1984
			100 ppm 6 hours/day, 5 days/week 13 weeks	Focal necrosis in nasal cavity in 2/20 females	
			250 ppm 6 hours/day, 5 days/week 13 weeks	Lesions of nose and mouth; after 7weeks in 8 animal's focal metaplasia and necrosis in nasal turbinate; after 13 weeks, increased severity and pneumonia	
11	60 males and 60 females' rats	Inhalation	10 ppm 6 hours/day, 5 days/week 104 weeks	Focal squamous metaplasia in 1/60 males; keratitis in 8/60 males and 2/60 females	Harbison *et al.*, 1989
			50 ppm 6 hours/day, 5 days/week 104 weeks	Turbinate necrosis: 6/60 males, 2/60females; focal squamous metaplasia of turbinate epithelium: 7/60 males, 2/60 females	
	57 males and 60 females	Inhalation	150 ppm 6 hours/day, 5 days/week For 104 weeks	Focal squamous metaplasia: 46/57males, 42/60 females; turbinate necrosis: 20/57 males, 35/60 females; keratitis, 18/57 males, 10/60 females	

12	Mice	Oral	0.25% morpholine oleic acid salt in drinking water for 96 weeks	No changes	Shibata *et al.*, 1987
			1% morpholine oleic acid salt in drinking water for 96 weeks	Significantly reduced body weight gain, squamous epithelial hyperplasia of the fore stomach, no significant tumors	
13	Rats and Hamsters	Oral	1000 mg/kg in diet during median 117 weeks	3/104 liver cell carcinoma; 2/104 angiosarcomas; 2/104 malignant brain gliomas in rats and no effects in Hamsters	Newberne *et al.*,1973 Shank *et al.*, 1976

Where ppm: parts per millions, Kg bw: kilogram body weight

Symptoms of morpholine exposure from the International Chemical Safety Cards (ICSC) are tabulated in Table 7b

Table 7b: Morpholine exposure and its effects

Type of Hazards	Acute Hazards/Symptoms
Fire	Flammable, Gives off irritating or toxic fumes (or gases) in a fire.
Explosion	Above 35°C explosive vapour/air mixtures may be formed.
Inhalation	Burning sensation, Cough, Laboured breathing, Shortness of breath
Skin	Redness, Pain, Skin burns, Blisters.
Eyes	Redness, Pain, Blurred vision, Severe deep burns.
Ingestion	Abdominal pain, Burning sensation, Cough/Vomiting, Diarrhoea, Shock
Carcinogenic	Nitrosated to the carcinogenic N-nitrosomorpholine by reactions outside, or within, the human body

As per 2008 summary report of the Health Hazard Assessment (HHA) on "morpholine in wax coatings of apples" by Health Canada, the chronic oral toxicity study in rat and mice- No Observed Adverse Effect Level (NOAEL) is an Acceptable Daily Intake (ADI) of 0.48 mg/kgbw/day. When not considering the potential for nitrosation, morpholine exposure for

children and adults is about 8% (0.0384 mg/kg bw/day) and 5% (0.024 mg/kg bw/day) of the ADI respectively, seems to be safe and not a cause for any concern. Although it is assumed that there is some probability of harm at any level of exposure to a genotoxic carcinogen, actual exposure may be so low that the health risk is practically negligible. Regarding the presence of NMOR on apples coated with wax containing morpholine--No NMOR was determined to be present on these apples, and even no NMOR was formed when morpholine and nitrite were combined in experiments conducted in the presence of apple flesh. From the estimated morpholine exposure, the possible endogenously formed NMOR was estimated to be 2.2 and 3.6 ng/kg bw/day for adults and children, which is less than the estimated safe dose of 4.3 ng/kg bw/day. Uncertainties in this estimate of NMOR formation include the physiological differences between humans and rats and actual levels of nitrite consumed. It is unreasonable to assume that these uncertainties would increase the estimated NMOR formation in the human gut. Since, no epidemiological studies of morpholine have been conducted no data is available from human studies on the carcinogenicity of morpholine. The overall IARC (International Agency for Research on Cancer) evaluation was that morpholine was not classifiable for its carcinogenicity to humans (IARC-1989). Although no direct data about in-situ formation of NMOR as a human carcinogen is available it was found that prevalence of liver cancer has risen after the introduction of morpholine in United Kingdom (UK). Morpholine was introduced in (UK) in late 1970 and gradually it was used intensively in different industries. Exposure of morpholine through various route results adverse health effect which might be one of the reasons (insufficient literature) other than physiological cause of higher incidence of liver cancer as shown in Fig 9 as per Cancer Research, UK report, 2010. Hence, in October 2010, UK Food Standards Agency had undertaken an initial risk-assessment of morpholine coated fruits/vegetables and found that morpholine at the levels detected, 0.03-0.3ppm, is likely to be of a low risk to consumer health. Therefore, the use of morpholine is prohibited in UK and European Union (EU) member countries. UK Food standards agency banned the import of apple from Chile because it was found to have about 2ppm of morpholine and advised that affected apples should not be on sale in the UK. The use of morpholine in cosmetics has also been forbidden in EU countries since 1986 (available from http://www.inchem.oeg /documents /hsg/hsg/hsg092.htm). In Germany, the use of morpholine in water-repellent food packaging material is forbidden (BUA, 1991).

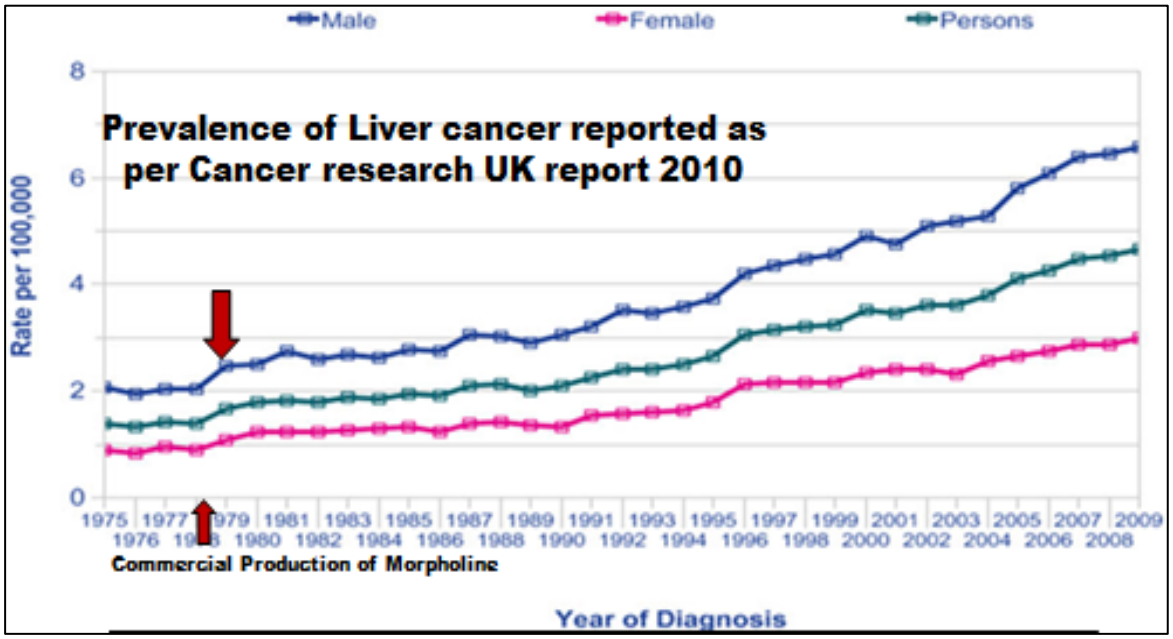


Fig. 9: Prevalence of liver cancer as per cancer research report, UK, 2010

2.5. Microorganisms for morpholine degradation: Free and immobilized microbes

2.5. 1. Acclimatization of microorganisms for degradation studies

The first step to biodegrade toxic substances in a wastewater treatment plant is the acclimation of the microorganisms. In a stress condition, when microorganisms are put in contact with toxic compounds, acclimation to these compounds may occur (Moreno-Andrade, I. and G. Buitrón 2004). In general, acclimatization is a selection criterion of a xenobiotic resistant bacteria from different geological sites to be used for bioremediation of polluted environment. Acclimatization enables the microorganisms to develop the capacity to degrade them faster, preventing the chemical stress. It is the process in which an individual microbe was grown in presence of xenobiotic to adjusts to this changed environment. Acclimatization takes shorter period from hours to weeks and during this process, it undergoes morphological, behavioral, physical, and/or biochemical changes in response to the concentration of xenobiotic or pollutants. The capacity to acclimatize in de novo environmental condition for different bacterial isolate has been well reported for degradation studies of a specific pollutants or xenobiotic. Several authors investigated the degradation efficacy of acclimatized microbes for biodegradation studies. Gupta *et al.*, 2015 showed the effective treatment of two pollutants, chromium (VI) and 4-chlorophenol

(4-CP) by acclimatization of anaerobic sludge and found that sulfate-reducing bacteria present in the anaerobic sludge helps to remove heavy metals. Ebtesam et al 2013, used Atrazine acclimatized bacteria (acclimatized period: 10 days) for biodegradation of Atrazine in the contaminated soils. However, Moreno-Andrade, I. and G. Buitrón 2004 conclude that a reduction in degradation time was noticed as the acclimation to 4-chlorophenol (4CP) process occurred in an aerobic automated sequencing batch reactor. The results showed, during acclimation for an initial concentration of 50 mg 4CP/L, degradation time was reduced from 40 h to 50 min after 10 cycles. Wong *et al.,* 2011 studied the acclimatization and performance study of anaerobic degradation process for palm oil mill effluents by anaerobic bacterial isolate and Bayle *et al.,* 2009 investigated the effect of acclimatization period for the microbial aerobic degradation of volatile organic compounds (VOCs). Other author like Zawani *et al.,* 2013 found the presence of acclimatized microbes in activated Sludge in Kenaf-Retting Wastewater. Özbelge *et al.,* 2007 noticed that microorganisms must be acclimated to heavy metals (Cu and Zn) or other toxic substances present in wastewaters before they are used in an activated sludge process. In last few decade, these approaches have been used for effective removal of pollutants, pesticides and heavy metals in waste water. However, the acclimatization and performance studies of microorganisms to degrade morpholine is not reported. So, in this present study, different bacterial isolates were investigated for their capacity to degrade morpholine degradation when acclimatized in presence of morpholine.

2.5.2. Morpholine degraders: Free cells or Microbial consortium

Form earth's six billion years' history, microbes ruled on the globe, from ocean floor to arctic permafrost, under extreme environmental conditions (Fulekar MH. 2010). The abundant biodiversity may be the key to helping the planet by putting these rich microbial communities to work for serving the needs of men and environment (MAE) through different application. One of the potential applications is environment cleanup of xenobiotics such as morpholine through bioremediation. This technology relies on promoting the growths of specific microflora or microbial community that are indigenous to the contaminants and able to perform desired activities (Agarwal, 1998). Bioremediation refers to the use of a biological system of micro-organism to break down the contaminants into harmless products. Like all living creatures,

microbes obtain nutrients, carbon and energy to survive and multiply from breaking down of these chemicals by enzymatic reactions. In this process, microorganisms use the contaminants as a source of nutrient Carbon and Nitrogen (C, N) and energy (Hess *et al.,* 1997; Agarwal, 1998, Subrahmanyam*et al.,* 1983) so that these microorganisms enzymatically attack the pollutants and convert them into harmless products. Many authors found that morpholine degraders are mainly *Mycobacteria sp*. isolated from activated sludge, soils and water in most cases (Cech *et al.,* 1988, Brown *et al.,* 1990) except some *Arthrobacter sp* and *Pseudomonas sp* (Table 8). There is no obvious reason why morpholine degraders almost exclusively belong to the genus *Mycobacterium.* It might be due to wide spread in nature. However, some *Mycobacterial* strains showed a great resistance to the toxicity of this amine (Mazure and Truffaut 1994). The basis of remediation is the enormous natural capacity of microorganisms to degrade organic compounds. In almost all cases, it will be individual strains or consortia of microorganisms that will act on pollutant molecules. The degradation of a range of compounds however has been shown to proceed more readily with mixed culture of organisms and the utilization of some compounds may not proceed at all in monocultures. The individual bacteria can produce a small quantity of enzyme. In the case of consortium, the enzyme production rate was high because more number of bacterial loads (Ajao *et al.,* 2011). However, till date there are no reports for successful removal of morpholine using co-culture/mixed culture or microbial consortium.

Table 8: Different microorganism used in morpholine degradation

SI. No	Morpholine degraders	Source	Morpholine conc.	Duration	References
1	*Mycobacterium gilvum*. strain HE5	Forest soil	10-15mM	10 hr.	Schrader *et al.,* 2000
2	*Mycobacterium. Strain* MorD and MorG	Activated sludge	10 mM	60 hr.	Knapp *et al.,* 1982
3	*Mycobacterium* aurum MO1	Culture in a reactor	10 mM	10 hr.	Cech et al 1988 Combourieu *et al.,*1998, 2000, Mazure and Truffaut 1994
4	*Mycobacterium* strain RP1	-	10 mM	50 hr.	Combourieu *et al.,* 1998; Mazure and Truffaut, 1994.
5	*Mycobacterium. strain MO1*	---	---	---	P. Poupin *et al.,* 1996

6	*Mycobacteriumchoroph enolicum* RP1	Activated sludge	10 mM	13 hr.	P. Poupin *et al.,* 1998
7	*M. aichiense* BM01 *M. diernhoferi* FM30 *M. komossense* RP1 *Mycobacterium* aurum LM20	Grassland soil Manure heap soil Activated sludge Lake sediments	10 mM	06 days	P. Poupin *et al.,* 1999
8	*Mycobacterium chelonae* MorG	-	-	-	Swain *et al.,* 1991 Franklin 1985
9	*Arthrobactersp*	-	-	-	Dmitrenko *et al.,* 1985
10	*Pseudomonas fluorescens*	--	---	--	Knapp *et al.* 1996 Chandra sekaran and Lalitha Kumari 1997
11	*Pseudomonas fluorescens*	Waste water	1.26 mM	10 days	Magda M. and Aly 2011
12	*Mycobacterium Butyricum*	Waste water	11.5 mM	10 days	Magda M. and Aly 2011
13	*Pseudomonas putida*	-	-	-	Aly MM (2004)
14	*Mycobacterium sp.*	-	-	-	Besse *et al.,* 1998

2.5.3. Immobilized microbes for morpholine degradation

Immobilization is the technique used for the physical or chemical fixation of cells, organelles, enzymes, proteins onto or into a solid support, or retained by a membrane, so that their stability is increased and increase the possibility for their continuous use. Immobilized cells have been defined as cells that are entrapped within or associated with an insoluble matrix. An immobilized cell possesses many benefits over free cells. The immobilization of whole cells tends to improve the stability of enzymes by retaining them in their natural surroundings during immobilization and subsequent continuous operation. Overall, the immobilized cell systems are more tolerant to local perturbations such as fluctuations in pH, temperature, presence of inhibitor compounds, etc. (Venkatasubramanian and Veith 1979, Tallur *et al.,* 2009). Besides being stabilized by immobilization, cells usually retain physiological activities like those of free cells. Most commonly used polymers are porous, and permit adequate diffusion of nutrients, dissolved gases,

and metabolic byproducts. Natural polymers such as alginate, agarose and others, usually used for microbial immobilization. They were chosen for their different properties (given below) as reported by Keweloh *et al.*, 1989, Cassidy et al. 1996, Feodorov *et al.*, 1993, Heinze and Rehm 1993, Lee *et al.*,1994.

- The simplicity of preparation and gelation under mild conditions (a process which does not cause stress in organisms)
- Biodegradability, non-toxicity, nonirritant in nature
- Ease of use and mixing,
- Cheap and good shelf life, good surface area,
- Controlled hardness with temperature and time,
- Slow release of biomass over time,
- Protection against xenobiotics compounds and/or enhance the degradation rates

Entrapment of cells in a semi permeable gel matrix of alginates is the most popular system of immobilization (Champagne *et al.*, 1994). The process is accomplished by mixing cells with a polymeric solution, applying feeding drops of suspension into a hardening solution which lets the drops harden to form expected spherical gel beads (Chibata and Wingard 1983, Hulst *et al.*, 1985, Linko and Linko1984). In many industrial applications, the use of immobilized morpholine degraders is preferable and many authors have reported its efficacy over free cells (Table 9). Moreover, immobilized microorganisms promise to be cost effective since they can be used several times without significant loss of activity (Rhee *et al.*, 1996; Devi and Sridhar, 2000). Therefore, immobilized microorganism technology has been explored as promising method for wastewater treatment in the past few decades and will continue to be used in near future (Zhou *et al.*, 2008).

Table 9: Immobilization of morpholine degrading microbes

Morpholine degraders	Immobilization matrix	Result/s	Remark/s	Reference
Mycobacterium aurum M01	K-Carrageenan (2.2%)	28% to 75% of morpholine removal in *5* days by using free and immobilized cells respectively	Morpholine degradation began earlier and faster than in free culture	Mazure, N. and Truffaut 1994

Mycobacterium sp.	Sodium alginate (2%)	11% to 23% of morpholine degradation by using free and immobilized cells respectively	Increased morpholine degradation compared with free cells	Magda M. and Aly 2011

2.6. Method of quantification of morpholine and its derivatives

The methods available for estimation of morpholine involve chemical reactions that are characteristic of the individual functional groups. Many less sensitive and indirect methods based on titration (Hassan *et al.,* 1985) and chemical conversion to oxides- chemical oxygen demand-COD (Cech *et al.,* 1988) have been used in the past 2-3 decades. Several methods based on analytical instruments such as capillary isotachophoresis (Alexandra Widhalm and Ernst Kenndler 1991, Aarts *et al.,* 1990) (Fig. 10), gas chromatography (OSHA-pv2123 1999, Rupak Kumar and Suman Kapur 2013 (Fig. 11a, 11b), high pressure liquid chromatography (Minoru Koga and Takashi Akiyamam 1985, M.Joseph *et al.*, 1993, Lindahl *et al.,* 2001), (Fig. 12a, 12b and 12c), ion/cation exchange chromatography (Isaeva *et al.,* 1983, www.dionex.com/en-us/.../114771-AN1062-IC-Morpholine-Linezolid_AN70702_E.pdf, Dionex, application note: 86, determination of trace cations in power plant waters containing morpholine, http://www.dionex.com) (Fig. 13a, 13b) and Spectrophotometric method (WH Stevens and Kirsten Skov. 1965, Giti Emtiazi *et al.,* 2001) are in use for quantification of morpholine. Each analytical method has its own advantage and limitation (Table 10), e.g. cation-exchanger resins have a high capacity for amines, but they do not work well in the presence of electrolytes e.g., NaCl, Na_2S0_4 (Pradip K. Pahari and Man Mohan Sharma. 1991). Morpholine determined by gas chromatography (GC- Fig. 11a, 11b, and 11c) require packed as well as capillary columns with detectors like flame ionization detector (FID), flame photometric detector (FPD), nitrogen selective detector (NSD), mass spectrometry (MS) and thermal energy analyzer (TEA). However, high-performance liquid chromatography (HPLC) needs UV, TEA, or refractive index detector. For determination of trace amounts of morpholine, derivatization is required in same analytical method (Table 10). These different methods reported here require sophisticated, expensive analytical instruments along with specific sample preparation steps and provide an opportunity to rapidly evaluate effluent composition/treatment for removal of morpholine.

Table 10: Different analytical method of estimation of morpholine or its derivatives

Analytical method	Characteristic feature	Detection limit	Specificity	Remark	Reference
Spectrophotometric	Inherent simplicity, high sensitivity, low cost and wide availability	1-10 mg/l	Primary and Secondary amine	Morpholine lacks a chromophore	WH Stevens and Kirsten Skov. 1965, Giti Emtiazi *et al.*, 2001
GC-FID	An amine specific column and Flame ionization detector with nitrogen/helium as carrier gas in the split mode injection method	µg/l to mg/l (0.001-1000 ppm)	Morpholine, Methyl morpholine	Amines tend to be strongly adsorbed and decomposed on the columns. It gives tailing and poor reproducibility	OSHA-pv2123 1999, Rupak Kumar and Suman Kapur 2013 Analysis of N-methyl morpholine and morpholine (http://younglin.com), Amines: gas chromatography, https://murdercube.com/.../AMINES%20%20GAS%20CHROMATOGRAPHY.pdf
GC-MS (Electron ionization)	Analyze morpholine based on generation of ion followed by its separation (differences in *m/z)* after passing through GC column	mg/l	Morpholine	Positive mode major m/z peaks at 57, 87,56	https://pubchem.ncbi.nlm.nih.gov/compound/morpholine
HPLC	Column of Supelco-sil-ODS with an isocratic mobile phase and UV detection at 280 nm		Morpholine and its derivative products	Derivatised with N-succinimidyl-p-nitrophenyl acetate	Minoru Koga and Takashi Akiyamam 1985, M.Joseph *et al.*, 1993, Lindahl *et al.*, 2001

Capillary isotachophoresis (ITP)	Coupled with conductimetry detection that allows the identification of the analytes based on the corresponding step height	30 mg/l	Morpholine and its derivatives products	The separation capillary is filled with a discontinuous buffer: one with leading electrolyte with ion of the highest mobility and the other with terminating electrolyte with ions of the lowest mobility	Alexandra Widhalm and Ernst Kenndler 1991, Aarts *et al.*, 1990 .
Ion/Cation exchange ion chromatography (IC)	IC with Dionex IonPac CG19 Guard column with suppressed conductivity detector Eluent: 7.5 mM with Methane sulfonic acid	µg/l	Morpholine	Flow Rate: 0.25 ml/min Inj. Volume: 100 µl Concentrator column Dionex IonPac TCC-ULP1	Isaeva *et al.*, 1983, www.dionex.com/en-us/.../114771-AN1062-IC-Morpholine-Linezolid_AN70702_E.pdf, Dionex, Application note: 86, Determination of Trace Cations in Power Plant Waters Containing morpholine, http://www.dionex.com
Titration	The acidimetric titration of the amines in accordance to standard procedure of Karl Fischer titration	Lower mg/l range	Morpholine	As a strong basic amine, it is titrated in acidified reagent solutions (Benzoic acid, EDTA, amine N-oxide) to a stable end point.	Hassan *et al.*, 1985 Metcalfe L.D. 1982
COD	The amount of a specified oxidant that reacts with the sample under controlled	230 mg/l = 423ppm COD	Morpholine	The specific COD of morpholine is 1.839 g/g	Cech *et al.*, 1988 US-APHA 1976

	conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence.				

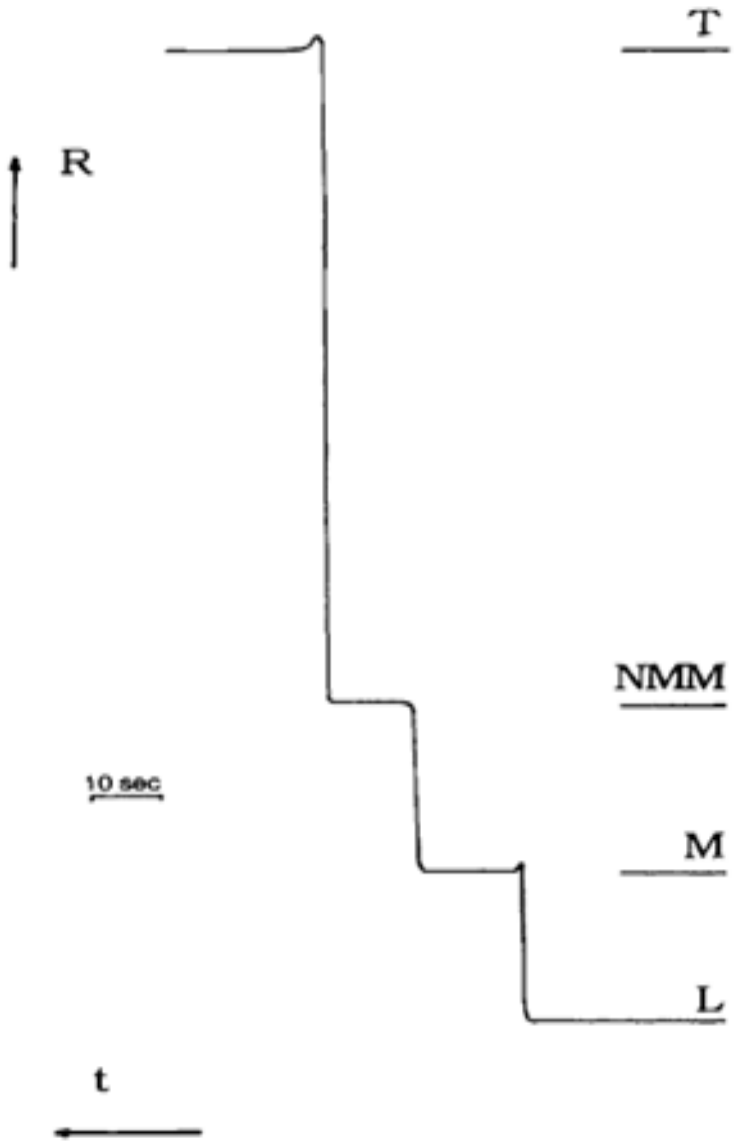


Fig. 10: Isotactophoresis of mixture of morpholine (M) and N-Methyl Morpholine (NMM). Where, L indicates the leading ion, T indicates the terminating ion, R indicates the resistance and t indicates the time (Alexandra Widhalm and Ernst Kenndler 1991)

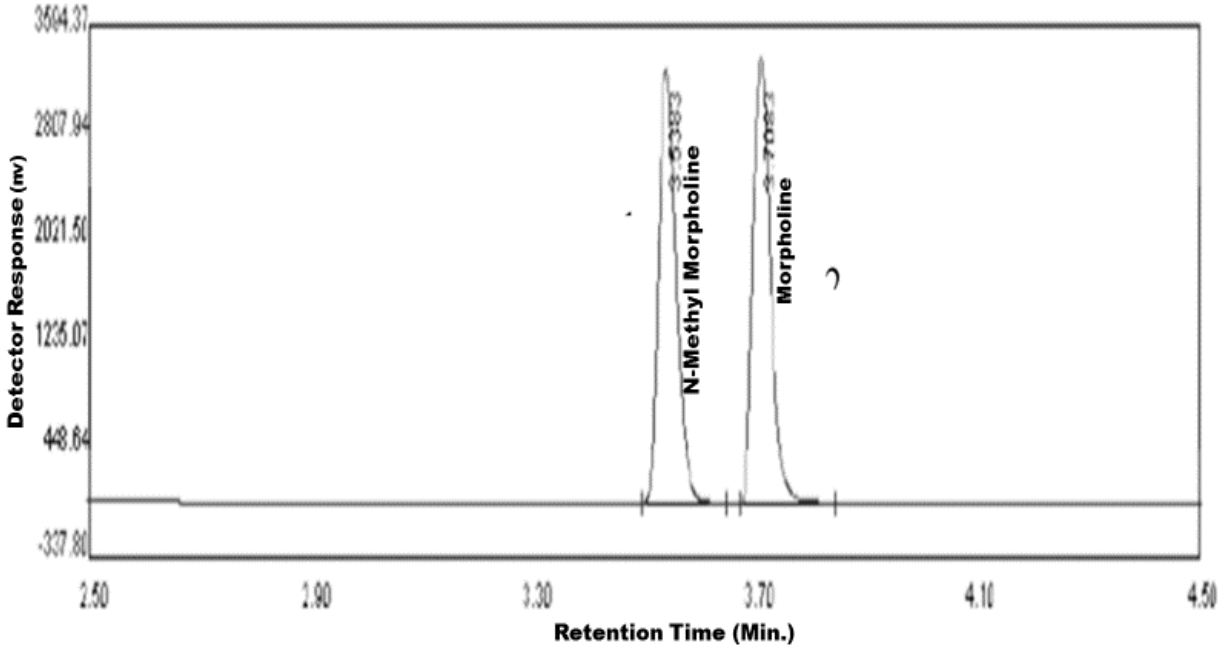


Fig. 11a: GC-FID Analysis of N-methyl morpholine and morpholine
(http://younglin.com)

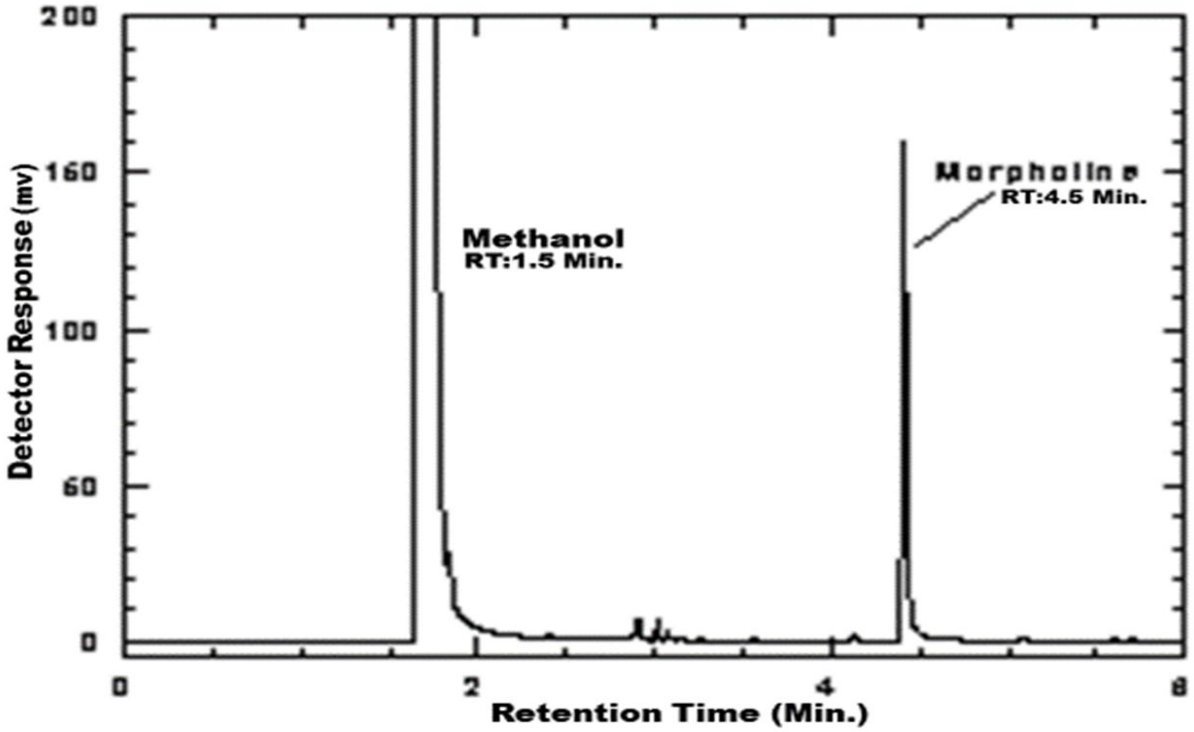


Fig. 11b: GC-FID analysis of morpholine
(OSHA- PV2123, 1999 https://www.osha.gov/dts/sltc/methods/partial/pv2123/pv2123.html)

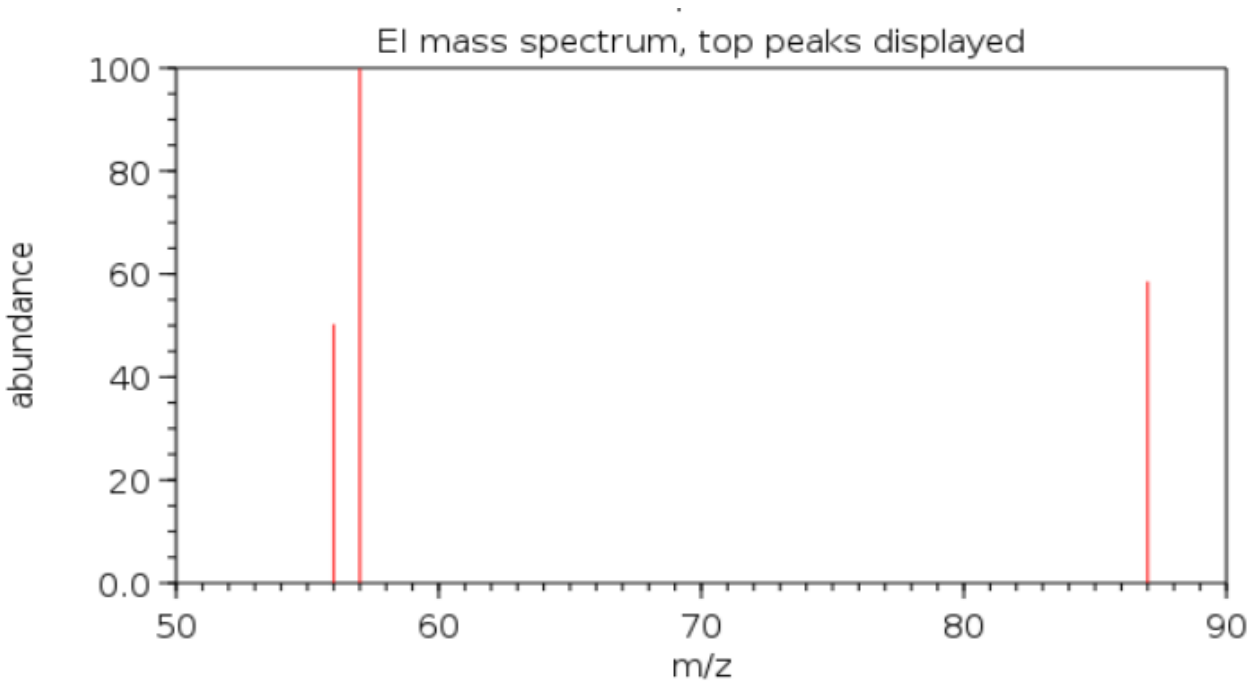


Fig 11c: GC-MS analysis of morpholine where top m/z top peak are at 57, 87 and 56 respectively (https://pubchem.ncbi.nlm.nih.gov/compound/morpholine)

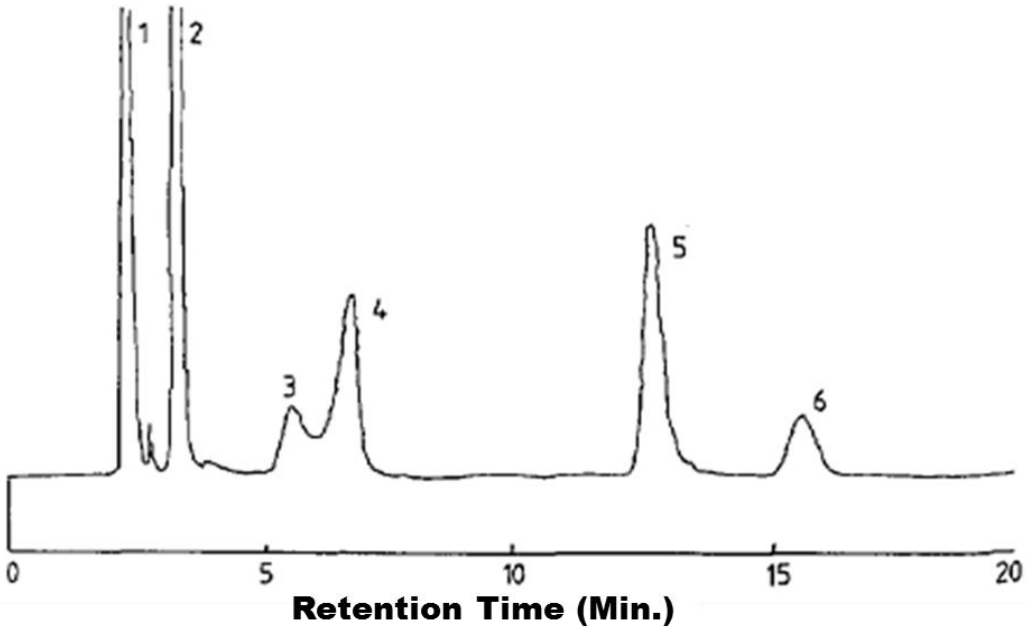


Fig. 12a: HPLC analysis of N-succinimidyl-p-nitrophenylacetate (SNPA) derivatized products. Where peak 1 = p-nitrophenylacetic acid, 2 = N-hydroxysuccinimide, 3 = Ethanolamine, 4 = Ammonia, 5 = Morpholine and 6= 2(2-aminoethoxy) ethanol (M. Joseph *et al.*, 1993)

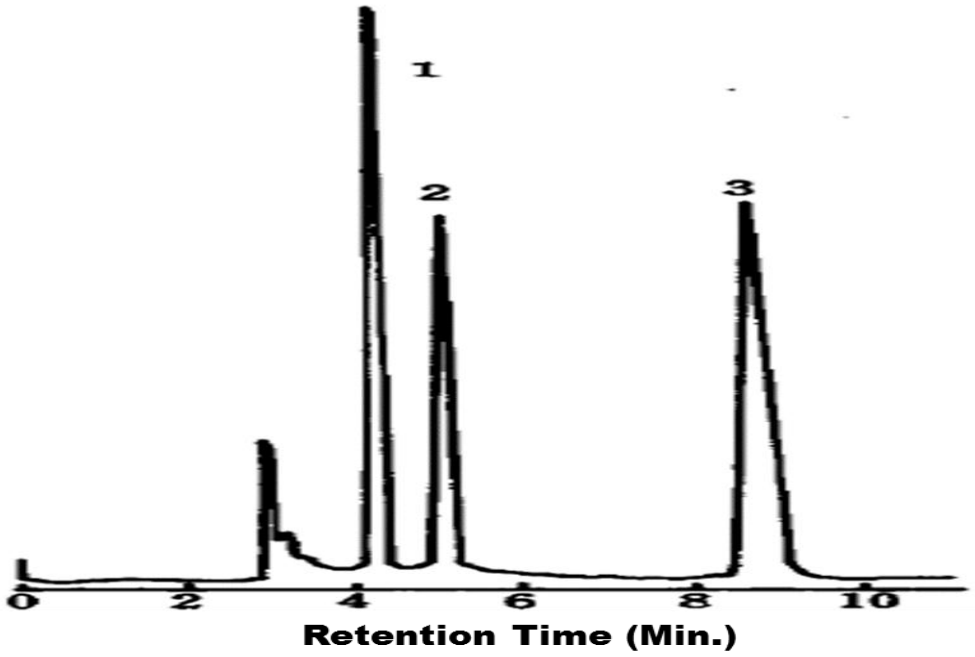


Fig. 12b: HPLC analysis of 1, 2-Naphthoquinone (NQ) derivatized products. Here**,** peak 1 = Morpholine-NQ, 2 = Pyrrolidine-NQ and 3 = Piperidine-NQ (Minoru Koga and Takashi Akiyamam 1985)

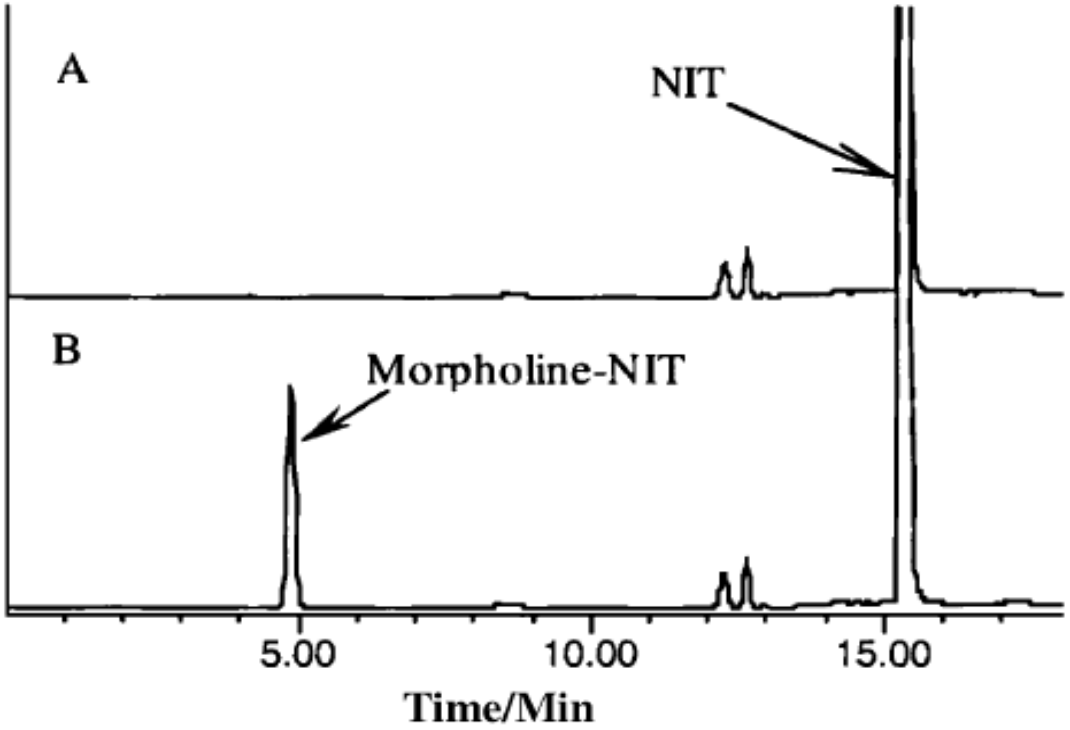


Fig. 12c: HPLC analysis of 1-naphthylisothiocyanate (NIT) derivatized products. Here A indicates the filter blank and B indicates the filter spiked with morpholine (Lindahl *et al.,* 2001)

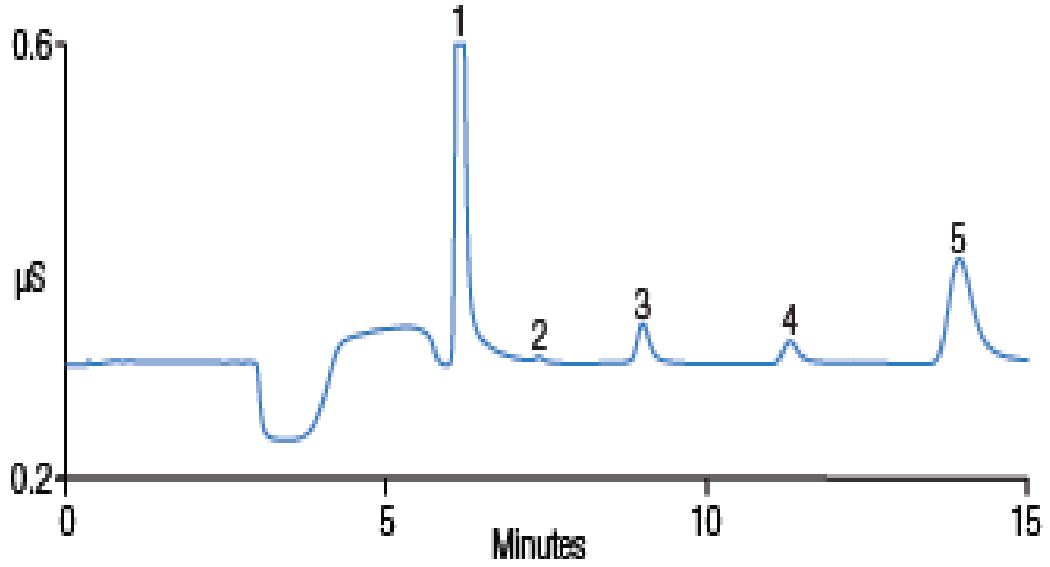


Fig. 13a: Ion chromatography of morpholine (20 µg/l morpholine in 10% methanol) using Dionex Ion Pac CS19 column. Where, peak 1= Ammonium salt, Peak 2= Potassium, peak 3= Morpholine, peak 4= Magnesium and peak 5= Calcium (www.dionex.com/en-us/.../114771-AN1062-IC-Morpholine-Linezolid_AN70702_E.pdf)

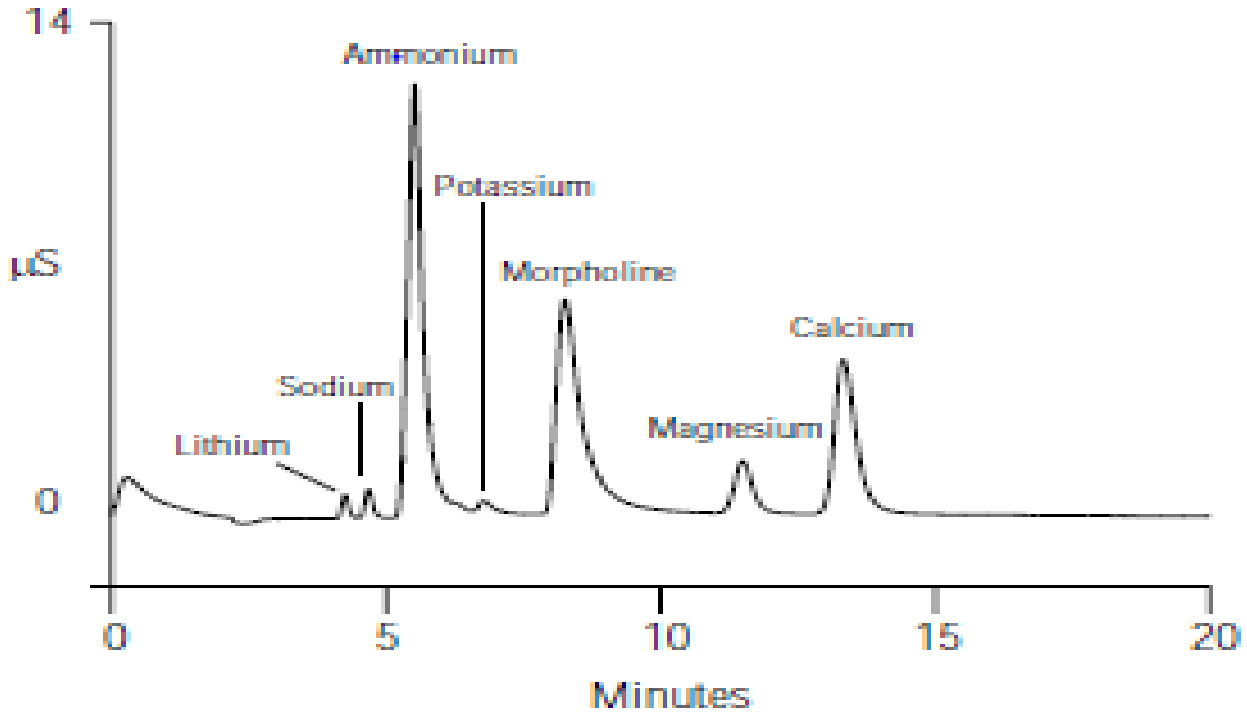


Fig. 13b: Ion chromatography of morpholine mix sample using Dionex Chromatography system (Dionex, Application note: 86, Determination of Trace Cations in Power Plant Waters Containing morpholine, http://www.dionex.com)

2.7. Biodegradation pathway of morpholine

Biodegradation is the process by which waste materials such as oil spill, herbicides, pesticides etc. are degraded by the action of microbial systems. Organic compounds may be biodegradable (transformed by biological mechanisms which might lead to mineralization), persistent (fail to undergo bioremediation in an environment or under specific set of experimental conditions) or recalcitrant (inherently resistant to biodegradation). Most naturally occurring (or biogenic) compounds are biodegradable while manmade (or xenobiotic) compounds may be biodegradable, persistent or recalcitrant. Xenobiotic chemicals, because they are manmade and have been developed recently, are present in the environment for comparatively shorter periods of time from a geological point of view. This in turn means that the microbial communities present in these environments may not have evolved specific mechanisms for their degradation. Many possible mechanisms exist, however, which one leads to active biodegradation differs from pollutant to pollutant. Some enzymes can bind analogous to their natural substrates which contain xenobiotic functional groups, if these do not greatly alter the charge of the active site. It is possible for the enzyme to catalyze a reaction with the xenobiotic as substrate. The success of this metabolism as a biodegradation mechanism depends on other factor also, such as the ability of the xenobiotic to act as an inducer and the nature of product formed. The large-scale annual usage of morpholine and its potentially carcinogenic effects motivate investigation of the metabolic pathways involved in its biodegradation**.** The toxicity or the contamination of chemicals can be reduced by the bioremediation as a solution with many intermediate or degradation product. However, to date, no tool for the direct detection of intermediates, or even of morpholine degradation product/s has been available. The metabolic pathway involved in biodegradation of morpholine has been very difficult to establish, because this chemical does not possess any chromophore and is highly soluble in water, which does not allow easy extraction. Consequently, no tool for direct detection of intermediates or even morpholine has been available. Only indirect strategies have been developed like COD, optical density, and ammonia (NH_3) measurements, growth on intermediates, and in-vitro enzymatic assays.

A few studies were carried out to understand the morpholine biodegradation process and its regulation by Swain *et al.* 1991, Mazure *et al.* 1994 and Shaikh *et al.*, 2009. They proposed a

hypothetical pathway for the biodegradation of morpholine that could proceed via 2-(2-aminoethoxy) acetate and glycolate and/or ethanolamine. Swain *et al.,* 1991 found proposed hypothetical pathway in *Mycobacterium chelonae* (Fig.14b). Mazure and Truffaut 1994 described the degradation of morpholine via the ethanolamine and glycolate routes in *M. aurum* MO1. Degradation of morpholine is likely to begin by the breakage of a bond between the hetero atom and an adjacent carbon atom and the enzyme responsible for the ring cleavage was a monooxygenase. Morpholine monooxygenase is an important enzyme in morpholine degradation (Sielaff *et al*., 2001). This enzyme catalyzes the biotransformation of morpholine to 2-(2-aminoethoxy) acetic acid and contains a cytochrome P450 catalytic subunit (Shaikh *et al.,* 2009, Poupin *et al*., 1999). Morpholine could serve as substrates for flavin-containing monooxygenase or cytochrome P450 (Knapp and Brown, 1988) which is associated with oxygen consumption (Knapp *et al*., 1996). Poupin *et al*., 1998 attributed the inhibitory effects of metyrapone on the degradation ability of *Mycobacterium*strain RP1 to the involvement of cytochrome P-450 in the bio-degradation of morpholine. Depending on the morpholine concentration in the medium, one pathway could be used while the other was inhibited. Recently, a new approach, in which ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy has been performed directly with culture supernatants to identify some metabolic intermediates of morpholine by *M. aurum* MO1 (Combourieu *et al.,* 1998, 2000). Many different species of *Mycobacterium* have been shown to degrade morpholine via this shared group of degradation reactions for cyclic amines. The degradation reaction mechanism and characterization of these reactions has been assayed by direct means but with little information about the enzymes involved in the reactions (Combourieu *et al.,* 1998) as shown in Fig.14a. Furthermore, the byproducts of microbial processes can provide indication for successful bioremediation. However, till date, no tool for the direct detection of intermediates, or even of morpholine, has been available. Consequently, only hypothetical pathways were proposed, and limited interpretations of various experiments have been made (Fig.14b).

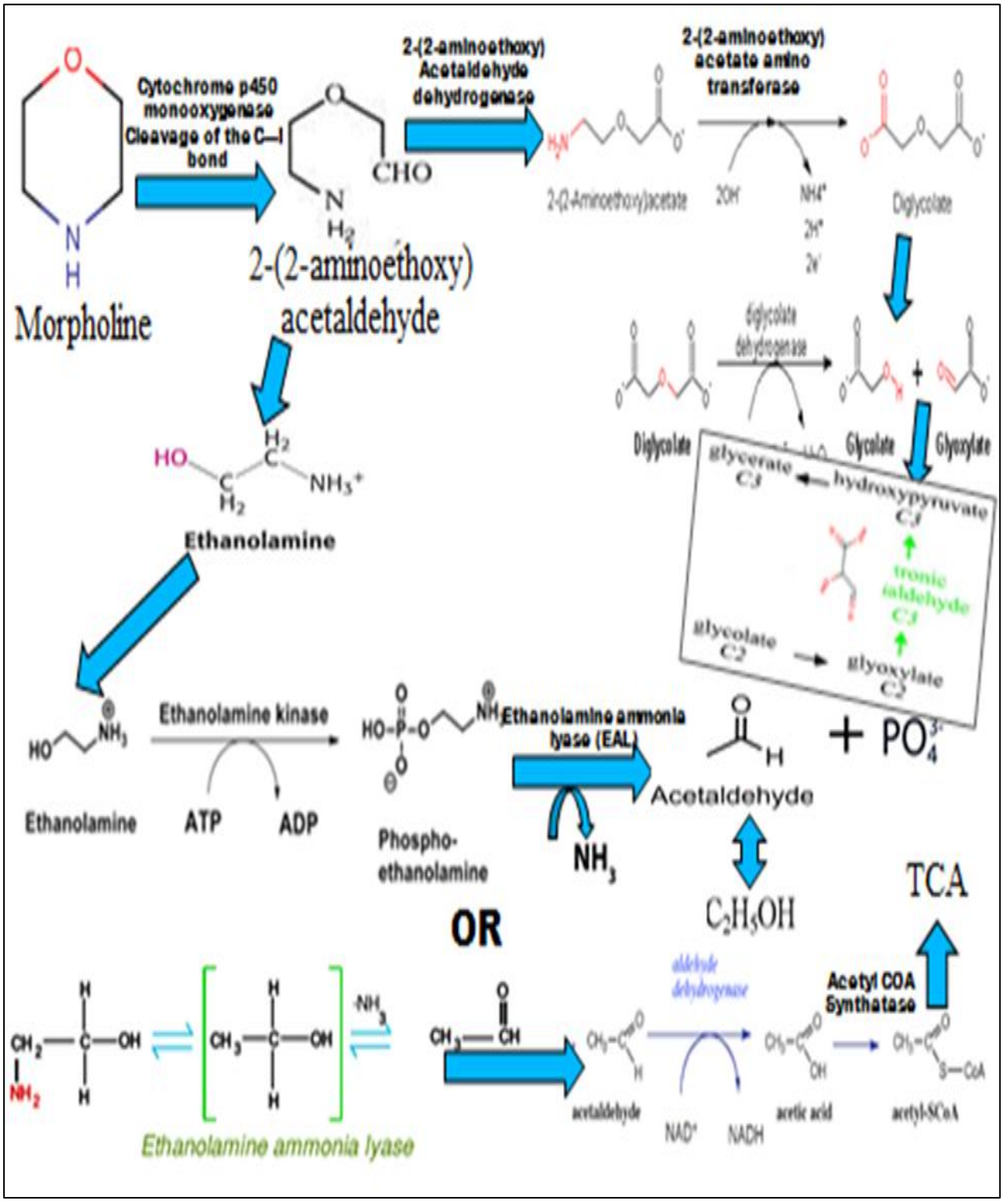


Fig. 14a: Elucidation of degradation pathway of morpholine
(Swain *et al.* 1991 and Mazure *et al.*, 1994)

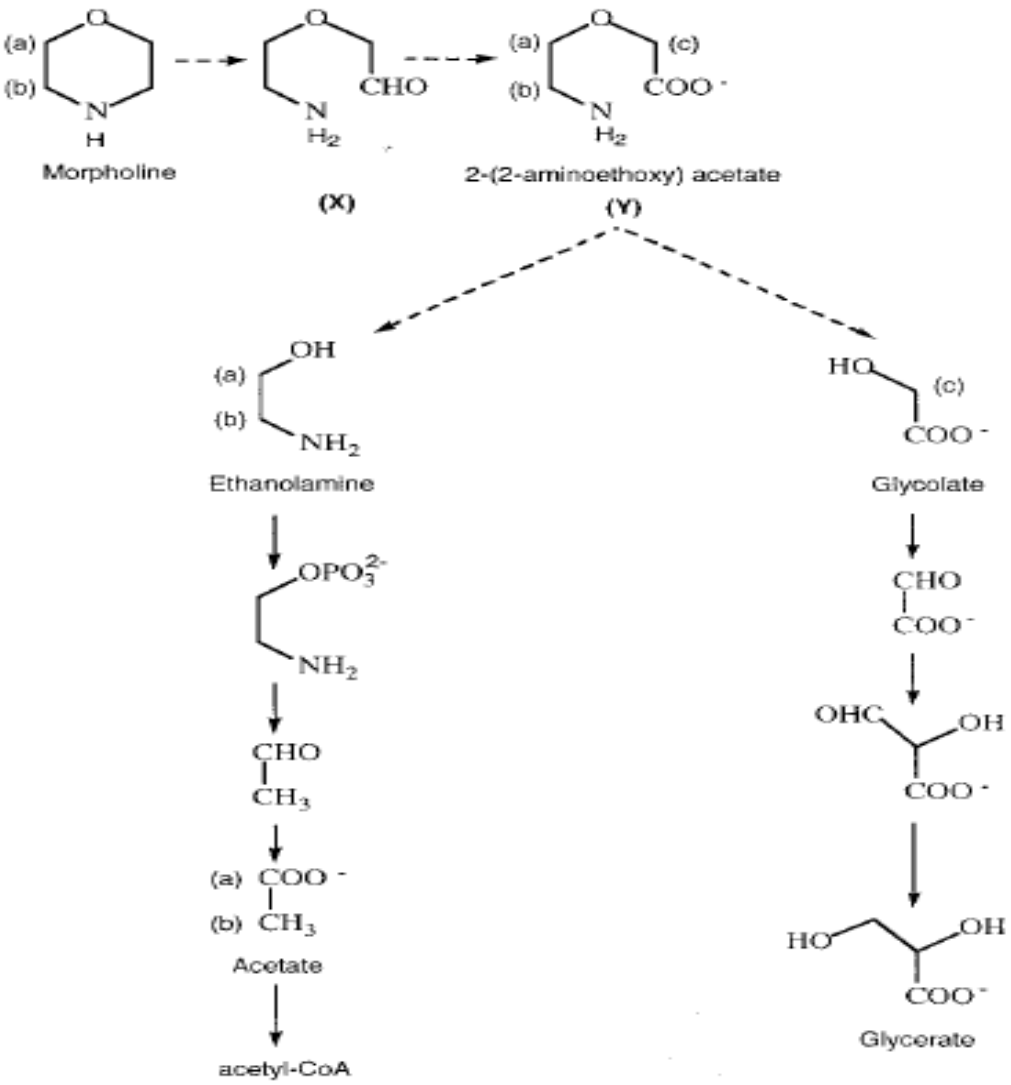


Fig. 14b: Hypothetical pathway of morpholine degradation

Where, X= 2(2-aminoethoxy) acetaldehyde, Y= 2(2-aminoethoxy) acetate and a, b, c indicates the position of carbon in the ring (Swain *et al.*, 1991).

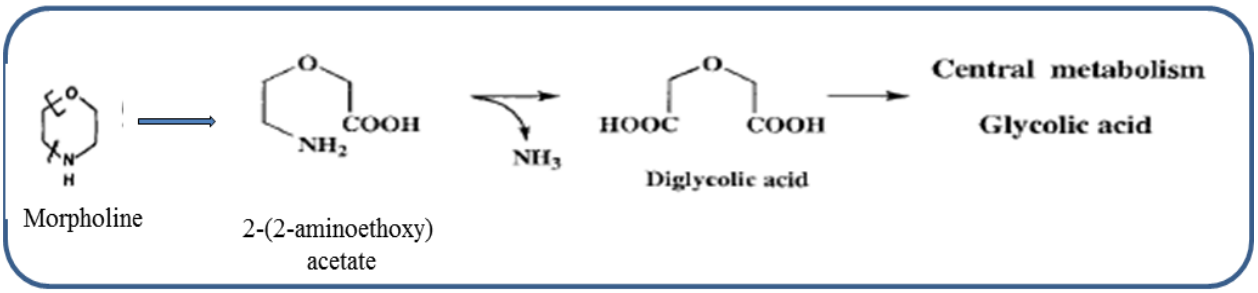


Fig. 14c: Postulation of morpholine degradation pathway after ^{1}H NMR and Ion spectroscopy analysis (Poupin *et al.*, 1998, Combourieu *et al.*, 1998, 2000)

2.8. Estimation of morpholine degradation products/intermediates

2.8.1. Estimation of monoethanolamine

One of the important degradation intermediate monoethanolamine was chemically tested with modified rimini test in which amine reacts with acetone in presence of aqueous methanolic solution of sodium nitroprusside to produce a colored complex. However, GC analysis of basic compounds such as monoethanolamine is relatively difficult to establish due to its basic nature and the hydroxyl group that can interact with silanols from the column resulting the peak tailing. This can be overcome by choosing the fused silica capillary column Rtx-35- (Restek) with an intermediate-polarity stationary phase to provide selectivity and reduce adsorption and peak tailing for amines (https://www.chromtech.net.au/pdf2/59380_Rtx-35%20Amine%20Capillary%20Columns%20from%20Restek.pdf). The response and peak shape of monoethanolamines was evaluated by Rtx®-35 amine column and exhibits excellent peak shape and significantly higher response (Fig.15)

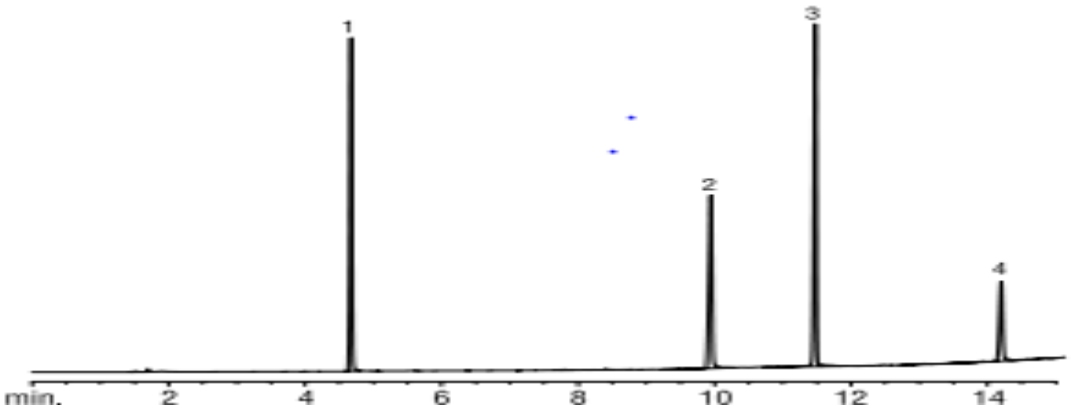


Fig. 15: Gas chromatography analysis of basic amines with RTX-35 column

Where 1= monoethanolamine, 2= Diethanolamine, 3= Triethyleneglycol monomethylether and 4= triethanolamine (https://www.chromtech.net.au/pdf2/59380_Rtx35%20Amine%20Capillary%20Columns%20from%20Restek.pdf)

2.8.2. Estimation of 2,2 aminoethoxy acetate and diglycolate

Recently, in situ ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy (Poupin *et al.,* 1998, Combourie *et al.,* 1998, 2000, Fig. 16a, 16b) and ion spray mass spectroscopy (Combourie *et al.,* 2000) were used to find out the intermediate of degradation pathway of morpholine. In ^{1}H-NMR

analysis (Fig. 16a, Poupin *et al*., 1998, Combourieu *et al*., 1998) *M. aurum* MO1 cells (5 gm of wet cells in 50 ml of enrichment MSS media or Knapp buffer were incubated with 10 mM morpholine at 30°C with agitation (200 rpm) for 72 hr. 1 ml sample was collected at different time point and after centrifugation 12000g for 5 min, the supernatants of these samples were analyzed by ^{1}H-NMR spectroscopy at 300.13 MHz (Table 11). Similar NMR spectra was found when *M. aurum* MO1 cells were incubated in 50 ml distilled water in the presence of 10 mM morpholine (Fig. 16b Combourieu *et al.*, 2000). ^{1}H-NMR analysis revels that *mycobacterium* cleaves the C-N bonds of morpholine ring, leads to formation of an amino acid, which undergoes deamination and oxidation and results in formation of glycolate (Fig. 14c). The pathway via ethanolamine has not been confirmed as ethanolamine has not been detected may be due to concentration below the limit for NMR detection (50µM). This approach was useful to directly investigate the degradation pathway of morpholine.

Table 11: Analysis of intermediate degradation products of morpholine

S. No	Morpholine degrader	Analytical method	Finding	Reference
1	*Mycobacterium* RP1 *Mycobacterium aurum* MO1	^{1}H-NMR	Chemical shift (ppm) 2.88(s), 3.72 (s): CH_2 of morpholine 3.96(s), 3.67(pt) and 3.05(pt): CH_2 of 2-(2-aminoethoxy) acetate, 3.95(s): CH_2 of glycolate	Poupin *et al.*, 1998, Combourieu *et al.*, 1998, 2000
2	*Mycobacterium aurum* MO1	Ion spray spectroscopy (Positive mode)	m/z 120: $[M+H]^+$ of 2-(2 aminoethoxy) acetate m/z 142: $[M+Na]^+$ of 2-(2 aminoethoxy) acetate m/z 158: $[M+K]^+$ of 2-(2 aminoethoxy) acetate	Combourieu *et al.*, 2000
		Ion spray spectroscopy (Negative mode)	M/z 133: $[M-H]^-$ anion of diglycolic acid M/z 155: $[M-2H+Na]^-$ Sodium adduct of anion of diglycolic acid M/z 171: $[M-2H+K]^-$ Potassium adduct of anion of diglycolic acid M/z 75: $[M-H]^-$ of glycolic acid	

(Where s= singlet, pt= pseudotriplet)

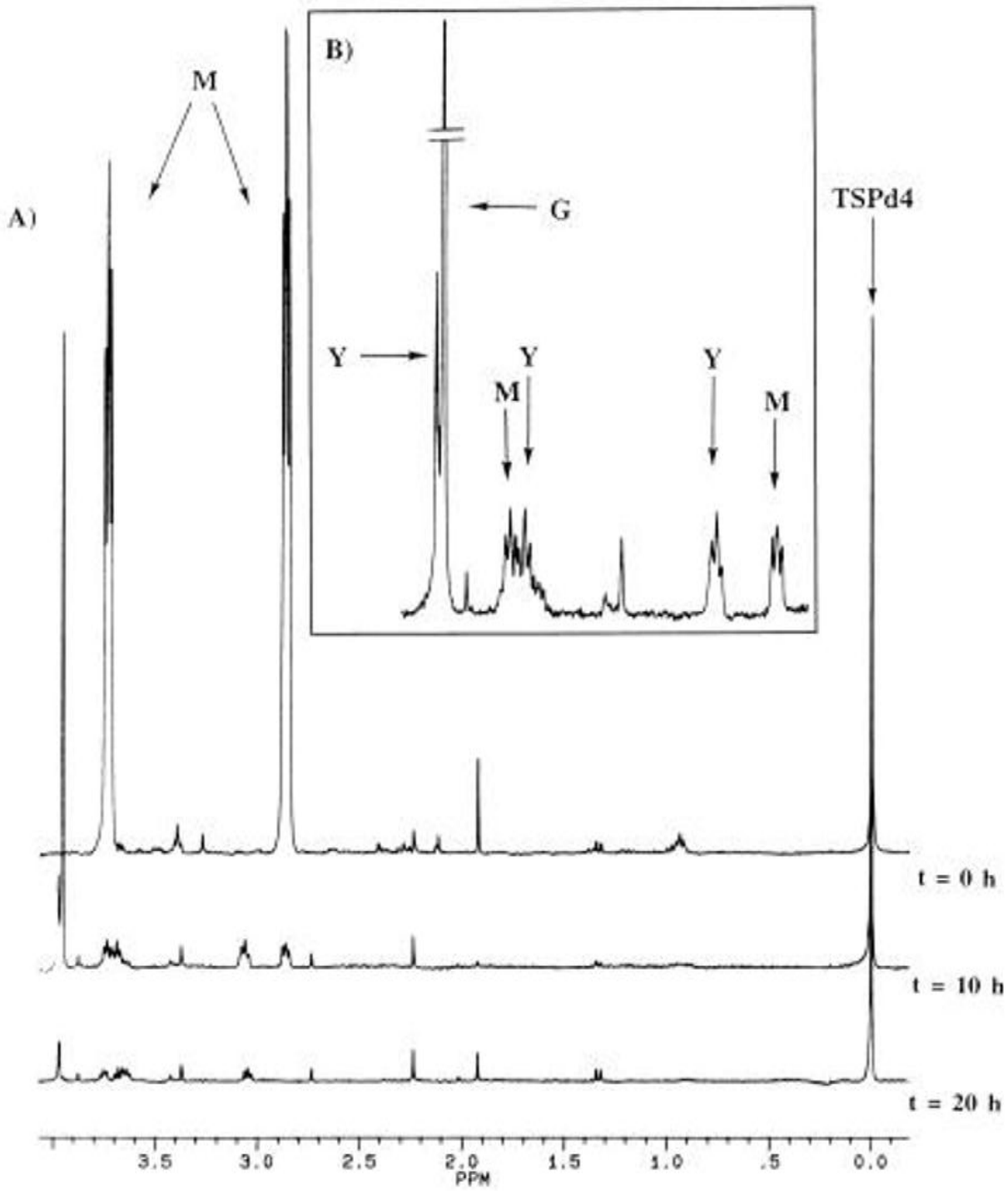


Fig. 16a: (A) Kinetics of morpholine degradation by *Mycobacterum* culture supernatant by ^{1}H-NMR spectroscopy at 0, 10 and 20 hr. (B). Expanded scale of nuclear shift from 2.60-3.98 ppm of the 10 hr spectrum. Where, M = morpholine; G= Glycolate; Y, 2-(2-aminoethoxy) acetate, $TSPd_4$= Sodium trimethylsilypropionate as internal standard reference for chemical shift to zero.

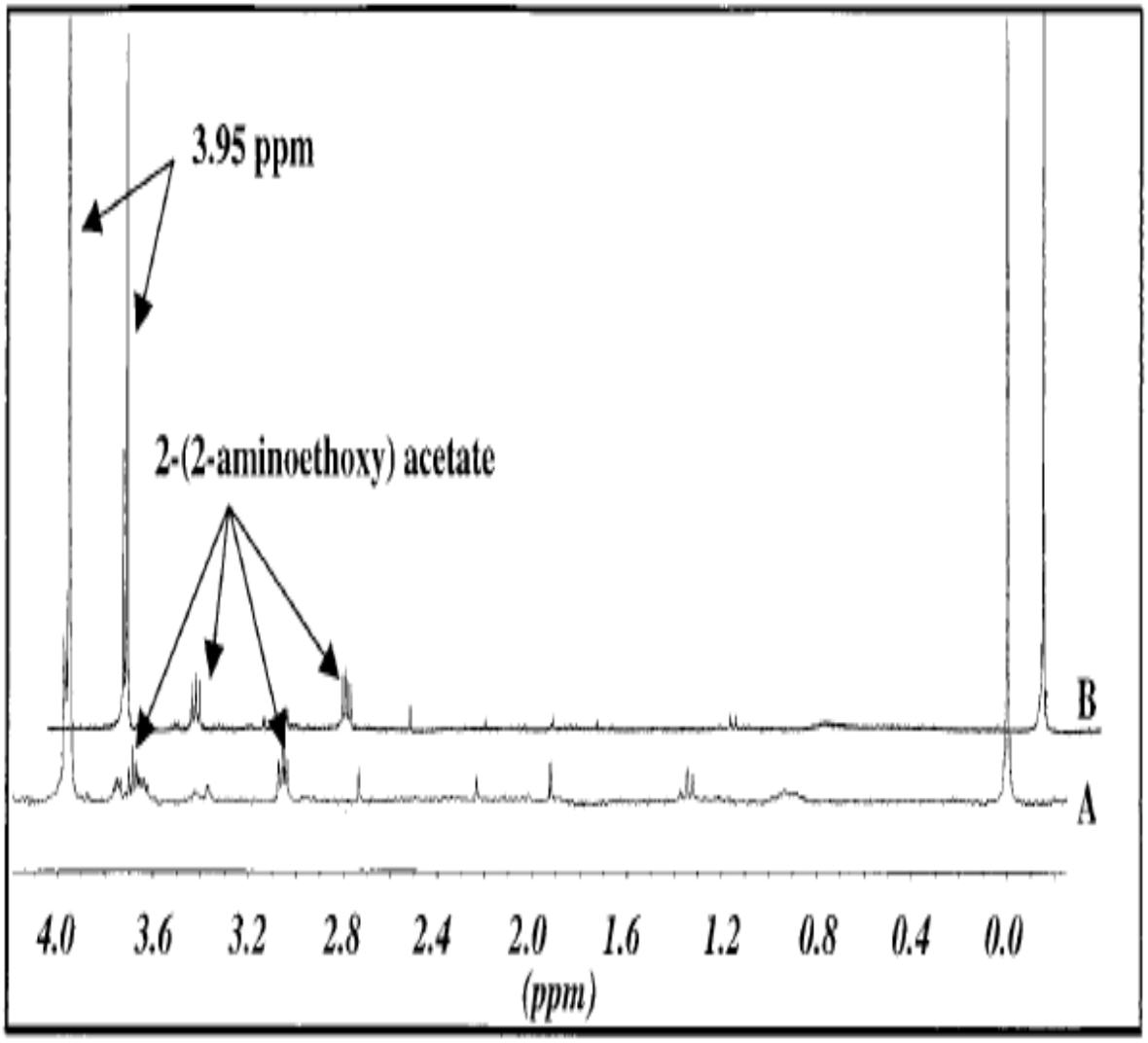


Fig. 16b: ^{1}H-NMR spectra of samples (*M. aurum* MO1 cells) in Knapp buffer/MSS (Spectrum A-12 hrs. incubation) and in distilled water (spectrum B-13 hrs.)

In ion spray mass spectrometry of the culture supernatants was also used to confirm the structures of some metabolites during morpholine degradation. In this study, *Mycobacterium* cells (5 gm wet weight of cells in 50 ml of distilled water) were incubated with 10 mM morpholine at 30°C and agitation (200 rpm) for 72 hr. 1 ml sample was taken at regular interval and centrifuged at 12,000 g for 5 min. The supernatants were filtered through a 0.22-mm-pore-size membrane to eliminate the cell debris before injection for mass spectrometer. Ionspray mass spectra in positive and negative mode as shown in Table 11 and Fig. 17. These signals (positive and negative mode m/z) were not present at time zero, indicating that these were corresponded to metabolites of

morpholine and are in complete agreement with ^{1}H-NMR finding. These experiments clearly showed that diglycolic acid is an intermediate in morpholine metabolism and further cleaved to form glycolic acid and is completely mineralized by the cells. Using this technique, new metabolite diglycolate (which was not detectable by ^{1}H-NMR) was identified and was essential for the proposal for a general pathway for heterocyclic compound degradation by *Mycobacterium* strains (Fig. 14c).

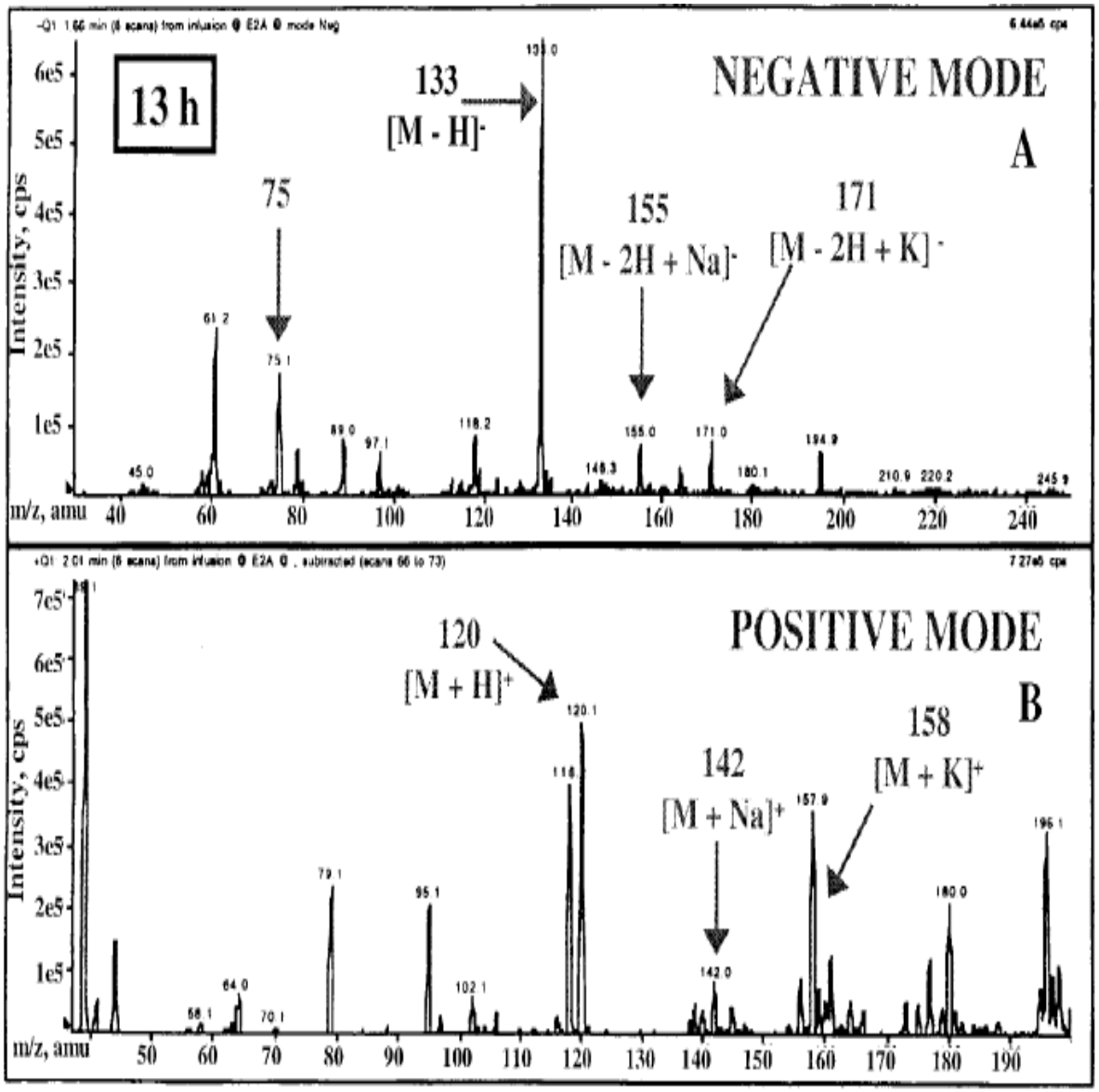


Fig 17: Ion spray mass spectra recorded under negative (A) and positive (B) ionization mode with an infusion pump for a sample collected after 13 h of incubation of *M. aurum* MO1 cells in distilled water supplemented with 10 mM morpholine

2.8.3. Estimation of ammonia/ammoniacal nitrogen

The product of degradation of morpholine results the generation of ammonia which increases the pH of growth medium. Accumulation of ammonia inhibits the bacterial growth and complete mineralization of morpholine. Presence of ammonia was also confirmed with increase in pH indicating the degradation and it is thus possible to link growth and degradation. Increased pH thus inhibits the bacterial growth. This could result in decrease of the degradation rate, as Mazure and Truffaut 1994 showed that ammonia was toxic to *mycobacterium aurum* MO1 strain. Ammonia was quantified either by Nessler reagent (Dmitrenko *et al.,* 1987) or modified phenol-hypochlorite method (Dilworth and Thorneley 1981) or coupled enzyme assay using glutamate dehydrogenase (S. Wakisaka 1987). As morpholine disappeared from the culture ammonia accumulated and molar conversion ratio of morpholine removed to NH_4^+ is represented in Table 12.

Table12: Molar conversion ratio of morpholine to ammonia by morpholine degraders

Method of quantification	Morpholine degraders	Morpholine concentration and duration	Molar conversion morpholine to ammonia	References
Nessler reagent	*Mycobacterium* sp.	1M 240 hr.	1:0.89	Magda M.Aly 2011
Nessler reagent	*Mycobacterium* sp.MO1	1M 235 hr.	1:0.82	Swain *et al*., 1991 N.Mazure and N.Truffat 1994
Coupled enzymic assay using glutamate dehydrogenase	*Mycobacterium* sp.HE5	60mM 48 hr.	1:0.5	Schrader *et al.,* 2000
Nessler reagent	*Mycobacterium* sp.MOD	----	1:0.87	Knapp *et al.,* 2000
Nessler reagent	*Mycobacterium* sp.MOG		1:0.94	
Phenol-hypochlorite reagent	*Pseudomonas fluorescens* CAS 102	25mM 120 hr.	1:0.82	S. Chandrasekaran and D. Lalithakumari 1998

CHAPTER 3

Materials and Methods

3.0. Materials and Methods

The complete work flow of methodology is represented in Fig. 18. The materials and methods followed for individual experiments are described below. Some of the common methods and culture media preparation are described in Annexure.

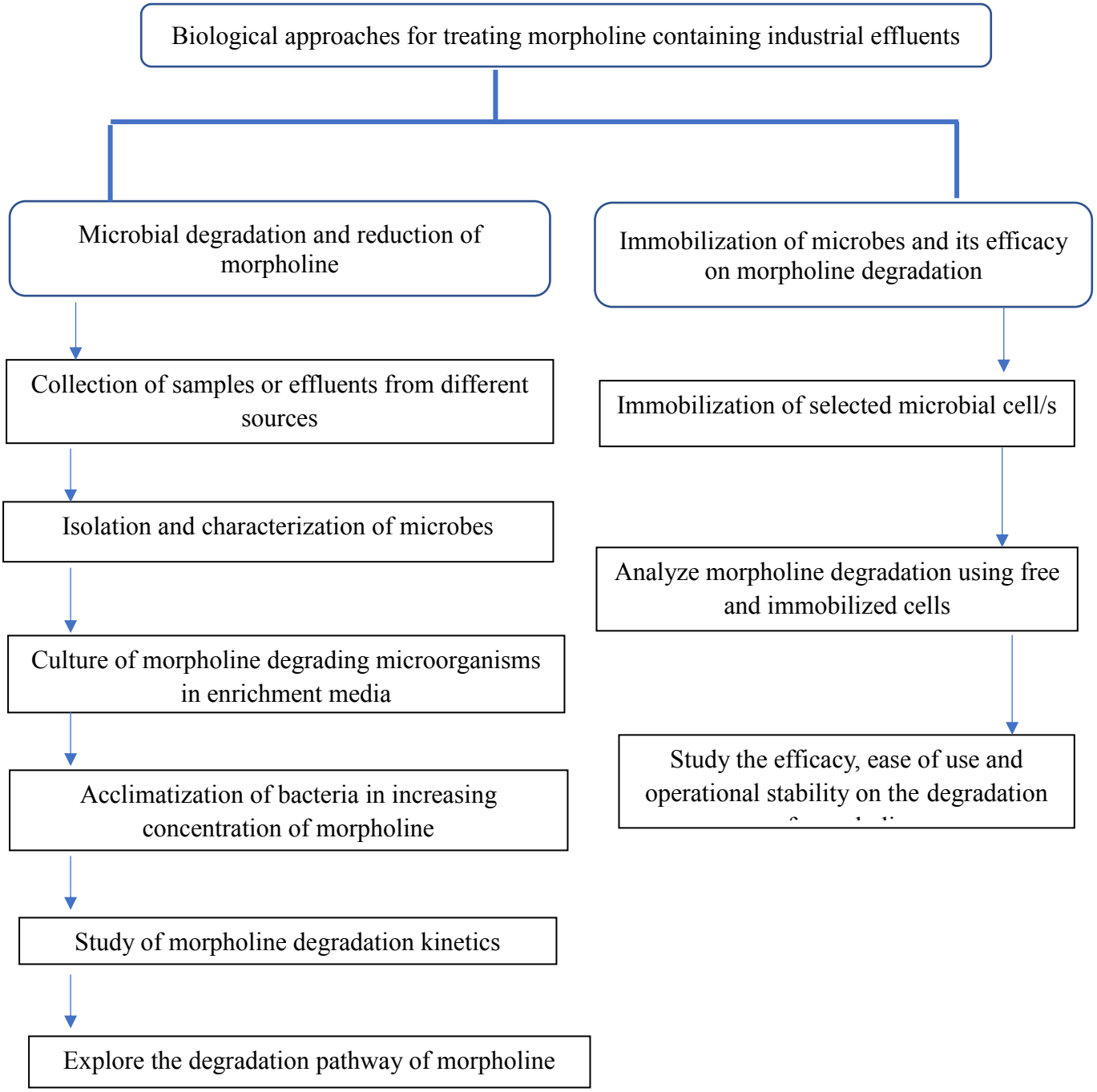


Fig. 18: Workflow of the methodology for the degradation studies of morpholine

Section I: Microbiological Methods

3.1. Source of samples and reagents

3. 1.1. Sample sites

The samples were collected from different geographical locations. Selection of geographical location is based on the availability and applicability of morpholine in different industries.

3.1.2. Source materials

Natural samples (soil and water) and industrial effluents are the terrestrial source of microbes used in this study. However biological fluid was also used to screen the microbes of interest (It was taken after approval of institutes ethical committee in accordance with the declaration of Helsinki. informed consent obtained from each person before taking the biological samples). The biological fluid used here was human urine from urinary tract infection (UTI) patients. All chemicals used were of analytical grade and were used as received without any further purification. Milli-Q water (Elix 3, conductivity 0.12 mho) was used in the preparation of aqueous solution of reagents. However autoclaved double distilled water was used for microbial culture purposes.

3.1.3. Collection of effluents and samples

Natural samples and industrial effluents were collected from different ecological location for the isolation of microbes. The bacteria were isolated and screened from water, soil and biological fluids. Due care was taken to ensure that the sample collection points were widely varying about geographical distribution. Samples were collected in a clean, sterile plastic container, transferred to the laboratory and stored at room temperature until further use for analysis and degradation studies. Proper measure for discard of unused samples or related plastic ware, etc., were used.

3.2. Selection of microorganisms for morpholine degradation

Microbes that have the specific ability to detoxify xenobiotic chemicals, production of enzymes that can catalytically convert the waste. This requires strategies to evolve and adapt microorganisms that can oxidize and breakdown toxic materials by their ability to use them as a carbon, nitrogen and energy sources.

3.2.1. Isolation of microorganisms

Microorganisms used for biodegradation of morpholine were isolated by standard microbial techniques. For the initial isolation and cultivation of microorganisms, luria bertani broth (LB) for bacteria and sabouraud dextrose agar (SDA) for fungus were used (Annexure 1, 4). Serial dilutions of samples were prepared with distilled water. Suitably diluted samples were pour plated using SDA for fungus and LB agar for bacteria. Upon incubation for 24-48 hr. pure colonies were isolated and preserved at -80^{0}C as glycerol stocks. Isolated microbes were further characterized using morphological, biochemical and molecular tests.

3.2.2. Characterization of isolated microbes

3.2.2.1. Morphological and biochemical characterization

Morphological characterization was achieved by visual observation of colony in term of appearance, shape, color, arrangement optical nature, margin, texture and elevation. The biochemical tests performed were acid fast staining, gram Staining, and growth on selective medium (Hi-Chrome Media M1353 and MacConkey Agar), citrate utilization, urease, indole, methyl red and voges-proskaeur tests to characterize bacteria (Annexure 3a). Specific colonies obtained from different natural sources were sub-cultured further to find the pure bacterial strain. Bacterial inoculums were used by suspending the freshly-grown bacterial colonies in 10 ml sterile LB and incubated at 37°C, which were then plated in both hichrome agar and macconkey agar plates and kept for incubation at 37°C for 24-48 hours for bacterial identification based on specific metabolism of chromogenic substrates. However, rest biochemical characterization was carried out as per standard methods reported in Buchanan, R.E. and Gibbons, N.R., Bergey's Manual of Determinative Bacteriology, 8th ed., Williams and Wilkins. Baltimore, 1974.

3.2.2.2. Molecular characterization

The identification of potential bacterial species for the removal of morpholine in waste water was performed by using molecular tools. 16S rRNA is a component of the 30S small subunit of prokaryotic ribosome. It is 1500 nucleotide long and highly conserved and hence amplicon sequencing method is used to identify, and phylogenetic comparison of bacteria present in a sample. Molecular characterization of selected isolated was outsourced at Yaazh Xenomics,

Chennai, Tamilnadu, India to confirm the bacterial species. Molecular characterization of the isolates (RK-11) was performed using PCR (Polymerase Chain Reaction) amplified 16S r-RNA restriction analysis followed by the generation of phylogenetic tree.

3.2.2.2.1. DNA extraction

- Bacterial Genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit (Bio-Rad Catalog # 732-6030) as per the kit instruction and procedure followed below
- An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. Centrifuged for 1 minute at 10,000–12,000 rpm to remove the supernatant
- 200 µl of Insta Gene matrix added to the pellet and incubated at 56 °C for 15 minutes
- Vortexed at high speed for 10 seconds and the tube was placed at 100 °C in heating block or boiling water bath for 8 minutes
- Finally, the contents were vortexed at high speed for 10 seconds and spun at 12,000 rpm for 2 minutes. After heating, the supernatant was used for PCR. 20µl of the supernatant was used per 50 µl PCR reaction.

3.2.2.2.2. PCR protocol

16S rRNA universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler (Bio-Rad PTC 200).

- Primer used for amplifying bacterial genomic DNA shown in detail in Table 13a.

Table13a: Primer used for amplifying bacterial genomic DNA

Primer Name	Sequence Details	Number of bases
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

- Added 1µl of template DNA in final volume of 20 µl of PCR reaction mix having 1 µl each of 10 µM stock 27F/1492R primers for polymerization reaction.
- PCR reaction was performed using following conditions (Rahamat Unissa *et al.*, 2015). DNA was denatured at 94°C for 5 min, followed by 35 cycles of amplification, each of 94°C for 45 sec (denaturation), 55℃ for 60 sec (annealing) and 72℃ for 60 secs, (extension) followed by 10 min 72℃ (final extension) (Table 13b).

Table13b: PCR reaction condition for amplifying bacterial genomic DNA

Steps		Temperature	Duration
Initial denaturation		94℃	2 min.
35 cycles amplification	Denaturation	94℃	45 sec.
	Re-annealing	55℃	60 sec.
	Extension	72℃	60 sec.
Final Extension		72℃	10 min.
Final Hold		4℃	Infinite

- DNA fragments were amplified, ~about 1,400bp in the case of bacteria. A positive (*E.coli* genomic DNA) and a negative control were included in the PCR.

3.2.2.2.3. Purification of PCR products

- Removed unincorporated PCR primers and dNTPs from PCR products by using SpinPrep™ PCR Clean-up Kit (Merck Millipore-70976-3, India)

3.2.2.2.4. Sequencing of PCR products

- The PCR product was sequenced using the 518F/800R universal primers (Table 14a).

Table14a: Primer used for sequencing the PCR product

Primer Name	Sequence Details	Number of Base
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

- Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq Gold® DNA polymerase (FS enzyme, Applied Biosystems Inc).
- Single-pass sequencing was performed on each template using below 16s rRNA universal primers (Table 14b).

Table14b: 16s rRNA universal primers

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

- The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol.
- The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730XL automated DNA sequencer (Applied Biosystems Inc USA).
- Sequence data was aligned and analyzed for identifying the bacterial isolate.

3.2.2.2.5. Phylogenetic analysis

- The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
- The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b.
- This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model.
- PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al*., 2008).

3.2.2.2.6. Sequence accession

The nucleotide sequence of the isolated bacterium was deposited in GenBank, NCBI and was assigned the accession number having 2 letters and 6 numerals. (https://www.ncbi.nlm.nih.gov/Sequin/acc.html)

3.3. Cultivation of bacteria for degradation of morpholine

3.3.1. Preparation of inoculums

Suitably diluted samples for the preparation of bacterial inoculums were pour plated on LB Agar (Annexure 1) and the selected pure colonies were further transferred aseptically to 10 ml enrichment media (Mineral Salt Solution/MSS-Composition of MSS used are listed in Annexure 2) supplemented with 0.1% morpholine. Cultures were incubated at 37^{o} C and 150 rpm for different time points and absorbance at 600 nm was taken as a measure of growth. The count of bacterial cells was adjusted to 1×10^{8} cells/ml (1-unit absorbance = 5×10^{8} cells) by varying incubation period.

3.3.2. Acclimatization: Microbial adaptation against morpholine

To study the morpholine degradation, the bacterial isolates, after morphological identification, were further cultivated in enrichment media amended with morpholine. All the tested bacteria grown in MSS broth supplemented with morpholine were plated in MSS Agar (containing 2% agar) with same concentration of morpholine to confirm the adaptation against morpholine stress. These were called as seeded acclimatized bacteria and further adapted to gradually increased concentration of morpholine. 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^{o}C and growth was monitored with absorbance at 600nm. Based on the growth when an OD of 0.5 was reached, a 1:100 dilutions was made, and culture was spread on MSS Agar + 0.1% morpholine. A total of 6 isolates out of 22 isolated bacteria grew on 0.1% morpholine and were used for further acclimatization by procedure in Annexure 6. The growing culture was centrifuged at 6500 rpm for 10 min and pellet was re-suspended in the MSS media with gradually increasing the concentration of morpholine up to 0.28%. For each acclimatization, culture was plated on the respective morpholine concentration and preserved as glycerol stocks. In the present study measurement of growth of bacteria at 600nm against media blank and GC analysis for left over morpholine were chosen as methods for confirming adaptation of bacteria.

3.4. Storage and further processing of samples

The culture of morpholine degrading bacteria was maintained on the MSS Agar with morpholine at 4^{0}C. For long term storage, pure isolated cultures were preserved at -80^{0}C in 15% (prepared in enrichment media with morpholine) final glycerol concentration as cryoprotectant for future use. It was done by making stock glycerol concentration of 60% (3 ml of neat glycerol mixed in 2ml of distilled water so that final volume should be 5 ml) which was further diluted with enrichment media (0.975 ml of media + 0.325 ml of 60% glycerol stock- for bacteria) to give final concentration of 15% and 10% for bacteria and fungus respectively (Annexure 7).

Section II: Analytical Method

3.5. Measurement of morpholine

The biological treatment studies included the microorganism/s capacity to degrade the morpholine (starting concentration 0.1%) and the remaining morpholine in culture supernatant was estimated by spectrophotometrically and by Gas Chromatography (GC) (Annexure 5). Percent degradation was calculated from the values obtained and the results are tabulated and presented in the results and discussion section.

3.5.1. Spectrophotometric estimation of morpholine (Die Away Test)

Preliminary availability of morpholine in culture supernatant was chemically analyzed and carried out as per reported method by Giti Emtiazi *et al.*, 2001 and Stevens *et al.*, 1965. The spectrophotometric method depends upon the nucleophilic substitution action of morpholine on the sodium salt of 1,2-naphtoquinone-4-sulphonic acid (Folin's reagent/ NPQ/NQS) under alkaline condition. A colored (orange/red) complex is formed, namely 4-(4-morpholinyl) naphthalene-1, 2-dione, which was read at 480nm against a reagent blank (Fig.19). The reaction was completed within 20 minutes. The rate of reaction between morpholine ion and NPQ ion is influenced greatly by alkalinity of the reaction mixture. Since it is light sensitive reaction, appropriate precaution was taken in consideration.

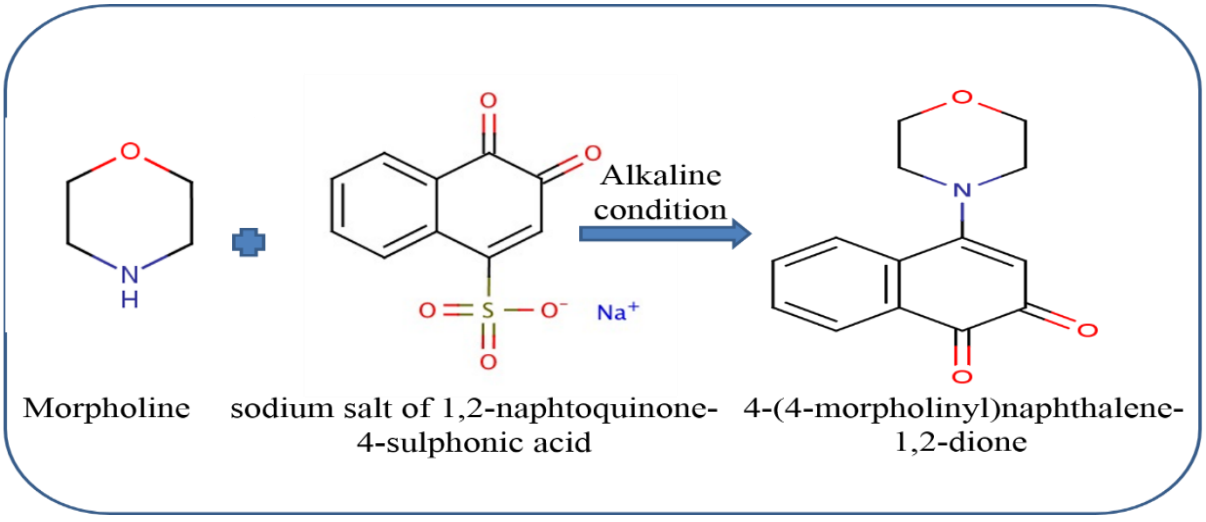


Fig 19: Nucleophilic substitution reaction of morpholine with sodium salt of 1,2-naphtoquinone-4-sulphonic acid (Folin's reagent/NPQ/NQS)

3.5.1.1. Instrument

An Elisa reader (ELX50/8MS BioTek India) provided with matched wavelength and light controller was used to quantify concentration of Morpholine for bio-remedial treatment of industrial effluents.

3.5.1.2. Reagents and solutions

All chemicals used were of analytical grade. Double distilled water was used for dilution and blanking purpose. 0.115M (3%) of NQS/NPQ reagent was prepared by dissolving 30 mg in distilled water and making the volume up to 1ml in micro centrifuge tube, this solution was prepared freshly as a daily procedure. The solution was stored in the dark at 4ºC. 1 Normal solution of sodium hydroxide (NaOH) was prepared by dissolving 4 gm in distilled water and making the volume up to 100 ml in a volumetric flask.

3.5.1.3. Standard solution

A standard solution of 1000 ppm (0.1% v/v) of morpholine was prepared separately and further diluted 1:10 by dissolving 200 µl of 0.1% in 2.0 ml of double distilled water in a micro centrifuge tube. Thus, the working solution concentration is 100 ppm (0.01%). The working solution was further diluted to get a series of standard solutions concentration (2-10 ppm) with double distilled water. A calibration curve was plotted and presented in result section (Table 15).

Table15: Preparation of standard solution for spectroscopic assay

Calibration level	Final standard concentration (ppm)	Volume of working standard added (µl)	Volume of solvent (µl)
1	2	6	294
	4	12	288
	8	24	276
	10	30	270

3.5.1.4. Methodology

Morpholine was analyzed spectrophotometrically. Suitable aliquots (300µl) of morpholine working solution were pipetted out into standard 96 well Elisa plates. 2 µl of 1N NaOH and 2 µl of 3% of NQS reagent were added to each of these solutions. The solutions were left for 20 min

at room temperature in dark. The absorbance of these solutions was measured at 480 nm against a reagent blank (Fig. 20). A calibration plot was constructed by plotting absorbance against different concentrations. Suitable aliquots of acclimatized bacterial culture supernatant (for bio-remedial treatment) sample were taken and the above process was repeated. Using the calibration curve, the concentration of morpholine was calculated in culture supernatant.

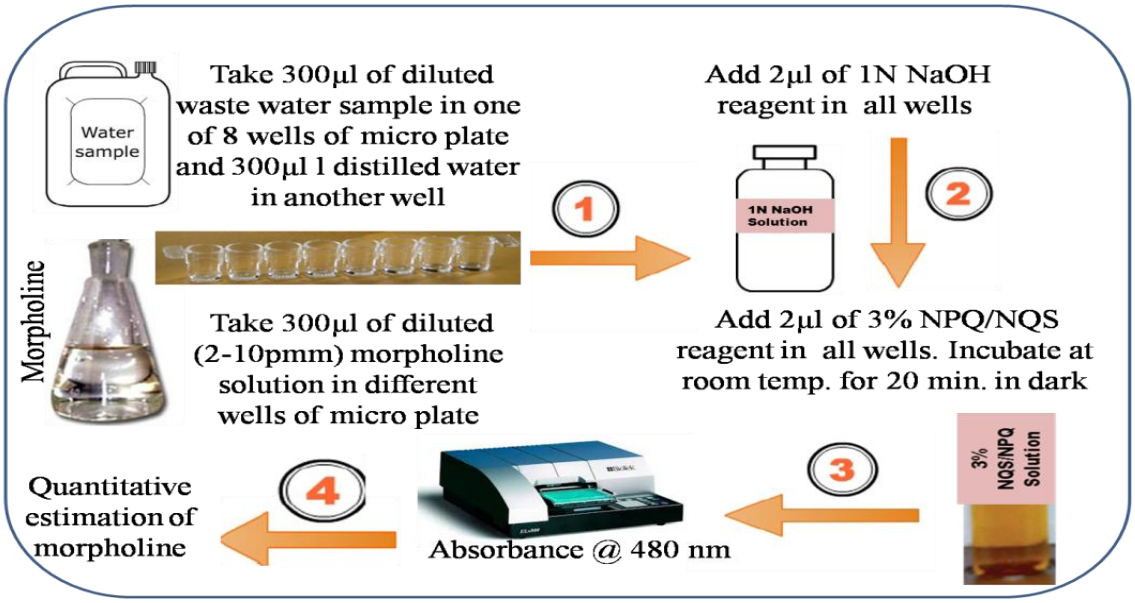


Fig 20: Flow sheet for the spectrophotometric assay for morpholine estimation

3.5.2. Mass spectroscopy estimation of morpholine

The concept behind mass spectroscopy is that a charged particle passing through a magnetic field is deflected along a circular path on a radius that is proportional to mass to charge ratio (m/z). In mass spectroscopy, a radical cation is formed by displacing an electron from the organic molecules known as molecular ion. If the molecular ion is too unstable then it can fragment to give other smaller ions. The collection of ions is then focused into a beam and accelerated into the magnetic field and deflected along circular paths according to the masses of ions. By adjusting the magnetic field, the ions can be focused on the detector and recorded. The analyte sample is introduced into mass spectrometer. The analyte molecule is then converted into ionic compounds by electron spray ionization (ESI) process; following ionization the ions are progressed to mass analyzer and ion detector/electron multiplier (Venn 2000) (Fig. 21).

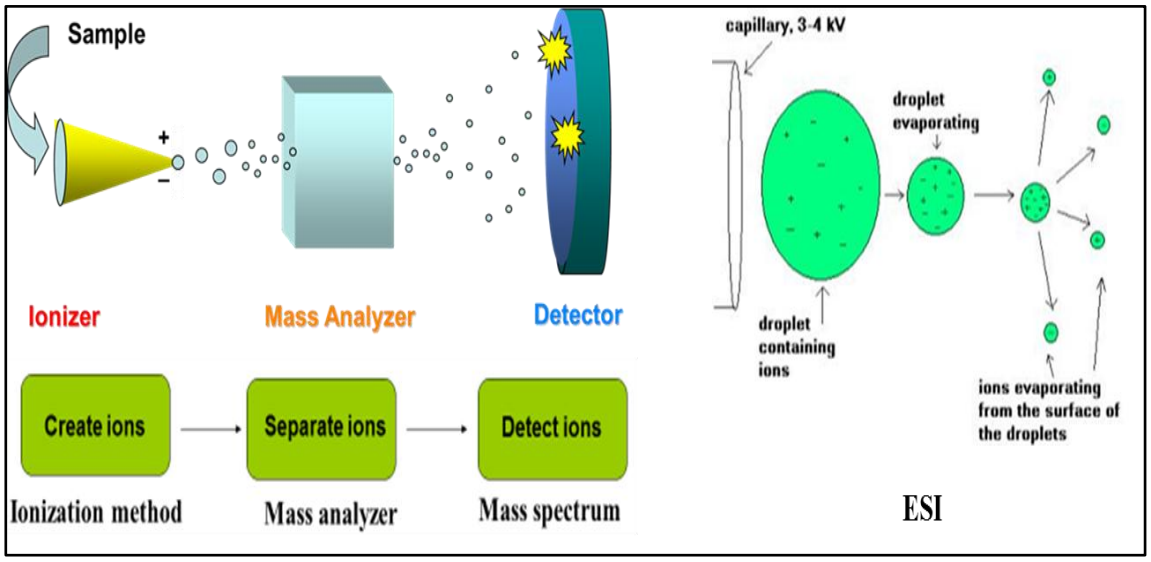


Fig.21: MS-ESI system for estimation of morpholine degradation products

3.5.2.1. Instrument and its parameters

MS system of an integrated LCMS (Liquid Chromatography Mass Spectroscopy) instrument (Shimadzu LCMS-2020) equipped with inlet interface, ion source, mass analyzer and detector has been used for analysis of degradation products of morpholine. The analytical parameters for estimation of morpholine degradation product are summarized in Table 16.

Table 16: Mass spectroscopy operating parameters

Parameter	Specificity
Ionization	ESI Needle voltage 4.5 kv
Interface temperature	350ºC
Heat block temperature	200ºC
Sheath/Drying gas flow rate	15 l/min
The nebulizer gas flow rate	1.5 l/min
Acquisition time	2 min
Acquisition mode	Positive/Negative
	Scan m/z 50-200
	Scan speed 52 unit/sec
	Sampling acquisition time 1.56 Hz (640 msec)
Detector	Electron multiplier
Software	Lab solutions
Workstation	Window 7

3.5.2.2. Preparation of samples

Culture sample was taken and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered (Nylon filter 0.22 µm pore size; Axiva Sichem Biotech, India) to avoid any bacterial cells. 1 ml filtrate was diluted with appropriate solvent (methanol/acetonitrile/acetone) and injected into MS. The culture media MSS was also prepared accordingly.

3.5.2.3. Sample analysis

Injected and processed all the samples as per given parameters. MS of culture supernatant was run against MSS blank.

3.5.2.4. Data Processing

A computer (with operating system window 7) interfaced with LCMS with software (lab solutions) was used for data acquisition, storage and processing of MS spectra.

3.5.3. Gas chromatography estimation of morpholine

Gas chromatography (GC) has been widely used for amine analysis because of its inherent advantages of simplicity, high resolving power, high sensitivity, short analysis time and low cost. A typical GC system consists of three major component of carrier gas inlet, column and detector (Fig. 22)

3.5.3.1. Instrument and its parameters

A GC system (Shimadzu GC-2010) equipped with standard oven for temperature ramping, split/split less condition, injection ports, flame ionization detector (FID), EB-1 column (30 mm x 0.25 mm x 0.25µm film thickness) with Nitrogen as carrier gas in the split mode by direct injection method has been used for analysis of morpholine. The analytical parameters for estimation of morpholine using GC technique are given in Table-17b as per OSHA Method 1999.

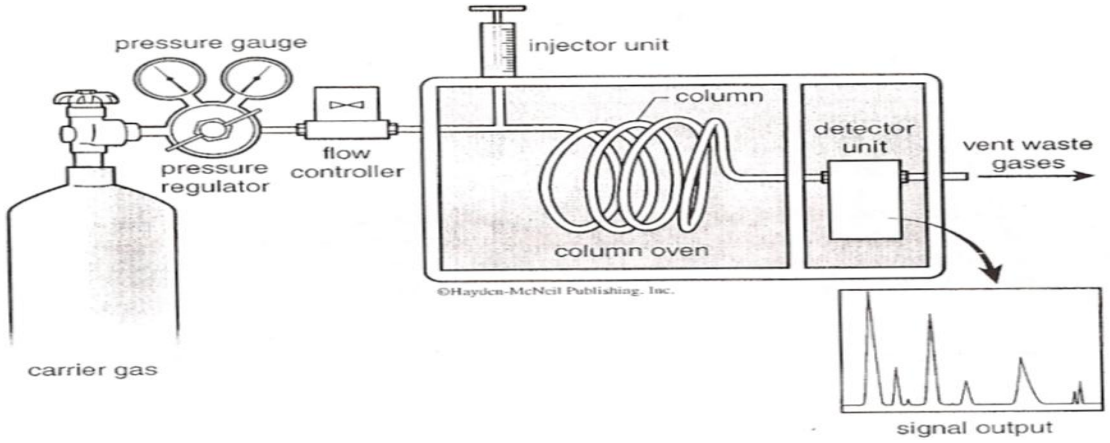


Fig 22: A Gas Chromatography system for analysis of volatile compound morpholine

3.5.3.2. Analytical procedure

The quantitative estimation of samples has been done using calibration curves. All dilution of the samples was done with analytical grade methanol. It details are described below:

3.5.3.3. Standard solution

Dissolved 50µl of neat morpholine to 1 ml methanol. This gave a 5% v/v solution, which was further appropriately diluted with different volume of methanol to obtain standard concentration of 0.05-2.5% (Table 17a).

Table17a: Preparation of standard curve for GC estimation

Calibration level	Final standard concentration (%v/v)	Volume of stock standard added (µl)	Volume of solvent (µl)
1	0.05	10	990
	0.1	20	980
	0.25	50	950
	0.5	100	900
	1	200	800
	2.5	500	500

3.5.3.4. Sample extraction

A measurable volume of sample was extracted with methanol. The culture was centrifuged, and supernatant was collected for morpholine quantification after dilution with methanol (1:10). Similarly, culture media MSS was also centrifuged and diluted with methanol (1:10) and served as MSS blank.

3.5.3.5. Instrument calibration

Gas chromatography system was set up to operate with parameter shown in Table 17b. First injection was a solvent blank each single time to equilibrate the column followed by calibration standard. Processed the calibration standard with instrument data system and prepared a calibration standard as presented in result section.

3.5.3.6. Sample analysis

All the samples including positive control and filtrate culture supernatant were injected and processed. GC was run against MSS blank and same % solution of morpholine in methanol as positive control in duplicate for reference.

3.5.3.7. Data processing

A computer interfaced with GC with software (GC Solution) was used to allow continuous acquisition, storage and processing of all chromatogram data (like selection of peak, integration of peak, removal of noise).

3.5.3.8. Qualitative and quantitative estimation

The most common quantification method used is area percent method. With each of the chromatograms obtained by injecting a constant volume of each standard, peak area/ area under curve (AUC) is calculated by machine. In similar way, culture supernatant chromatogram is recorded under same condition for the measurement of % degradation of morpholine. Morpholine was tentatively identified by gas chromatographic retention time in bacterial spent media.

3.5.3.9. Interferences

Most organic interference in sample can be removed by performing a cleanup step or filtration with 0.22µm filter. Reagent solvent blank was used for correction in case of any interference. Interference of noise is manually removed with GC solution software.

Table 17b: Gas chromatography operating parameter for morpholine

Parameter	Specificity
Column configuration	EB-1 30m x 0.25 mm x 0.25µm
Oven/column temperature	Initial 50ºC Hold: 3 minutes Ramping Rate: 10ºC/min Final temperature: 110ºC Final Hold time: 06 min
Injector Port	Temperature: 200ºC Split ration: 10:1 Injection volume: 1µl
Carrier Gas (Mobile phase)	Column gas flow: 2 ml/min Purge flow: 1ml/min Hydrogen: 40ml/min Zero air: 400ml/min Nitrogen: 15ml/min
Stationary phase (Liquid)	dimethyl polysiloxane
Detector	Flame ionization detector
Analysis time	9 min
Software	GC solution
Workstation	Window 7

Section III: Degradation and Pathway Studies

3.6. Experimental design for degradation studies (free cells)

Gas chromatography analysis were chosen for quantitative estimation of morpholine in the biological sample under study. GC analytical method was developed as per the parameters listed in the Table 17b. In the biological treatment studies, the remaining morpholine in culture supernatant was estimated by GC with respect to positive control (PC) of morpholine (starting conc. of 0.1%). Percent degradation was calculated from the values obtained and the results are tabulated and presented in the results and discussion section.

3.6.1. Degradation kinetics with Gas Chromatography

The most common quantification method used was percentage reduction in area under curve (AUC) by assuming that the detector responds identically to all compounds present in the sample. With each of the chromatograms obtained by injecting a constant volume of each standard solution (0.05– 2.5% v/v morpholine prepared in methanol); Peak area/ Area under curve was

calculated by machine and standard curve was plotted which is shown in result section. Subsequently culture supernatants were extracted as described in the methods section and morpholine was measured using the standard curve obtained. Degradation of morpholine was calculated in terms of % reduction and is shown in result section.

3.7. Elucidation of degradation pathway of morpholine

3.7.1. Growth on different hypothetical degradation intermediate compounds

The growth of isolates on various substrate (intermediate degradation products) were investigated by adding the corresponding compounds (0.15%) to mineral salt solution. The pH of the medium was adjusted to 7 and growth was carried out at 37^0C and 150 rpm at different time points by measuring the absorbance (optical density, OD). This experiment was undertaken to establish that these degradation products might have formed and would support the growth of the isolated bacteria.

3.7.2. Chemical tests of intermediate/s

Chemical test of degradation products mainly monoethanolamine was carried out by Simon- 1 (Rimini Test) and Simon-2 (Modified Rimini Test) to distinguish primary and secondary amine. Respectively. The amine undergoes nucleophilic addition reaction with nitroprusside in presence of acetaldehyde or ketone to give characteristic color of primary amine (blue) and secondary amine (violet) (Fig. 23)

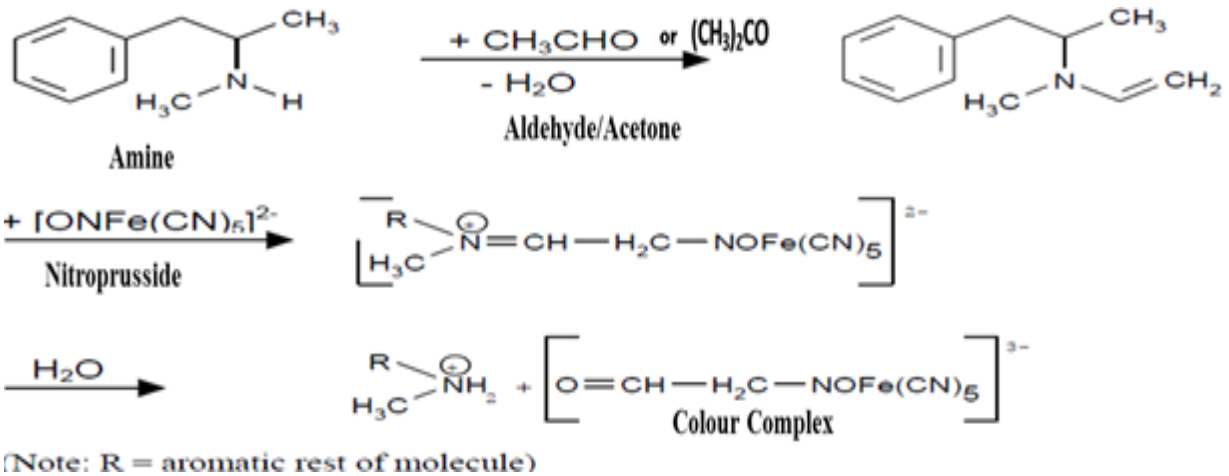


Fig.23: Simon test for primary and secondary amine

3.7.2.1. Sample analysis

Presence of primary amine (Monoethanolamine/MEA or ethanolamine) and secondary amine (Morpholine) was carried out as per method discussed in Table 18.

Table 18: Simon test for available primary and secondary amines in culture supernatant

<table>
<tr><td colspan="3">Simon test</td></tr>
<tr><td>Simon- 1 (Rimini test)</td><td colspan="2">Simon-2 (Modified Rimini test)</td></tr>
<tr><td rowspan="2">For secondary amine (Morpholine) (Qualitative)</td><td colspan="2">For primary amine (Monoethanolamine)</td></tr>
<tr><td>Qualitative</td><td>Quantitative</td></tr>
<tr><td rowspan="2">50 µl of amine + 50 µl of acetaldehyde + 3ml of water + 50 µl of 1% Sodium nitroprusside</td><td rowspan="2">50 µl of amine in 1ml of acetone + 3 ml of water + 50 µl of 1% Sodium nitroprusside</td><td>100µl amine+ 100µl of acetone + 50µl of 1%Sodium nitroprusside Color was developed and read at 490 nm against reagent blank</td></tr>
<tr><td>Standard primary amine of concentration 0.25-3 mg/ml (in water) was processed similarly to find out the concentration of primary amine in test samples</td></tr>
<tr><td>Blue color</td><td colspan="2">Voilet color</td></tr>
</table>

3.7.3. Gas Chromatography (GC) studies of degradation intermediate/s

3.7.3.1. Instrument and its parameters

A GC system as discussed in section 3.5.3 was used for the estimation of intermediate of degradation pathway mainly monoethanolamine/ethanolamine. The analytical parameters for analysis of monoethanolamine was summarized in Table 19a as per method (modified with change in column and its parameter- section 2.8.1) reported by Gerster FM *et al.*, 2012.

Table 19a: GC parameters for estimation of monoethanolamine

Parameters	Specificity
Column and its configuration	RTX-35 30mx0.32mmx1µm
Oven/Column temperature	Initial 60 ^{0}C Hold: 1min Ramping rate: 30°C/min Final temperature: 240°C hold for 3 minutes Linear velocity: 37.6cm/sec (for nitrogen)
Injector port	Temperature: 200°C Split Ratio: 30:1 Injection Volume: 1µl
Carrier gases (Mobile Phase)	Column Gas Flow: 2 ml/min Purge Flow: 1ml/min Hydrogen: 40ml/min Zero air: 400ml/min Nitrogen: 15ml/min
Stationary phase	60% Dimethyl polysiloxane and 35% Diphenyl polysiloxane
Detector	Flame ionization detector @ 300°C
Analysis time	10.0 min
Software	GC solution
Workstation	Window 7

3.7.3.2. Sample analysis and data processing

Standard solution of monoethanolamine 0.125 to 0.5% (Table 19b) was injected along with processed culture supernatant as per method developed (Table 19a). GC of test samples were run against Media blank and positive control.

Table 19b: Standard solution for estimation of monoethanolamine

Calibration level	Final standard concentration (ppm)	Volume of standard stock solution added (µl)	Volume of solvent (µl)
1	1250	250	750
	2500	500	500
	5000	1000	--

A computer (with operating system window 7) interfaced with GC with software (GC Solution) was used to allow continuous acquisition, storage and processing of all chromatogram data (e.g. selection of peak, integration of peak, removal of noise).

3.7.4. Mass spectroscopy (MS) studies of degradation intermediate/s

3.7.4.1. Sample processing

A MS system as discussed in section 3.5.2 was used for the estimation of degradation intermediates. Here sample for injection was prepared without solvent system as per Combourieu *et al.*, 2000. Culture sample (5 ml) was centrifuged and supernatant was filtered through 0.22 µm pore size nylon filter (Axiva Sichem Biotech, India) to avoid any bacterial cells. 1 ml filtrate was directly used to inject into MS. Similarly, culture media MSS was also prepared accordingly.

3.7.4.2. Data processing

Culture supernatant was run against MSS blank. A computer interfaced with LCMS with lab solutions software was used for data acquisition, storage and processing of MS spectra. Peak of intermediates was found based on their molecular mass and possible adducts with cation or anions.

3.8. Estimation of ammonia

Ammonia is a colorless, gaseous compound with a sharp distinctive odor. It is highly soluble in water where it exists in a molecular form associated with water and in an ionized form as $NH4^{+}$. This method involves the coupling of ammonium to Nessler's reagent to produce a yellow color under strong alkaline condition (Fig. 24). A resulting yellow color was formed in proportion to ammonium (NH_4^{+}) concentration and was measured at 425 nm using spectrophotometer against reagent blank. The ammonia level in terms of ammonium nitrogen is expressed as mg/l (or ppm).

Fig 24: Chemical reaction of Nessler's reagent with ammonia

3.8.1. Instrument

An Elisa reader (ELX50/8MS BioTek India) provided with matched wavelengths was used to quantify concentration of NH_4-N^{+} present in culture supernatant.

3.8.2. Reagents and solutions

All chemicals used were of analytical grade. Double distilled water was used for dilution and blanking purpose. Standard NH_4^+-N Solution was prepared by dissolving 3.819 gram of anhydrous ammonium chloride (dry in oven 100ºC for 1 hour) in 1 liter of double distilled water. This corresponds to 1000 ppm of NH_4^+-N solution. 50% m/v K-Na Tartarate: (Rochelle salt solution) was prepared by dissolved 500 mg Rochelle salt in 1 ml of double distilled water. It was boiled to expel the ammonia, if any, in water. Commercially available Nesseler reagent from sigma Aldrich, India was used.

3.8.3. Standard solution

A standard solution of 10 ppm of NH_4^+-N were prepared by dissolving 4.773 mg ammonium chloride in 125 ml of double distilled water and further diluted to make 1-5ppm NH_4^+-N as given below (Table 20). A calibration curve was plotted and is presented in the results section.

Table 20: Standard solution for estimation of ammonia-nitrogen

Standard NH_4Cl concentration	NH_4^+-N concentration
3.819 gm/1000ml	1000 ppm
38.19 gm/10 ml	1000 ppm
4.773 mg/125 ml	10 ppm

Standard solution final concentration (ppm)	Volume of standard solution added (µl)	Volume of solvent (µl)
1	25	225
2	50	200
3	75	175
4	100	150
5	125	125

3.8.4. Methodology

NH_4^+-N was analyzed spectrophotometrically. Suitable aliquots (250µl) of NH_4^+-N working solution were pipetted into microfuge tubes. 5 µl of 50% K-Na Tartarate and 5 µl of Nesseler reagent were added to each well. A suitable aliquot of culture supernatant was treated similarly as above. The solutions were left for 5 min at room temperature. Absorbance of these solutions was measured at 425 nm against a similarly treated reagent blank. When quantifying ammonia, the concentration of ammonia in the mineral salt medium was subtracted from the values

obtained to show only the liberated ammonia content. Using the calibration curve, the concentration of NH_4^+-N was calculated in culture supernatant (Fig. 25). From this, molar conversion ratio of morpholine to ammonia was also measured.

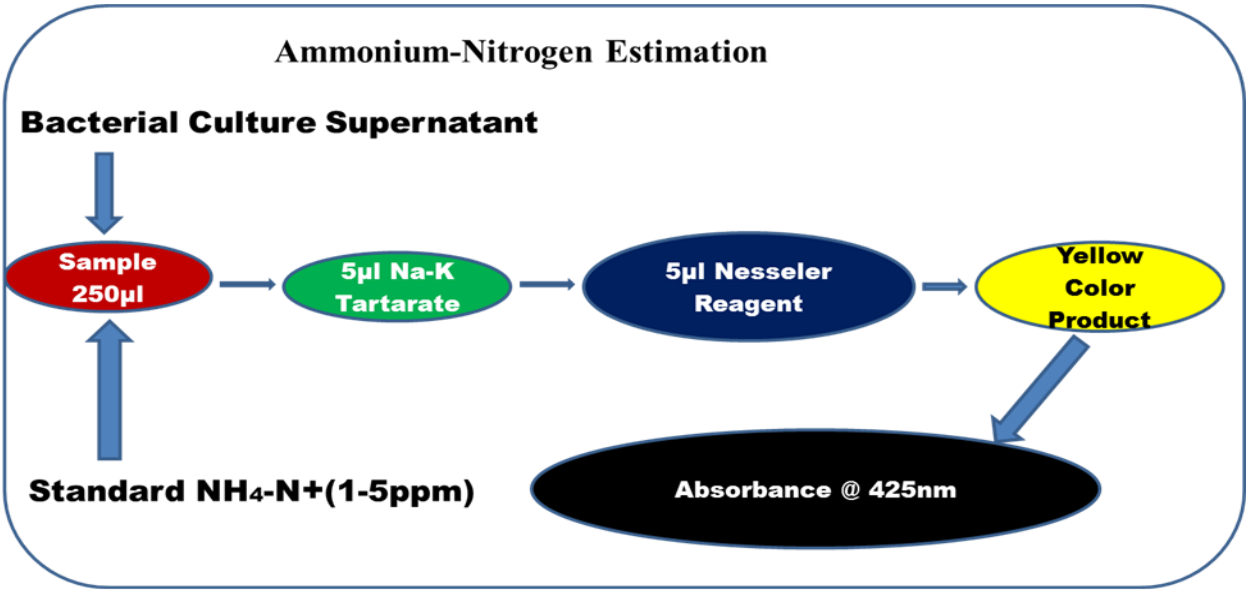


Fig 25: Estimation of ammonium nitrogen by Nessler's method. The color intensity of the solution determines the ammonia concentration

Section IV: Immobilization Studies

3.9. Immobilization of microbial cells

Immobilization of microbial cells is a biological process which occurs either as a natural phenomenon or through artificial process. This technique is used especially with eukaryotic cells where the whole metabolic machinery is often required for their specific application. It is noticed that microbial cell growth can break the bead and is generally considered undesirable beyond what is needed to compensate for the endogenous decay. That is the reason immobilized cells ideally should have just entered the stationary phase. One of the limitation is that the substrate must diffuse to the reaction site and inhibitory or toxic products must be removed from the environment. Oxygen transfer is often the rate limiting step in an immobilized cell culture (A. Blandino *et al.*, 2003). In this study microbes are immobilized by a process called encapsulation (Fig. 26) in which

- Cells are entrapped in semipermeable alginate polymer spheres
- The cells are uniformly distributed throughout the mixed solution
- The process is accomplished by mixing cells with a polymeric solution, applying a force that separates the polymer/cell mixture into generally spherical particles, and allowing the polymeric material to solidify
- The particles size ranges from small (approx. 2-10 µm diameter) to large (approx. 3 mm).

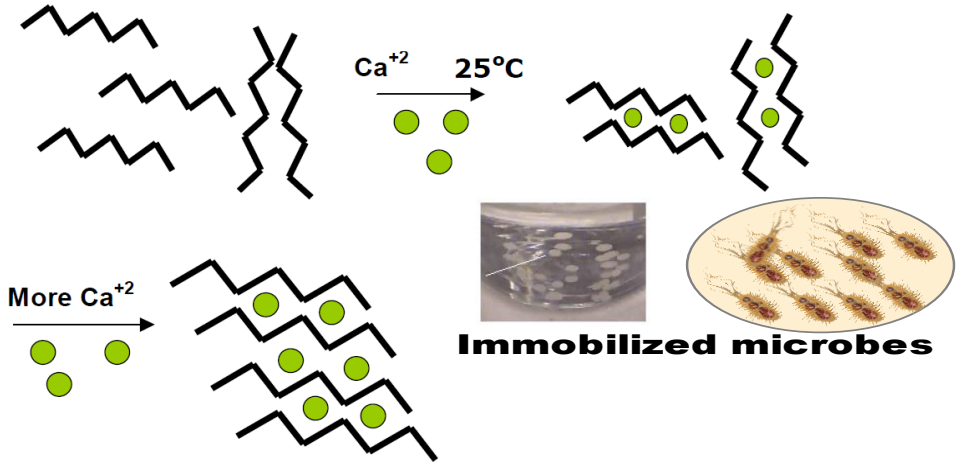


Fig 26: An illustration of encapsulation of bacteria (green) with the addition of calcium ions in alginate matrix (⩘)

3.9.1 Procedure of immobilization

The methodology followed was as per Haroldo *et al.*, 2011 with some modifications (Fig. 27). The procedure followed are given below

- ❖ Acclimatized microbes in MSS media were centrifuged at 13000 rpm for 10 minutes at room temperature.
- ❖ The pellets were washed twice with sterile distilled water and resuspended in 1.5ml of sterile distilled water.
- ❖ Mixed the cells with 2% sodium alginate solution in 1:1 ratio and stirred for 15 minutes.
- ❖ Dropped this solution from a height of 20 cm with 0.6mm needle into 1% or 0.2M $CaCl_2$ solution. The beads were left for 1-2 hour at room temperature.
- ❖ Free Ca^{++2} was washed with distilled water twice followed by transferring the gel beads to a growth media (MSS) having different concentration of morpholine.
- ❖ Measured the bead size and other physical parameters.

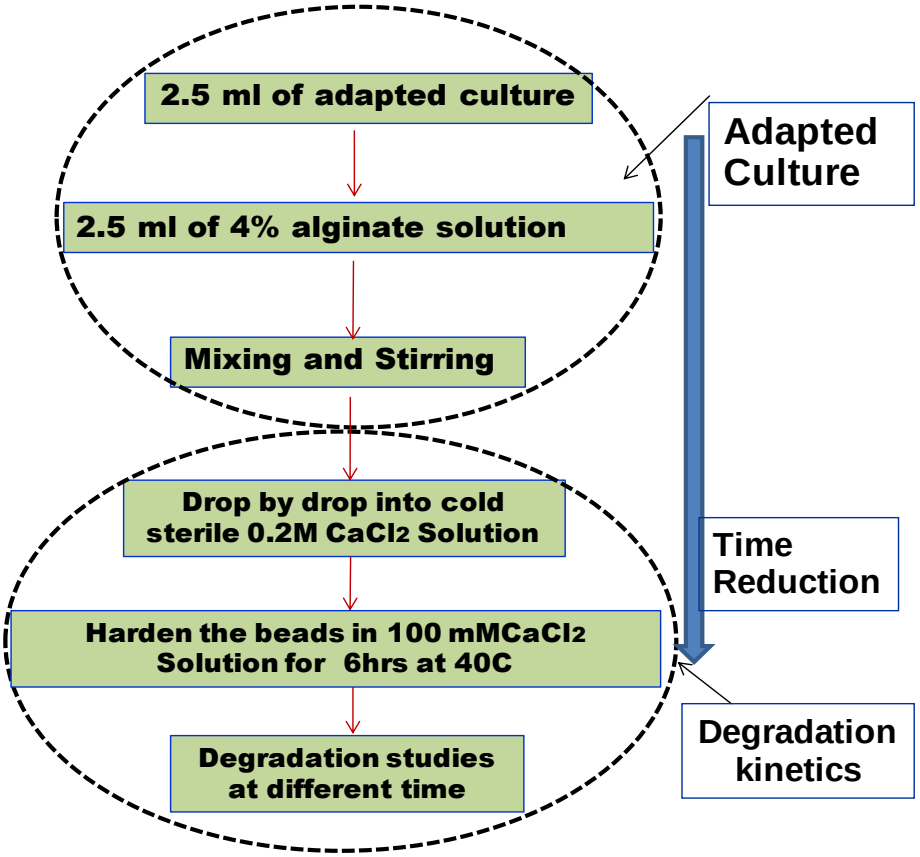


Fig 27: Flow sheet of preparation of alginate beads for degradation studies of morpholine

3.9.2. Characterization of immobilized beads

Single encapsulated bead was crushed aseptically in sterile distilled water and spread on LB agar plate to find the number of viable cells in each bead. Another physical characteristic feature measured was average bead size and durability of beads.

3.9.3. Degradation studies by immobilized cells

Spectrophotometric method of analysis was chosen to study the degradation kinetics of immobilized cells. Availability of morpholine in culture supernatant was chemically measured as per Giti Emtiazi *et al.*, 2001, reported in section 3.5.1. Morpholine was measured in appropriately diluted culture supernatant using the standard curve against media blank and respective positive control sample. Degradation of morpholine was calculated in terms of % reduction. This alternative method is simple, sensitive, rapid, reliable and economical for quantitative estimation of morpholine.

CHAPTER 4

RESULTS AND DISCUSSION

4.0. Result and Discussion

This study focused on the scope of indigenous microbes isolated from different geographical locations for their capacity to degrade morpholine, which is released as effluent from industries like rubber and textile. The results obtained of the entire study are presented and discussed below.

4.1 Collection of samples

The sample used in the present biological treatment was collected (in a clean, sterile plastic container) from industrial effluent and natural sources (soil and water) from different geographical locations as shown in Fig. 28a, 28b. The highlighted circle in figure represents the sample collection sites and 1 or 2 represents the natural and industrial sources respectively.

Fig. 28a: Sample collection sites at different geographical sites of Nepal. (http://nepalmap.facts.co/nepalmapof/nepalmap.php) Where 1 indicates natural sources (soil and water)

Fig. 28b: Sample collection sites at different geographical sites of India
(https://commons.wikimedia.org/wiki/File:Map_of_india_TB.gif).
Where 1 indicates natural sources (soil and water) and 2 indicates industrial effluent.

4.2. Isolation and characterization of morpholine degrading bacteria

4.2.1 Isolation of microbes

A total of 22 isolates were obtained from 7 different sites during Feb 2012 to December 2015 (Fig. 28a, 28b). Two different microbes, isolated from effluents from textile industry, Nagda, MP, India were named SK-1 and SK-2. One isolate obtained from human biological fluid was named SK-3 and another isolate obtained from a water sample collected from Husain Sagar Lake, Hyderabad, Andhra Pradesh (AP), India was named SK-5. Two more microbes, isolated from hydro thermal plant effluent Kataiya, Sapaul, Bihar, India were named BAC-1 and BAC-2. The isolates obtained from natural sources from Kathmandu, Nepal were named RK-1 to RK-7. The isolates obtained from natural sources from Bhubaneswar and Puri, Orissa, India were named RK-8 to RK-10. Microbes isolated from the effluents samples from Durgapur Steel Plant, West Bangal (WB), India was named RK-11 to RK-16. Isolates from different sample sites represented in Fig. 29. Out of 22 isolates, 06 isolates (SK-5, BAC-1, BAC-2, RK-1, RK-2, and RK-11) were used for morpholine degradation studies**.**

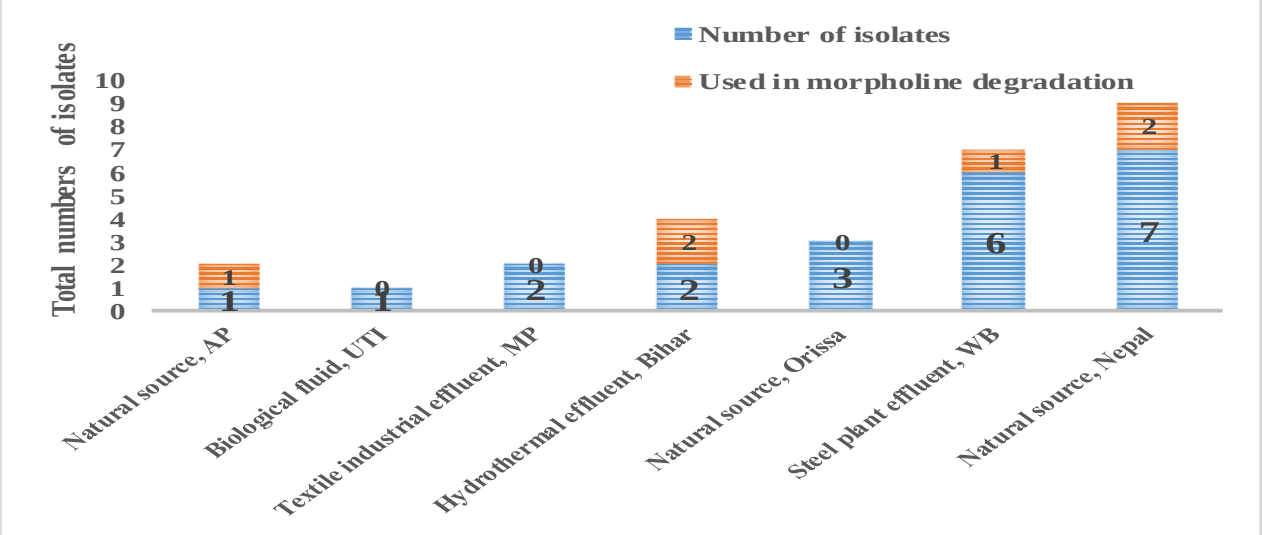


Fig. 29: Screening of isolates for the degradation of morpholine

4.2.2 Morphological, biochemical characterization of isolated microbes

Pure colonies were isolated and characterized using morphological, physiological and biochemical tests. The results of important morphological, physiological and biochemical characterization of isolates are summarized in Table 21.

Table 21: Morphological and biochemical characterization of isolates

	Isolates								
Characteristics	SK-1	SK-2	SK-3	SK-05	BAC-1	BAC-2	RK-1	RK-2	RK-11
Morphology Color Shape Arrangement Appearance Optical Margin Texture Elevation	 White Rod Straight Dull Opaque Edge Smooth Convex	 White Round Paired Dull Opaque Edge Smooth Convex	 White Rod Straight Dull Opaque Edge Smooth Convex	 White Rod Straight Dull Opaque Edge --- ---	 Yellow Round Coiled Bright Light Edge Sticky ----	 Yellow Rod Straight Bright Light Edge Sticky ---	 White Round Group Dull Opaque --- --- Convex	 White Rod Chain Dull Opaque --- --- Convex	 White Rod Single Dull Opaque --- --- Smooth Convex
Gram Staining	-	+	-	-	+	-	-	-	-
Time for visible colonies (days)	1-2	1-2	1-2	1-2	1-3	1-5	1-2	1-2	1-2
Gram morphology	Pink rods	Blue round	Pink rod	Pink rod	Blue round	Pink rod	Pink round	Pink rod	Pink Rod
Motility (Hanging drop)	+	nd	+	+	-	+	+	+	+
Acid fastness	-	-	-	-	-	-	-	-	-
Growth on Selective media (Hichrome M1353)	+	-	+	+	+	+	+	+	+
Citrate utilization	+	nd	nd	+	nd	nd	nd	nd	Nd
Urease	-	nd	nd	-	nd	nd	+	nd	Nd
Indole test	+	nd	nd	nd	nd	nd	-	nd	Nd
LB medium	+	+	+	+	+	+	+	+	+
SDA	-	-	-	-	-	-	-	-	-
Enrichment MSS medium	nd	nd	nd	+	+	+	+	+	+
Most likely organisms	*Pseudo monas*	nd	*Pseudo monas*	*Entero bacter*	*Staphylo coccus*	*Pseudo monas*	nd	nd	*H.blutaparo nensis*

Where +: positive, -: negative, V: variable, nd: not determined

4.2.3. Molecular identification of morpholine degrading bacterium

4.2.3.1. 16S rRNA-based phylogeny

The primary sequence of the 16s RNA from bacterial isolate RK-11 were determined (Annexure 3(2)). The sequence homology was obtained using NCBI BLAST search with known and identified cultures of the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) database. A comparative analysis of this sequence with the sequence representative of *E. coli* gave the phylogenetic position of this isolate. The isolate was found to be closely related to *Halobacillus blutaparonensis* as shown in Fig.30a.

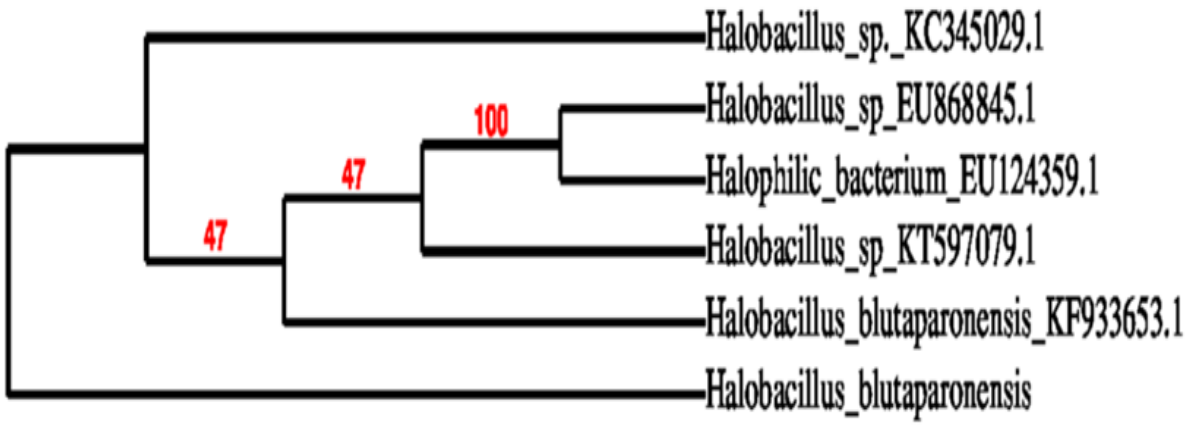


Fig. 30a: Molecular phylogeny of 16sRNA sequenced and sequences from identified bacteria in the database. The sequence of *E.coli* served as the outgroup for rooting the tree.

4.2.3.2. Sequence accession

Nucleotide sequence accession was assigned by Gene Bank, NCBI, an accession number of KC345029 (Fig. 30b). For the current study, this isolate was found closely related to *Halobacillus blutaparonensis.*

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Halobacillus sp. LB216 16S ribosomal RNA gene, partial sequence	1223	2397	98%	0.0	90%	KC345029.1
☐	Halobacillus blutaparonensis strain HNI94 16S ribosomal RNA gene, partial sequence	1223	2391	98%	0.0	90%	KF933653.1
☐	Halobacillus sp. S105-2 16S ribosomal RNA gene, partial sequence	1223	2397	98%	0.0	90%	EU868845.1
☐	Halophilic bacterium QW1015 16S ribosomal RNA gene, partial sequence	1219	2393	98%	0.0	90%	EU124359.1
☐	Halobacillus sp. ZV10-2 16S ribosomal RNA gene, partial sequence	1218	2373	98%	0.0	90%	KT597079.1
☐	Halobacillus sp. ID9 16S ribosomal RNA gene, partial sequence	1218	2391	98%	0.0	90%	KT695857.1
☐	Halobacillus sp. AMETH250 16S ribosomal RNA gene, partial sequence	1218	2391	98%	0.0	90%	KR150255.1
☐	Uncultured bacterium clone X628 16S ribosomal RNA gene, partial sequence	1218	2373	98%	0.0	90%	JX872358.1
☐	Halobacillus sp. SMK24-1 gene for 16S rRNA, partial sequence	1218	2386	98%	0.0	90%	AB695096.1
☐	Halobacillus sp. NSW13-5 gene for 16S rRNA, partial sequence	1218	2391	98%	0.0	90%	AB695095.1
☐	Halobacillus trueperi strain 14M_1428 16S ribosomal RNA gene, partial sequence	1218	2391	98%	0.0	90%	JN993994.1
☐	Halobacillus sp. NY-15 16S ribosomal RNA gene, partial sequence	1218	2391	98%	0.0	90%	FJ587216.1
☐	Halophilic bacterium QW109 16S ribosomal RNA gene, partial sequence	1218	2391	98%	0.0	90%	EU124356.1

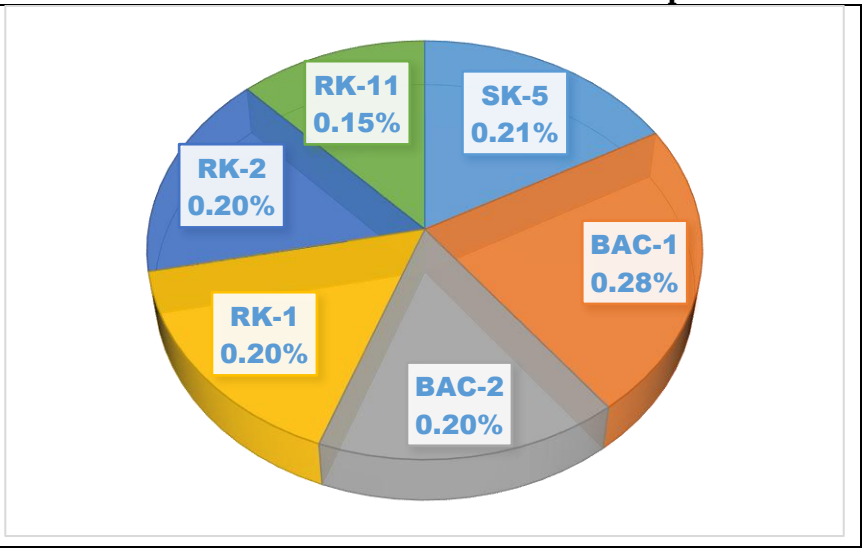
Fig. 30b: Nucleotide sequence accession number of closed identified bacteria

4.3. Acclimatization of microbes in increased concentration of morpholine

Different isolates have inherent capacity to metabolize morpholine to a certain concentration. They have further acclimatized up to 0.10-0.28% morphine as shown in Table 22.

Table 22: Acclimatized isolates with different concentration of morpholine

Isolate	Acclimatized (% morpholine)
SK-5	0.21
BAC-1	0.28
BAC-2	0.20
RK-1	0.20
RK-2	0.20
RK-11	0.15



4.3.1. SK-5 isolate

SK-5 isolate was acclimatized up to 0.21% of morpholine gradually. The number of colony forming unit (CFU) to each increasing concentration of morpholine was represented in Fig 31.

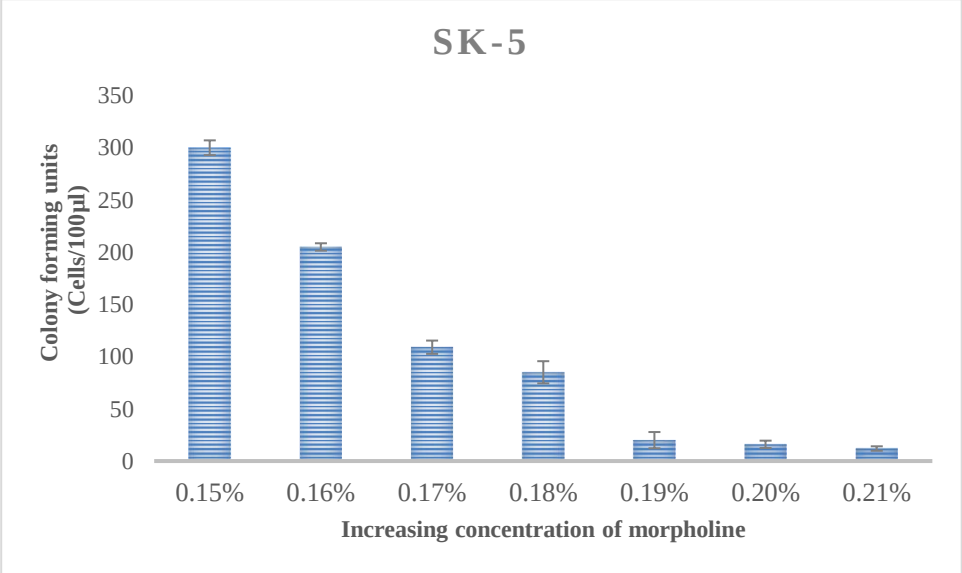


Fig. 31: Adaptation studies of isolate SK-5 isolate

4.3.2. BAC-1 and BAC-2 isolates

Two distinct colonies were grown on LB agar. The large and small one was designated as BAC-1 and BAC-2 respectively (Fig. 32). These two separate bacteria were acclimatized in increasing concentration of morpholine.

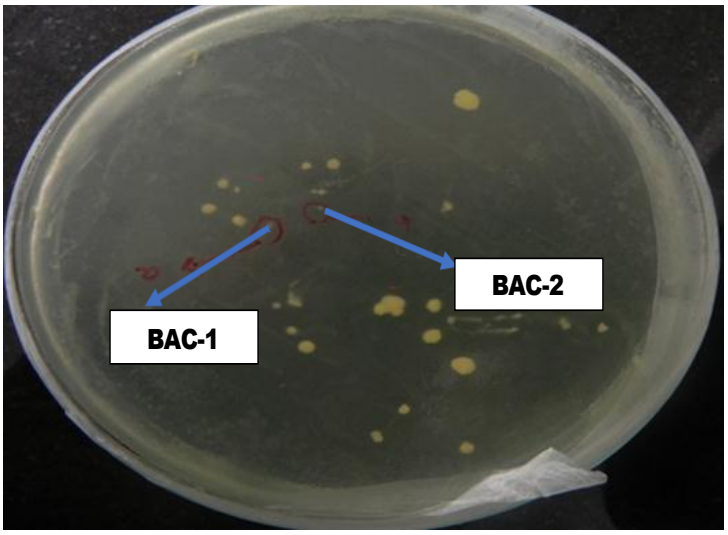


Fig. 32: BAC-1 and BAC-2 isolate

4.3.2.1. BAC-1 isolate

BAC-1 isolate was acclimatized up to 0.28% of morpholine and the number of colony forming unit (CFU) to each increasing concentration of morpholine was represented in Fig 32a.

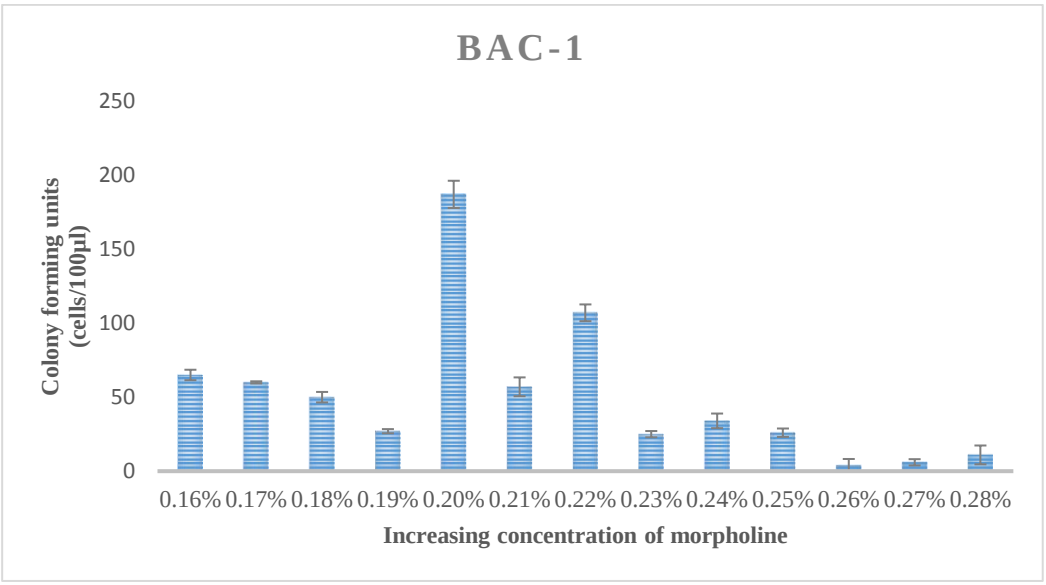


Fig. 32a: Adaptation studies of BAC-1 isolate

4.3.2.2. BAC-2 isolate

BAC-2 isolate was acclimatized gradually to 0.20% of morpholine and the number of colony forming unit (CFU) to each increasing concentration of morpholine was represented in Fig 32b.

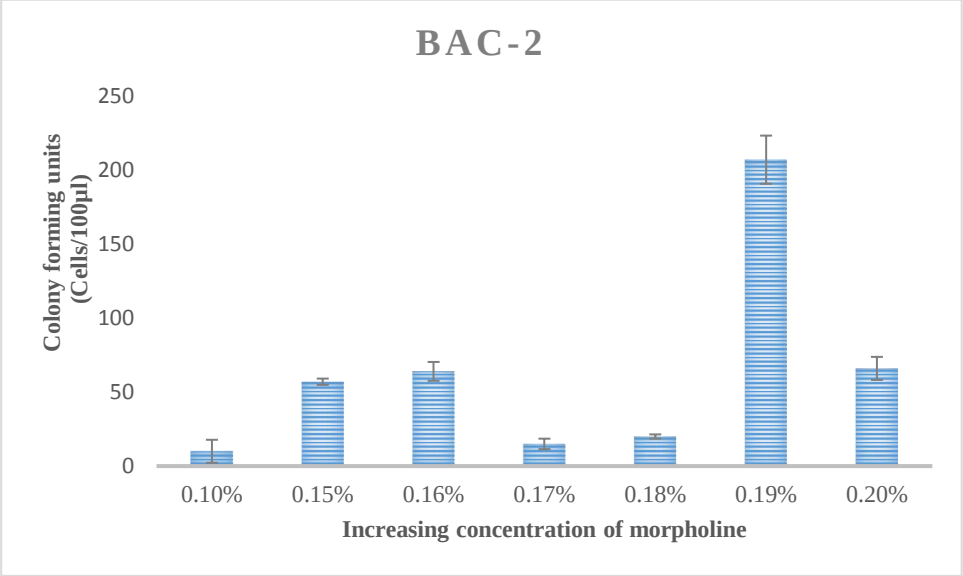


Fig. 32b: Adaptation studies of BAC-2 isolate

4.3.3. RK-1, RK-2 and RK-11 isolates

RK-1 and RK-2 isolate were acclimatized up to 0.20% of morpholine. However, RK-11 isolate could be acclimatized only up to 0.15% only. (Fig.33, Fig.34).

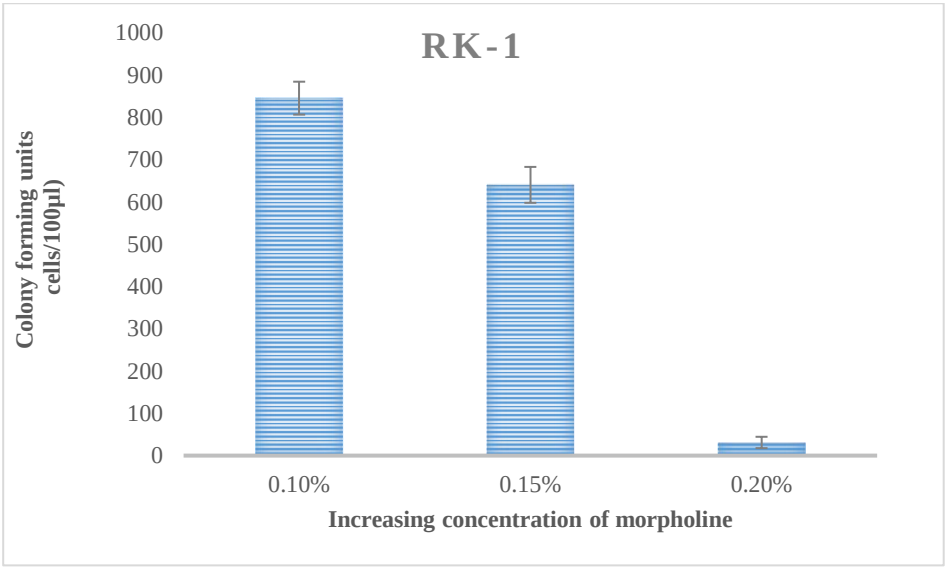
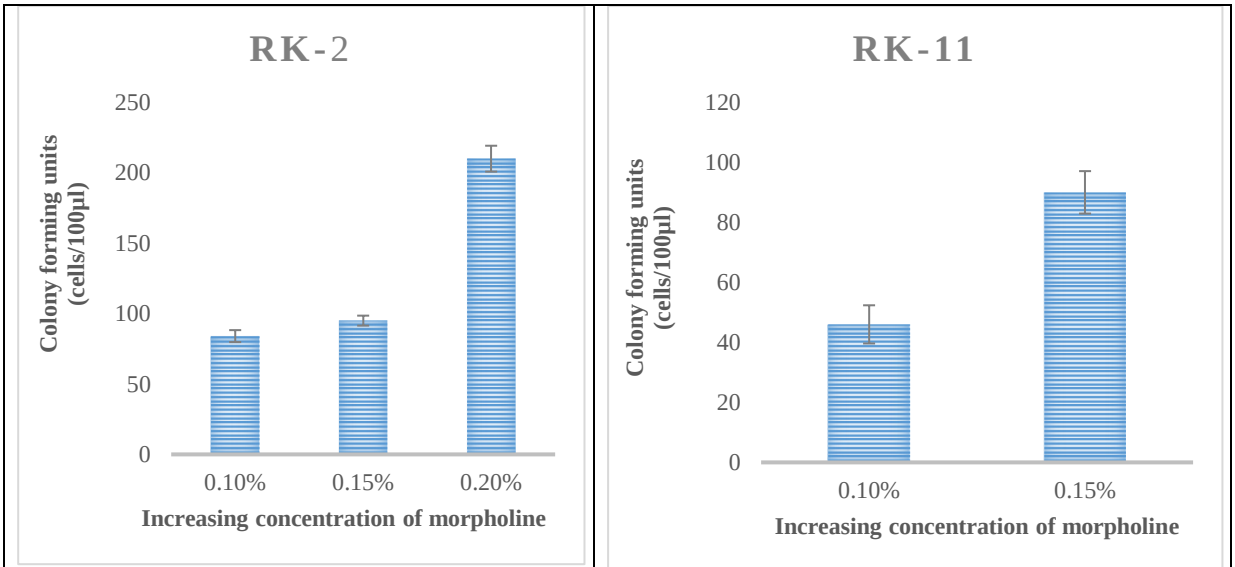


Fig. 33: Adaptation studies of RK-1 isolate



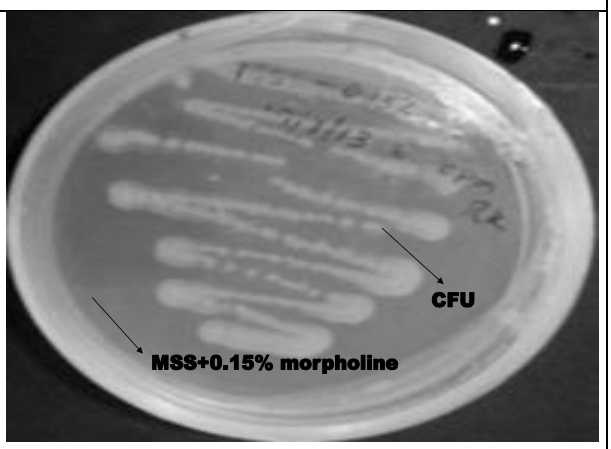
Adaptation studies of RK-2 **Adaptation studies of RK-11**

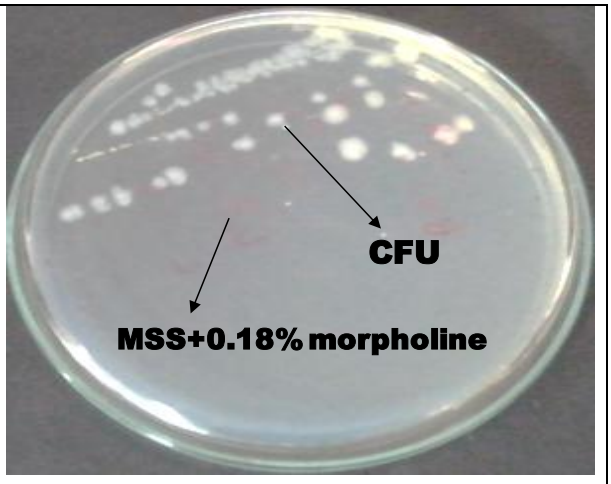
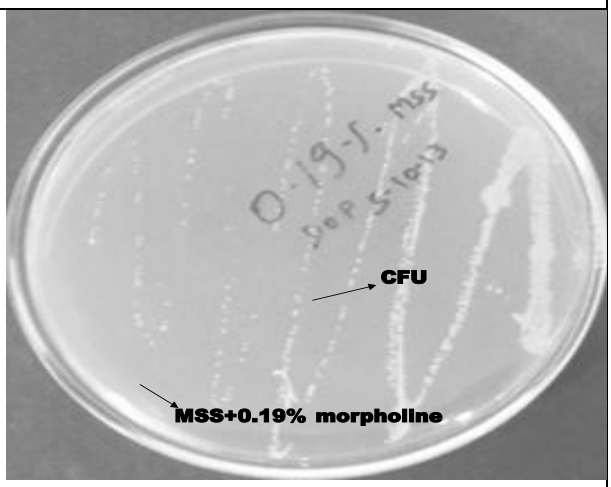
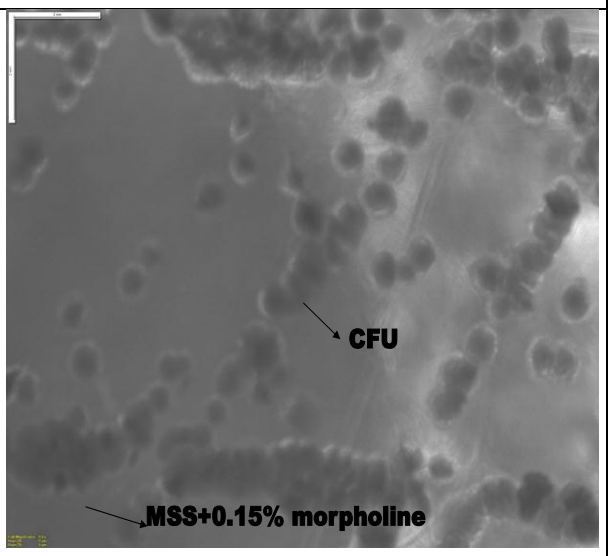
Fig. 34: Adaptation studies of RK-2 and RK-11 isolates

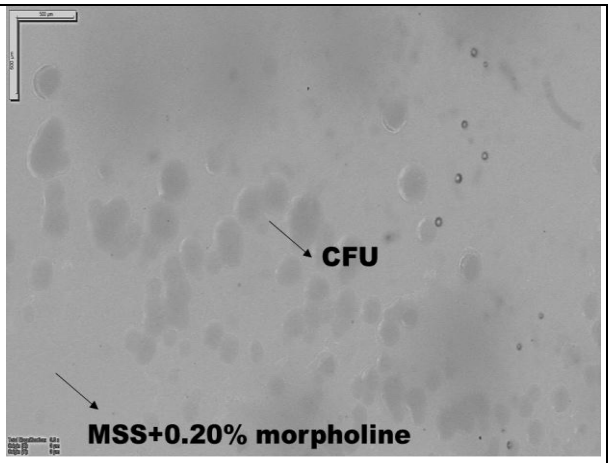
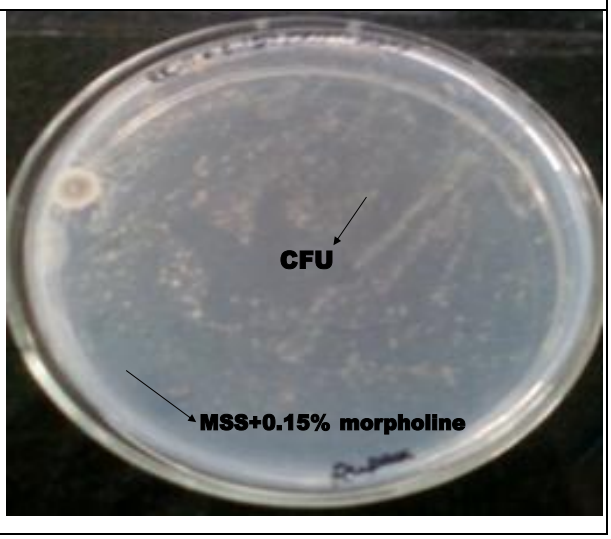
4.3.4. Acclimatization summary of microbial adaptation

The overall summary of the acclimatization of different potential isolates was shown in Table 23 with selected representation of the colonies for each isolate

Table 23: Summary of acclimatization of six potential isolates in gradual increasing concentration of morpholine

Potential isolates	Growth in % morpholine	No. of colonies (CFU)	Selected representation
SK-5	0.15	300	
	0.16	205	
	0.17	109	
	0.18	85	
	0.19	20	
	0.20	16	
	0.21	12	

BAC-1	0.16 0.17 0.18 0.19 0.20 0.21 0.22 0.23 0.24 0.25 0.26 0.27 0.28	65 60 50 27 187 57 107 25 34 26 4 6 11	
BAC-2	0.10 0.15 0.16 0.17 0.18 0.19 0.20	10 57 64 15 20 207 66	
RK-1	0.10 0.15 0.20	845 640 31	

RK-2	0.10 0.15 0.20	84 95 210	
RK-11	0.10 0.15	46 90	

Out of 6 isolates, all isolates i.e. 100% (n=6) showed successful growth till 0.15%; whereas 83% (n=5) stopped growing at 0.20% and 17% (n=1) of the isolates could metabolize more than 0.20% morpholine (Fig.35).

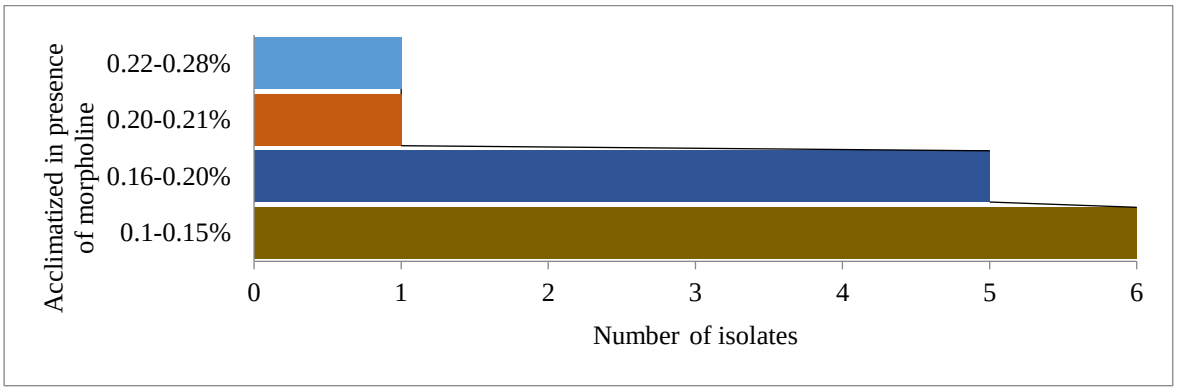


Fig. 35: Microbial adaptation against morpholine

4.4. Degradation studies

4.4.1. Quantification of morpholine in culture supernatant by gas chromatography

In the present study, gas chromatography analytical tool was used to estimate the available morpholine present in spent culture supernatant. The quantification method used was percentage reduction in AUC for the spent culture supernatant with respect to positive control (PC). Standard curve was plotted to quantitate available morpholine in culture supernatant (Fig 36). In a typical chromatogram, the retention time of the methanol and morpholine have been found to near 1.4 and 3.5 to 3.7 minutes respectively.

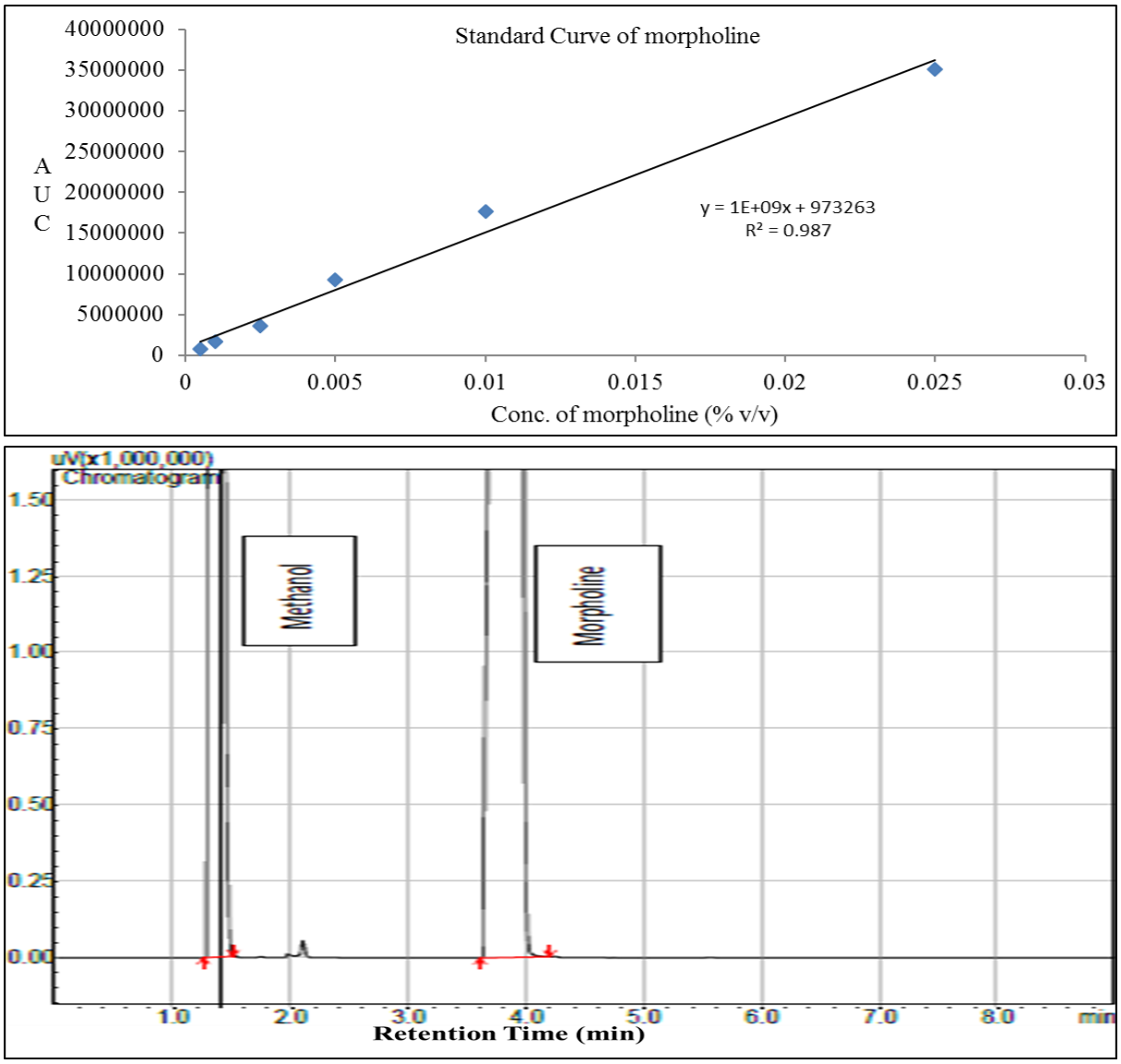


Fig.36: Standard curve and chromatogram of morpholine by GC

4.4.1.1. Degradation kinetics with SK-5

The corresponding retention time and area under curve of each concentration of morpholine shown in Table 24 and Fig. 37

Table 24: GC estimation of morpholine in SK-5 culture supernatant

Vial	RT	Compound	AUC	% degradation
Methanol Blank	1.415	Methanol	525913402.2	
Media Blank (1:10)	1.396	Methanol	472962013.7	
PC 0.15%	1.415 3.717	Methanol Morpholine	522973465.3 708045.4	
SK-5+0.15% (1:10)	1.395 3.628	Methanol Morpholine	474288188.3 68.7	99.9
PC 0.16%	1.413 3.700	Methanol Morpholine	521560287.3 1016276.8	
SK-5+0.16% (1:10)	1.395 3.634	Methanol Morpholine	473021672.7 21.0	99.9
PC 0.17%	1.413 3.694	Methanol Morpholine	518894322.3 1128677.8	
SK-5+0.17% (1:10)	1.396 3.637	Methanol Morpholine	472512360.1 177.9	99.9
PC 0.18%	1.412 3.690	Methanol Morpholine	520736208.7 1283996.4	
SK-5+0.18% (1:10)	1.396 3.369	Methanol Morpholine	473058510.2 141.4	99.9
PC 0.19%	1.412 3.688	Methanol Morpholine	518889190.3 1470783.5	
SK-5+0.19% (1:10)	1.396 3.545	Methanol Morpholine	471756655.0 12.1	99.9
PC 0.20%	1.412 3.679	Methanol Morpholine	539654144.4 1648380.9	
SK-5+0.20% (1:10)	1.396 3.738	Methanol Morpholine	469841588.8 77.8	99.9
PC 0.21%	1.412 3.680	Methanol Morpholine	526187607.5 1756720.4	
SK-5+0.21% (1:10)	1.337 3.515	Methanol Morpholine	582247912.8 295.2	99.8

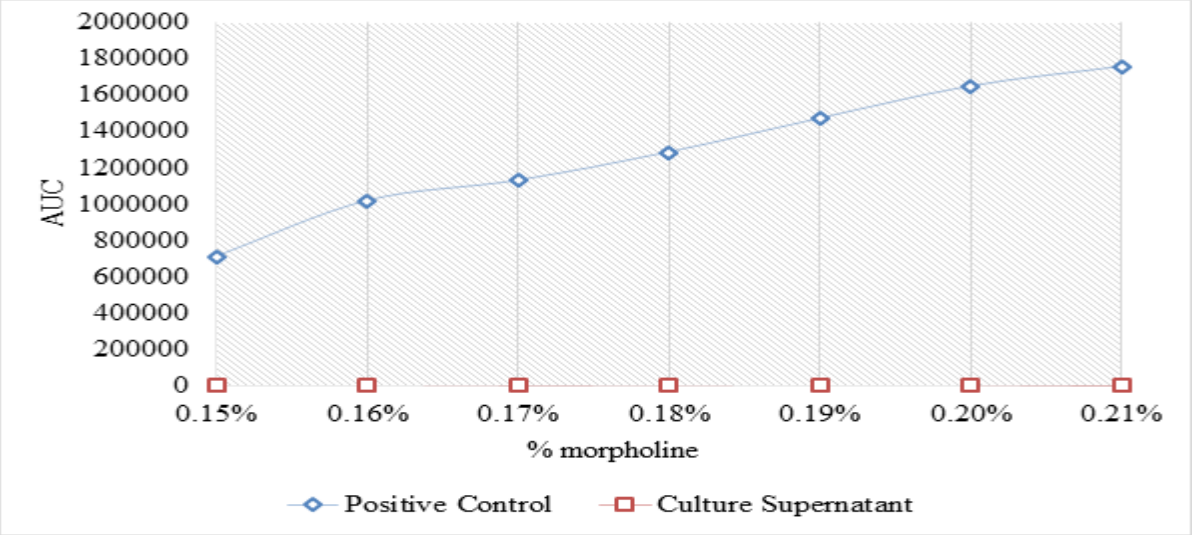


Fig. 37: Degradation kinetics of SK-5 isolate

4.4.1.2. Degradation kinetics with BAC-1

The corresponding retention time and area under curve of each concentration of morpholine shown in Table 25 and Fig. 38

Table 25: GC estimation of morpholine in BAC-1 culture supernatant

Vial	RT	Compound	AUC	% degradation
Methanol Blank	1.415	Methanol	587774262.8	
Media Blank (1:10)	1.396	Methanol	503356042.3	
PC 0.15%	1.415 3.717	Methanol Morpholine	588789244 694215.9	
BAC-1+0.15% (1:10)	1.393 3.781	Methanol Morpholine	522395429 52	99.9
PC 0.16%	1.413 3.700	Methanol Morpholine	592948509 777744.4	
BAC-1+0.16% (1:10)	1.396 3.766	Methanol Morpholine	532224259 64	99.9
PC 0.17%	1.413 3.694	Methanol Morpholine	591738319 806234.2	
BAC-1+0.17% (1:10)	1.396 3.753	Methanol Morpholine	533644096 78.5	99.9
PC 0.18%	1.412 3.690	Methanol Morpholine	520736208.7 873090	
BAC-1+0.18% (1:10)	1.401 3.785	Methanol Morpholine	602903602.6 51.1	99.9
PC 0.19%	1.412 3.688	Methanol Morpholine	533904104.9 904615.2	

BAC-1+0.19% (1:10)	1.337 3.657	Methanol Morpholine	546537409.2 558.7	99.93
PC 0.20%	1.412 3.715	Methanol Morpholine	524107425.2 1006608.3	
BAC-1+0.20% (1:10)	1.337 3.657	Methanol Morpholine	541013033.2 487.7	99.95
PC 0.21%	1.377 3.728	Methanol Morpholine	514948208.5 1703637.0	
BAC-1+0.21% (1:10)	1.350 3.560	Methanol Morpholine	454090095.4 556.1	99.96
PC 0.22%	1.369 3.725	Methanol Morpholine	501420656.8 1918758.4	
BAC-1+0.22% (1:10)	1.354 3.555	Methanol Morpholine	457890413.7 812.6	99.95
PC 0.23%	1.369 3.726	Methanol Morpholine	503086906.3 1991100.7	
BAC-1+0.23% (1:10)	1.353 3.562	Methanol Morpholine	453686622.1 784.3	99.96
PC 0.24%	1.370 3.722	Methanol Morpholine	4976676150.0 2072055.3	
BAC-1+0.24% (1:10)	1.388 3.551	Methanol Morpholine	440294623.0 722.3	99.96
PC 0.25%	1.370 3.722	Methanol Morpholine	498219843.7 2142953.1	
BAC-1+0.25% (1:10)	1.353 3.558	Methanol Morpholine	450862268.5 881.0	99.95
PC 0.26%	1.363 3.724	Methanol Morpholine	496361849.3 2177381.3	
BAC-1+0.26% (1:10)	1.353 3.561	Methanol Morpholine	446123276.9 842.6	99.96
PC 0.27%	1.366 3.724	Methanol Morpholine	496738136.3 2358169.4	
BAC-1+0.27% (1:10)	1.349 3.554	Methanol Morpholine	452954609.3 797.8	99.96
PC 0.28%	1.369 3.722	Methanol Morpholine	500517702.7 2658722.0	
BAC-1+0.28% (1:10)	1.349 3.570	Methanol Morpholine	444584428.2 698.7	99.97

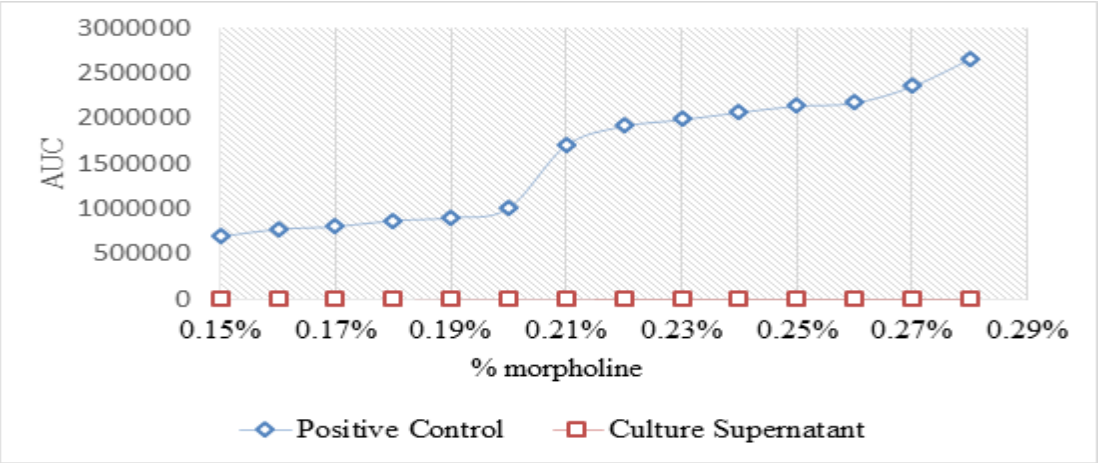


Fig. 38: Degradation kinetics of BAC-1 isolate

4.4.1.3. Degradation kinetics with BAC-2

The corresponding retention time and area under curve of each concentration of morpholine shown in Table 26 and Fig. 39

Table 26: GC estimation of morpholine in BAC-2 culture supernatant

Vial	RT	Compound	AUC	% degradation
Methanol Blank	1.355	Methanol	509038500.8	
Media Blank (1:10)	1.392	Methanol	468375385.4	
PC 0.10%	1.365 3.867	Methanol Morpholine	522835489.7 246507.0	
BAC-2+0.10% (1:10)	1.351 3.873	Methanol Morpholine	464109026.2 45.0	99.98
PC 0.15%	1.365 3.768	Methanol Morpholine	522505798.7 534926.6	
BAC-2+0.15% (1:10)	1.352 3.547	Methanol Morpholine	457886621.6 580.2	99.89
PC 0.16%	1.369 3.765	Methanol Morpholine	521628252.7 629917.8	
BAC-2+0.16% (1:10)	1.355 3.551	Methanol Morpholine	464002306 719.0	99.88
PC 0.17%	1.368 3.757	Methanol Morpholine	518073800.3 660696.4	
BAC-2+0.17% (1:10)	1.345 3.546	Methanol Morpholine	441465224.3 515.5	99.88
PC 0.18%	1.369 3.752	Methanol Morpholine	521625008.0 740278.9	
BAC-2+0.18% (1:10)	1.355 3.542	Methanol Morpholine	464849424.7 584.8	99.92

PC 0.19%	1.365 3.735	Methanol Morpholine	503734112.2 862159	
BAC-2+0.19% (1:10)	1.394 3.549	Methanol Morpholine	465367925.1 407.3	99.95
PC 0.20%	1.372 3.753	Methanol Morpholine	512508132.2 1006608.3	
BAC-2+0.20% (1:10)	1.393 3.657	Methanol Morpholine	4772051599 707.7	99.95

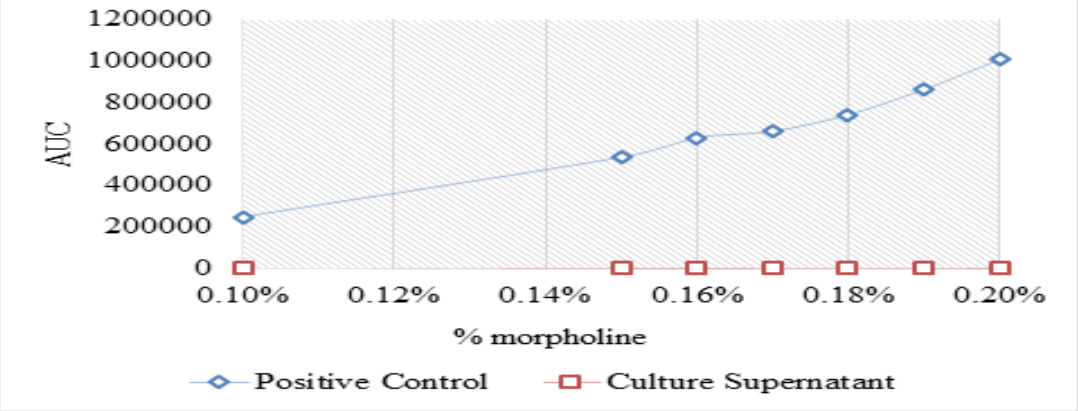


Fig. 39: Degradation kinetics of BAC-2 isolate

4.4.1.4. Degradation kinetics with RK-1

The corresponding retention time and area under curve of each concentration of morpholine shown in Table 27 and Fig. 40.

Table 27: GC estimation of morpholine in RK-1 culture supernatant

Vial	RT	Compound	AUC	% degradation
Methanol Blank	1.345	Methanol	509010779.2	
Media Blank (1:10)	1.335	Methanol	464444522.2	
PC 0.10%	1.352 3.742	Methanol Morpholine	499009767.7 164583.8	
RK-1+0.10% (1:10)	1.335 3.528	Methanol Morpholine	457273604.0 562.1	99.98
PC 0.15%	1.318 3.792	Methanol Morpholine	121422987.1 246723.8	
RK-1+0.15% (1:10)	1.316 3.758	Methanol Morpholine	112091177.0 27.8	99.98
PC 0.20%	1.316 3.752	Methanol Morpholine	122536168.9 388281.6	
RK-1+0.20% (1:10)	1.316 3.760	Methanol Morpholine	111123646.6 20.8	99.99

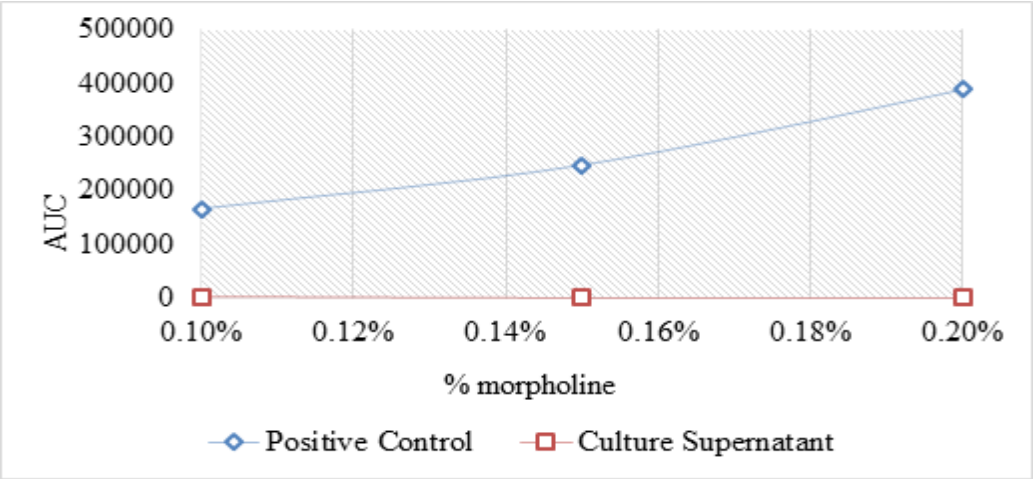


Fig. 40: Degradation kinetics of RK-1 isolate

4.4.1.5. Degradation kinetics with RK-2 and RK-11

The corresponding retention time and area under curve of each concentration of morpholine shown in Table 28 and Fig. 41.

Table 28: GC estimation of morpholine in RK-2 and RK-11 culture supernatant

Vial	RT	Compound	AUC	% Degradation
Methanol Blank	1.320	Methanol	122494814.8	
Media Blank (1:10)	1.317	Methanol	112809639.1	
PC 0.10%	1.316 3.887	Methanol Morpholine	123210583.3 122593.8	
RK-2+0.10% (1:10)	1.315 3.711	Methanol Morpholine	110507338.4 51.8	99.9
RK-11+0.10% (1:10)	1.315 3.728	Methanol Morpholine	120507338.4 62.1	99.98
PC 0.15%	1.318 3.792	Methanol Morpholine	121422987.1 246723.8	
RK-2+0.15% (1:10)	1.313 3.769	Methanol Morpholine	110427661.0 18.7	99.98
RK-11+0.15% (1:10)	1.316 3.758	Methanol Morpholine	112091177.0 27.8	99.98
PC 0.20%	1.316 3.752	Methanol Morpholine	122536168.9 388281.6	
RK-2+0.20% (1:10)	1.316 3.798	Methanol Morpholine	105534352.0 16.6	99.99

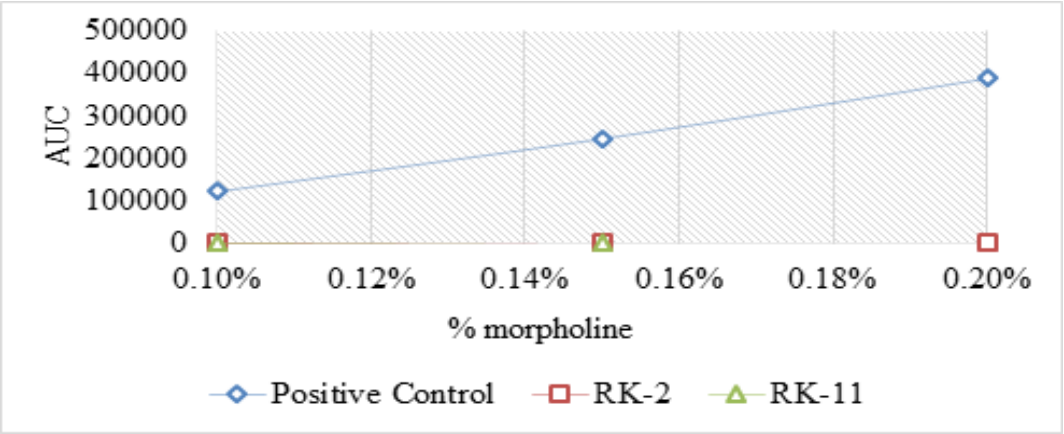


Fig. 41: Degradation kinetics of RK-2 and RK-11 isolates

4.4.2. Degradation summary of morpholine: GC analysis

Overall complete degradation of % available morpholine by potential isolates was shown in Fig. 42. The Degradation efficacy of the isolates follows the BAC-1 > SK-5/ BAC-2/ RK-1/ RK-2 > RK-11.

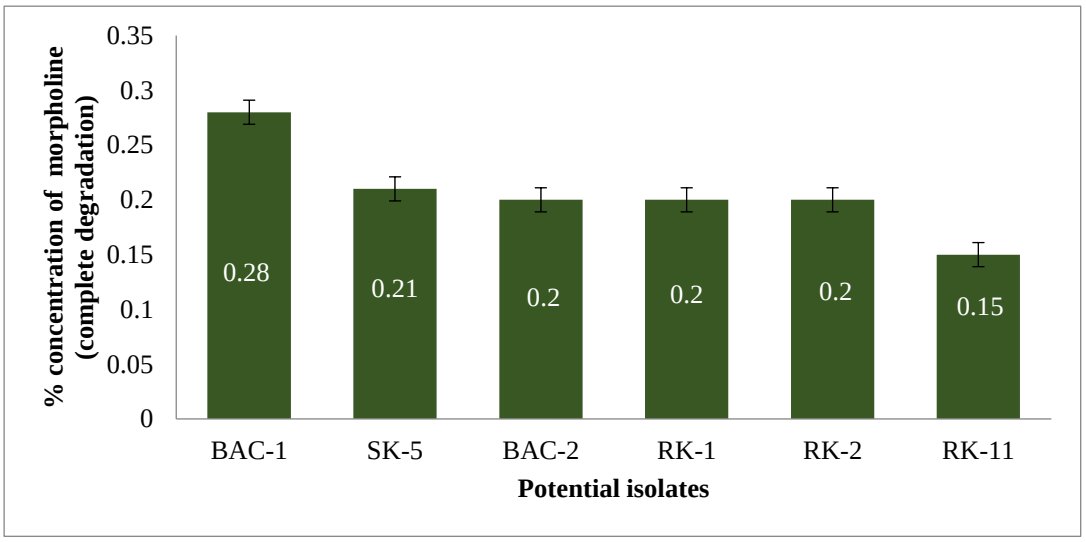


Fig. 42: Degradation studies of potential isolates

4.5. Elucidation of morpholine degradation pathway

The metabolic pathway involved in biodegradation of morpholine has been very difficult to establish. Indirect strategies have been developed like COD estimation, ammonia measurement, growth on intermediates and in vitro chemical assay of intermediates. However direct approach includes ^{1}H-NMR, GC and MS studies with culture supernatants to identify some metabolic

intermediates of morpholine. In this study, following approaches were used to elucidate the metabolic pathway follow either the ethanolamine or diglycollic route of degradation.

4.5.1. Growth on intermediates

Selected isolate RK-11 showed growth on morpholine and different intermediate (used as carbon source). However, no growth was recorded in presence of ethanolamine in culture media as shown in Table 29 and Fig. 43.

Table 29: Growth of RK-1 in presence of intermediates of morpholine degradation

Parameter (0.15% substrate)		Growth (absorbance@ 600nm)				pH
		4Hr.	17 Hr.	24 Hr.	41 Hr.	
Media control		0.002	0.002	0.002	0.002	7.0
Morpholine	Control	0.002	-0.013	-0.001	-0.002	7.0
	Test	0.152	0.356	0.677	0.380	
Aminoethoxy ethanol	Control	0.003	-0.008	-0.002	-0.003	7.0
	Test	0.294	0.512	0.737	0.685	
Ethanolamine	Control	0.005	-0.013	-0.003	-0.002	7.0
	Test	0.006	0.002	0.008	0.0057	

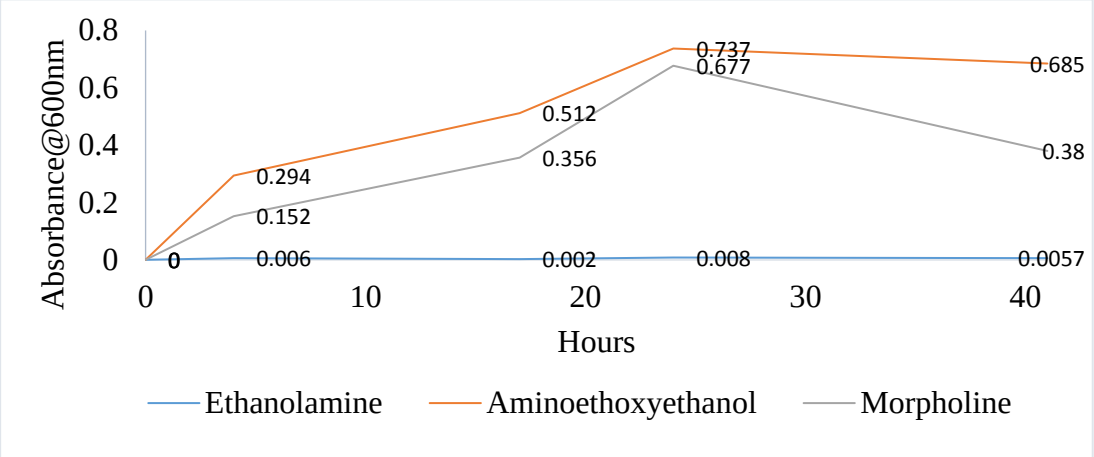


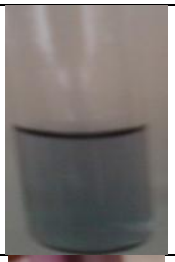
Fig. 43: Growth kinetics of RK-11 in presence of intermediate of morpholine degradation

4.5.2. Chemical assays for intermediate presence of intermediates of morpholine degradation

4.5.2.1. Qualitative estimation of monoethanolamine in culture supernatant

Simon test was conducted to find out the presence of monoethanolamine and morpholine in the culture supernatant as shown in Table 30 given below.

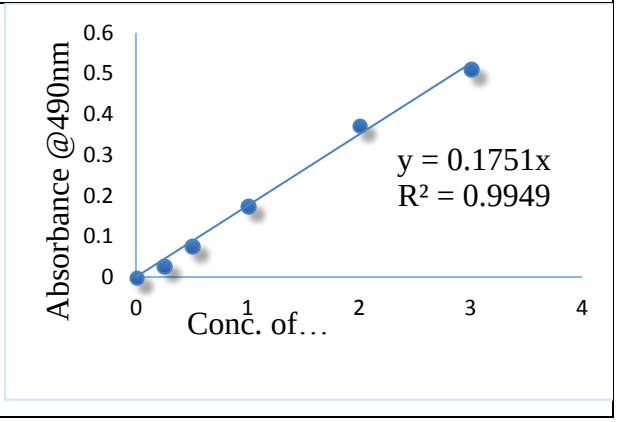
Table 30: Simon test of primary amine (Monoethanolamine), secondary amine (Morpholine) and culture supernatant

Sample	Test	Feature	Remark	Result
Morpholine (Secondary amine)	Simon 1	Characteristic blue color with secondary amine		Morpholine positive
Monoethanolamine (Primary amine)	Simon 2	Characteristic violet color with primary amine		Monoethanolamine positive
Culture media (MSS)	Simon 1	No characteristic blue color		Morpholine negative
	Simon 2	No characteristic violet color		Monoethanolamine negative
Culture Supernatant	Simon 1	No characteristic blue color		Morpholine negative
	Simon 2	No characteristic violet color		Monoethanolamine negative

4.5.2.2. Quantitative estimation of monoethanolamine in culture supernatant (Simon-2)

There was no detectable monoethanolamine in culture supernatant. The quantitative results of monoethanolamine in culture supernatant was negative and shown in Table 31.

Table 31: Quantitative estimation of monoethanolamine in culture supernatant

Conc. of monoethanolamine (mg/ml)	Absorbance @490nm
WB	0
0.25	0.029
0.50	0.077
1.0	0.176
2.0	0.374
3.0	0.512
Media Blank	0.092
Culture supernatant (1:100)	0.089
Standard curve (Regression equation)	

4.5.3. Analytical assay of presence of intermediated in culture supernatant

4.5.3.1. GC studies of monoethanolamine (MEA) in culture supernatant

GC was performed using culture supernatants along with different concentration of standard monoethanolamine (MEA) solution. Table 32 and Fig. 44 represents the retention time of monoethanolamine and was found to be 2.2 min which was absent in diluted culture supernatant.

Table 32: GC analysis of monoethanolamine in culture supernatant

Vial	Retention time (min)	AUC	Compound
Methanol	1.331	378534920.9	Methanol
5000 ppm	1.333	366649701.7	Methanol
MEA	2.218	2748948.5	Monoethanolamine
2500 ppm	1.331	374551161.2	Methanol
MEA	2.216	2397300.9	Monoethanolamine

1250 ppm MEA	1.331 2.211	378803557.4 1149593.1	Methanol Monoethanolamine
Culture Supernatant (1:10)	1.334 2.21	310947764.4 No peak	Methanol No Monoethanolamine

GC analysis revealed that there was no monoethanolamine (not getting the retention time of monoethanolamine) in culture supernatant supporting that bacteria prefer the glycolic route of degradation of morpholine. It was further confirmed by MS analysis.

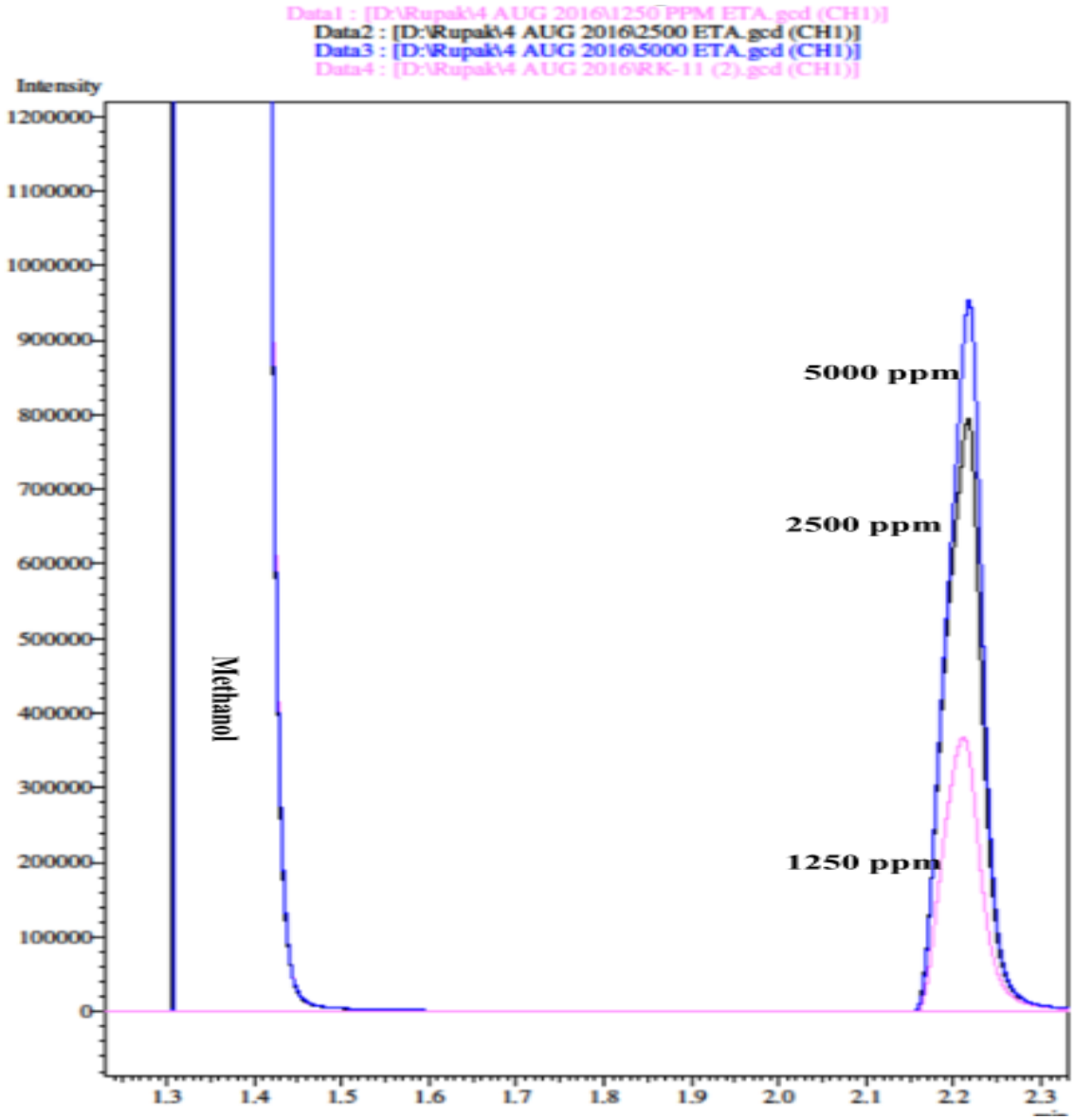


Fig. 44: GC (RTX-35)-FID chromatogram of monoethanolamine of 1250, 2500 and 5000 ppm concentration

4.5.3.2. MS studies of culture supernatant

4.5.3.2.1. MS analysis of culture supernatant (with solvent)

Water, methanol and acetonitrile were chosen to dissolve the culture filtrate and result is tabulated in Table 33a and 33b.

Table 33a: MS analysis of culture supernatant (with different solvent)

Sample	Solvent	Major m/z (+ Mode)	Major m/z (- Mode)
Positive Control (0.20% morpholine in MSS)	Methanol	88.10, 106.10, 120.10	75.10, 173.05, 191.10
	Acetonitrile (ACN)	88.10, 129.10, 170.10	77.15, 119.10, 130.10, 157.05, 171.10, 195.00, 217.00, 255.25, 272.15, 283.30
		88.10, 111.15, 129.15, 131.15, 170.00	77.15, 89.10, 100.10, 115.00, 119.10, 130.10, 171.10, 192.05, 212.90, 217.10
	Water	88.10, 106.15, 116.15, 129.10	61.10, 79.10, 116.10, 130.15, 131.10, 148.10, 217.10
Culture filtrate-1	Methanol	88.10, 120.10	89.10, 129.05, 157.05, 173.05, 194.95, **232.90**
	Acetonitrile (ACN)	88.10, 110.10, 129.10, 130.15, 170.10	77.10, 97.05, 119.10, 157.05, 177.00, 194.95, 217.10, 255.25
		88.10, 103.15, 111.15, **121.00,** 129.15, 170.10	77.15, 89.10, 97.05, 105.10, 119.10, 138.05, 157.05, 176.00, 185.05, 194.95, 217.10, **232.90**
	Water	88.10, 106.15, 129.10, 169.00, 174.90,	79.10, 89.10, 97.05, 115.05, 129.10, 143.05, 171.00, 190.85, 194.95, 217.10, **232.90**

Culture filtrate-2	Methanol	77.10, 88.10, 92.10, 120.10	**75.10,** 119.10, 157.05, 173.05, 194.95, 232.95, 330.90, 368.85, 428.90, 466.90, 488.85
	Acetonitrile (ACN)	88.10, **121.00**, 129.10, 170.10, 171.10	97.05, 138.05, 175.10, 194.95, 217.10,255.25, 283.30
		88.10, **121.00,** 129.10, 170.10	77.15, 97.05, 119.10, 138.05, 194.95, 217.10, **232.90**
	Water	88.10, **121.05**, 129.10, 174.90	79.10, 88.90, 97.05, 115.05, 194.95, 217.10, **232.90,** 241.05,

It was observed that morpholine forms adduct with methanol (m/z =120), acetonitrile (m/z= 129.00) and water (106.15) in positive ion mode. However, m/z = 121.00 indicates the 2, 2 amino ethoxy acetic acid (having formula $C_4H_9NO_3$ and molecular weight = 119.119) as $[M+2H]^+$ which is a degradation product of morpholine when dissolved in acetonitrile. Similarly, m/z = 232.90 indicates the di glycolic acid (having formula $C_4H_6O_5$ and molecular weight = 134.09) as $[M-2H+2K+Na]^-$ which is also a degradation product of morpholine when dissolved in acetonitrile. It is also noticed that m/z = 75 indicates the glycolic acid (having formula $C_2H_4O_3$ and molecular weight = 76) as $[M-H]^-$ which is also a degradation product of morpholine when dissolved in methanol but not seen in acetonitrile solvent system. These fragments are not present in the positive control indicating successful degradation of morpholine by bacteria using the glycolic acid pathway.

Table 33b: Expected intermediated in MS analysis of culture supernatant (with different solvent)

Sample	Solvent	m/z (+ Mode)	m/z (- Mode)	Remark
Culture supernatant	ACN	121.00	---	$[M+2H]^+$ 2,2 amino ethoxy acetic acid
		--	232.90	$[M-2H+2K+Na]^-$ di glycolic acid
	Methanol		75.00	$[M-H]^-$ glycolic acid

4.5.3.2.2.MS analysis of culture supernatant (without solvent)

The m/z peak in different solvent does not give a clear explanation of presence of degradation intermediate. So, MS was performed directly with filtrate of culture supernatant. Each sample was performed separately with two range of m/z namely 50-100 and 100-150 in positive and negative mode both (Table 34a, 34b and Fig. 45).

Table 34a: MS analysis of culture supernatant (without solvent)

Vial	Positive mode		Negative mode	
	m/z (50-100)	m/z (100-150)	m/z (50-100)	m/z (100-150)
MSS filtrate	52.20 57.0 59.10 65.10 71.05 80.05 82.05 92.10 97.10 98.05	118.10 136.90 147.10	96.95	103.0 110.65 114.95 124.95 129.0 132.70 135.00 138.75 143.00 149.0
Culture filtrate	88.10	106.10, **120.10** 129.10 136.90		113.0 115.0 129.0 **133.0** 139.0 143.05 147.05 149.0

Table 34b: Expected intermediated in MS analysis of culture supernatant (Without solvent)

Sample	m/z (+ Mode)	m/z (- Mode)	Remark
Culture filtrate	120.10	---	$[M+H]^+$ 2,2 amino ethoxy acetate
	--	133.0	$[M-H]^-$ Anion of glycolic acid

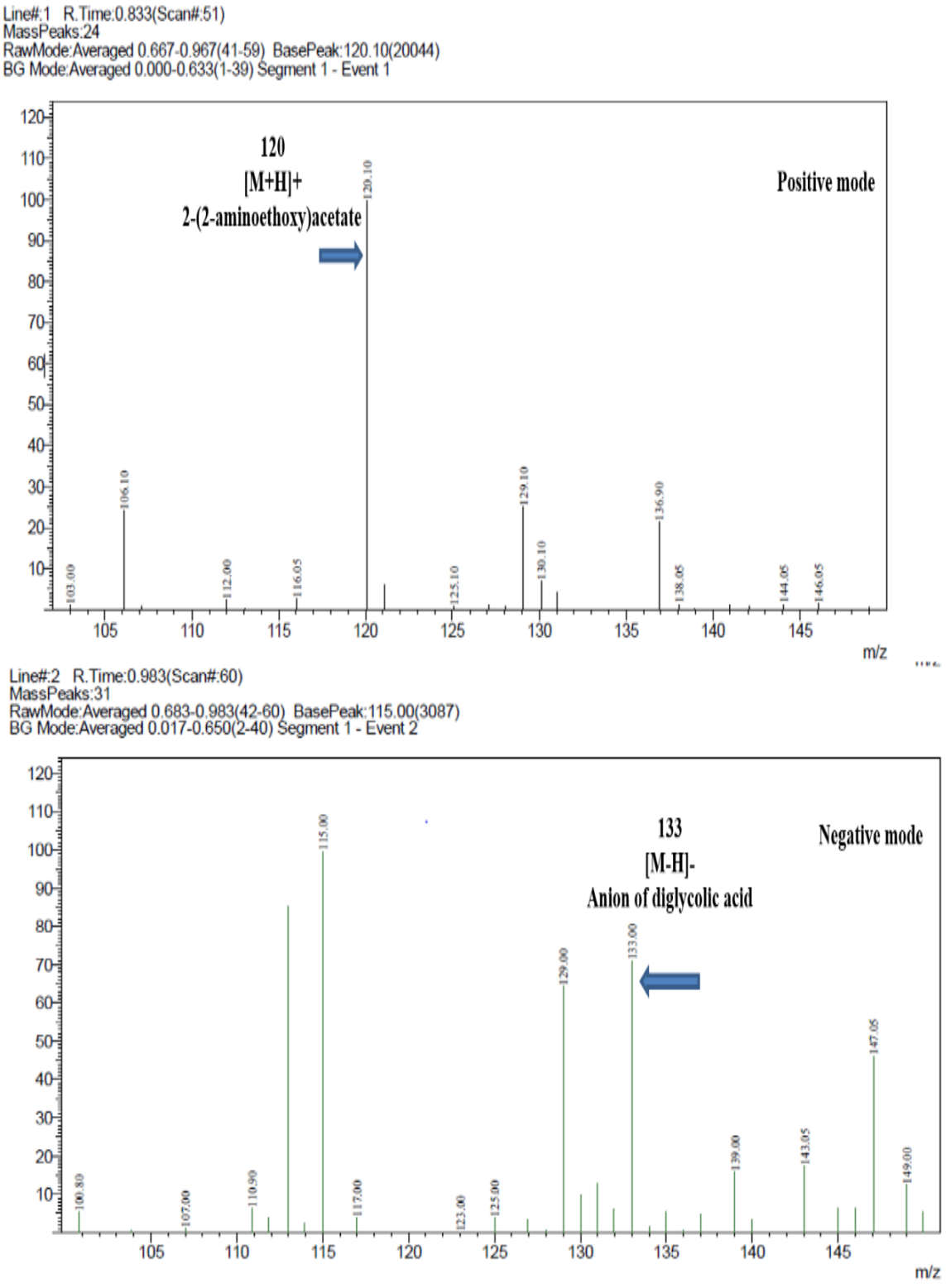


Fig. 45: ESI mass spectra recorded under positive and negative ionization of culture supernatant supplemented

4.5.3.2.3. Results of MS studies: Glycolate degradation pathway

MS analysis supports the GC findings and it is envisaged that as ethanolamine might be inhibitory to bacteria. Hence, it prefers the glycollic route of metabolic pathway. Similar results were also found by Combourieu *et al.*, 2000 by ion spray spectroscopy and supports the fact that in presence of morpholine, one of two branches of morpholine biodegradation pathway was induced while the other branch was inhibited. The illustrated degradative pathway might be starting with the cleavage of the C-N bond; this leads formation of an intermediary amino acid, which is further followed by deamination and oxidation of this amino acid to a diacid as shown in Fig. 46.

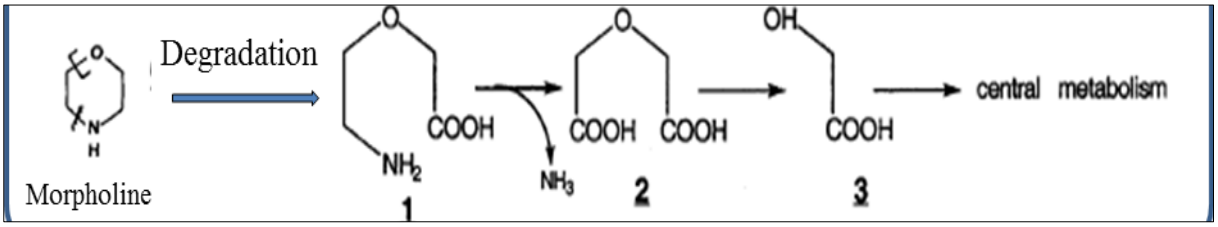


Fig.46: Glycolate pathway of morpholine degradation. Where, 1: 2-(2-aminoethoxy) acetate, 2: diglycolic acid and 3: glycollic acid

4.6. Ammonia release: As end product of morpholine degradation

Morpholine can be degraded by bacteria, with the liberation of ammonia, and that it can be used as a nitrogen source. Whatever the pathway bacteria prefer, ammonia is produced as a product of degradation of morpholine. As morpholine disappeared from the culture, ammonia accumulated that will increase the pH of the medium. Ammonia was quantified by Nessler reagent as shown in the Table 35 and Fig. 47.

Table 35: Estimation of ammonia by Nessler reagent

Well	10PPM Stock NH4-N^+ (µl)	Milli Q water (µl)	Culture Media (µl)	50% Na-k Tartarate (µl)	Nessler's Reagent (µl)	Absorbance @405nm	Net Absorbance @405nm
WB	--	250	---	5	5	0.106	0
Media Blank	--	--	250	5	5	2.630	0
1ppm	25	225	---	5	5	0.197	0.091
2ppm	50	200	---	5	5	0.390	0.284
3ppm	75	175	---	5	5	0.459	0.353
4ppm	100	150	---	5	5	0.658	0.552
5ppm	125	125	---	5	5	0.831	0.725
Culture supernatant	250		---	5	5	3.355	0.725

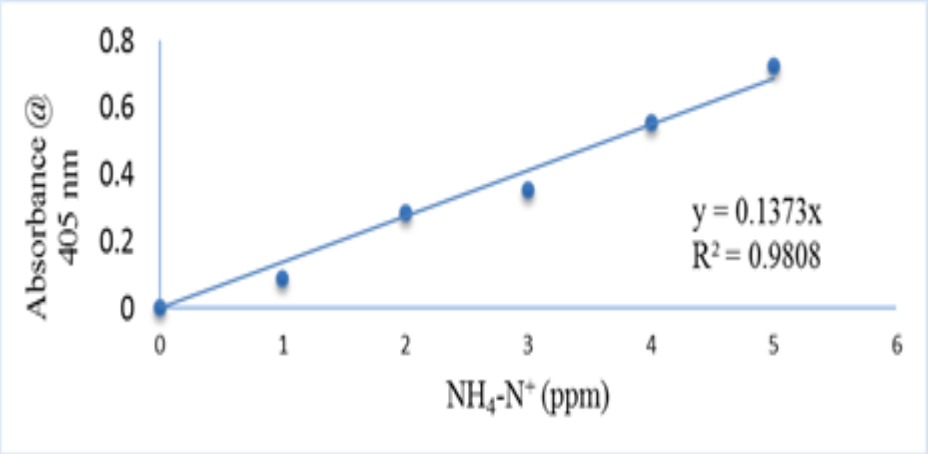


Fig. 47: Standard curve of ammoniacal nitrogen by Nessler reagent

4.6.1. Calculation of ammoniacal nitrogen: concluding remark

Ammoniacal nitrogen produced: 0.725/0.1373 = 5.2 ppm. Initial morpholine concentration in culture supernatant (before degradation): 2000 ppm. The molar conversion ratio of morpholine to ammonia was found to be 1:0.014. It was shown that the final pH of the media throughout the experiment did not change supporting the fact that low concentration of ammonia was released as end product of morpholine degradation.

4.7. Immobilization of microbes for degradation of morpholine

RK-1 isolate was immobilized in alginate polymeric matrix to ease the applicability in the degradation studies of morpholine. Fig. 48a represents the entrapped alginate beads of isolate in comparison with free cells. It was found that alginate beads were uniform in size and are denser than they are in free state. These beads were put in in 2000 ppm (0.2%) of morpholine for degradation studies at an interval of 24 hr. time points. Within 48 hrs., it was shown that beads itself degraded which might be due the inherent alginase activity of bacteria or reductive amination reaction in external environmental condition. Due to presence of dissolved or trace alginate in culture supernatant, the GC based degradation analysis was replaced with spectrophotometric based analysis of available morpholine in culture supernatant. Similar finding was observed with other isolates.

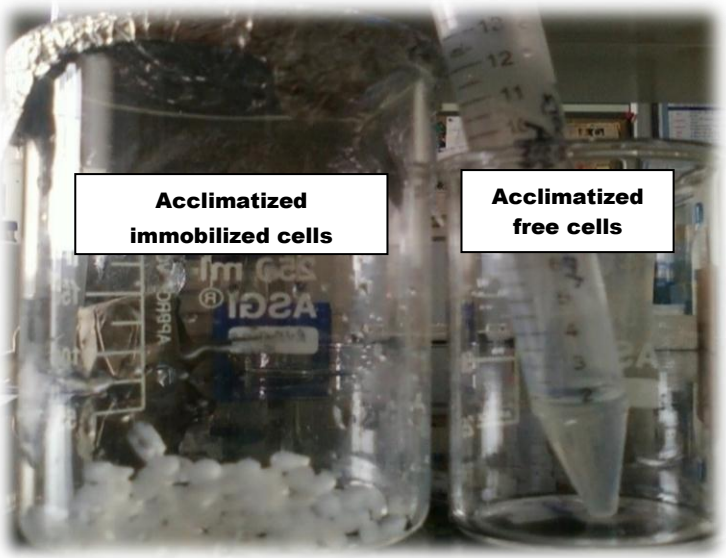


Fig. 48a: Immobilized alginate beads in comparison with free cells of a morpholine degrader

4.7.1. Characterization of beads

The characteristic features of beads (e.g., average size, enumeration of viable cells and degradation studies) was summarized in Table 36 and shown in Fig.48b.

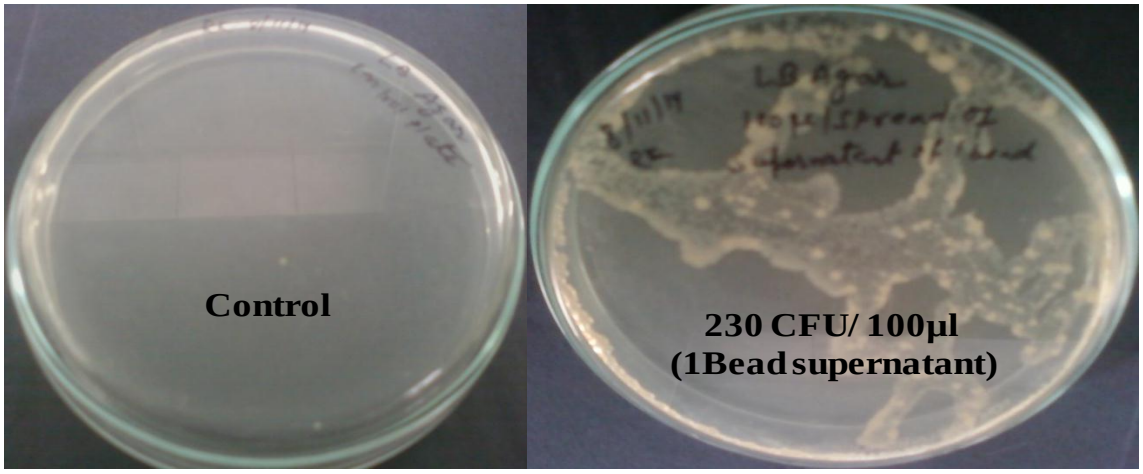


Fig. 48b: Enumeration of viable cells in single encapsulated alginate beads

Table 36: Characterization of alginate beads

Feature	Preparation	Average size(mm)	Cell viability enumeration	Degradation kinetics
Result	Easy	3	230 CFU/bead/100µl	Spectrophotometric analysis

4.7.2. Spectrophotometric degradation studies by immobilized cells: concluding remark

An excellent linear relationship was existed between the absorbance and the concentration of morpholine (r^2= 0.987, slope of 0.0309, Table 37a). Subsequently culture supernatants at different time points (at an interval of 24 hrs.) were collected to measure available morpholine using that standard curve against media blank. The results were shown in Table 37a, 37b and represented in Fig. 49.

Table 37a: Spectroscopic estimation of morpholine available in culture media

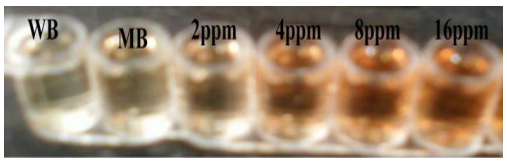
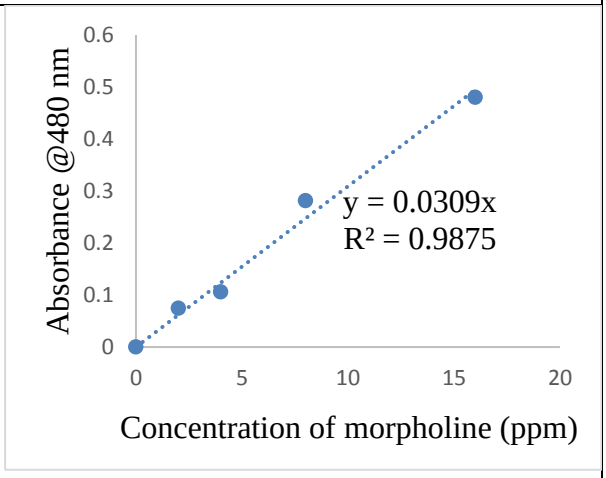
S. N.	Name	Net average OD@ 480nm
A	Water Blank	0
B	Media Blank	0
C	2 ppm	0.075
D	4 ppm	0.106
E	8 ppm	0.2815
F	16 ppm	0.480
G	0 hrs.	0.137
H	24 hrs.	0.072
I	48 hrs.	0.044
	 Where; WB: Water blank and MB: Media blank,	

Table 37b: Degradation kinetics of morpholine by immobilized isolate

Immobilized cells	Average net OD@ 480 nm	Initial concentration	Concentration after degradation	% degradation	% Error
1/400 diluted (0 hr.)	0.137	2000ppm	4.43X400 =1773 ≅ 1800	Almost no degradation	10%
1/400 diluted (24 hr.)	0.072		2.33X400 = 932	48%	
1/400 diluted (44 hr.)	0.044		1.32X400 =569.5 ≅ 570	69%	

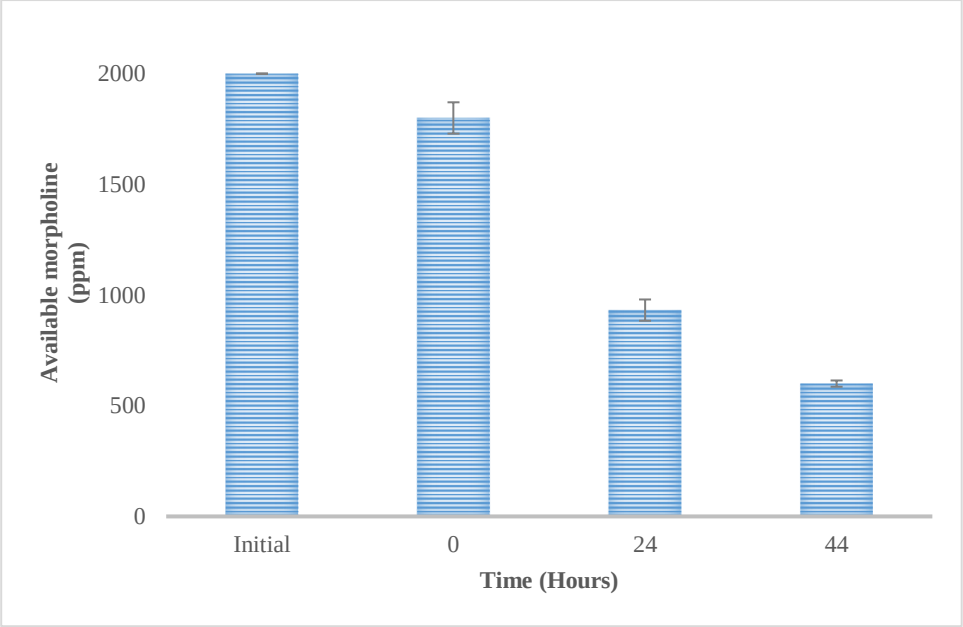


Fig. 49: Spectroscopic degradation studies by immobilized isolate

Table 38: Morpholine degradation by free and immobilized cells

Morpholine concentration (ppm)	Time for acclimatization	% degradation	
		Free cells	Immobilized cells
2000	2 weeks	100%	⇄ 70 %

Immobilized acclimatized cells of average bead size of 3 mm showed almost 70% of degradation of 0.20% of initial morpholine concentration at room temperature within 48 hours. However, free cells take 2-3 weeks' time to acclimatize itself for complete degradation of morpholine (Table 38). Within 48 hours, the immobilized beads itself degrade which might be due to fact that the isolate either itself produce alginase or other possibility is that the available amine in culture media itself reacts with alginate and undergoes reductive amination. Dalheim, M. *Ø et al.*, 2016 found that alginate undergoes reductive amination by coupling of amine to form Schiff base which further subsequently reduced. Due to this limitation, we are looking for other suitable hydrophillic polymeric matrix to evaluate the degradation studies for industrial purpose, which is future scope of the present study.

4.8. Discussion

Rapid and wide spread environmental, economic and social changes have led to urgent global questions of biological solutions of manmade chemical pollution. Many industrial processes generating wastewater containing toxic compounds are characterized by their variability. Because of the high variations in flow and concentration of contaminants in industrial wastewater, usual preliminary, primary, secondary and tertiary treatment processes have been carried out to obtain satisfactory removal efficiencies. Besides, due to contaminant toxicity, secondary treatment (biological treatment) of industrial wastes is difficult. This can be overcome by using the acclimatization approaches to adapt the native microbes in that environment conditions. The first step to biodegrade toxic substances in a wastewater treatment plant is the acclimation of the microorganisms. In a chemical stress environment condition, when microorganisms are put in contact with toxic compounds, acclimation to these compounds may occur (Aelion *et al.,* 1989). Wiggings et al. 1987 described different mechanisms to explain the acclimation phase and suggested that there is a selection and multiplication of specialized microorganisms during this phase and physiological transformations occur in the metabolic system of the microorganisms by alterations of enzymatic level, its regulation, production, and finally mutations, etc. Further also stated that in aerobic microbial communities, the acclimation periods range from several hours to several days. After wards, this strategy was widely used to treat different industrial effluent containing toxic contaminates or heavy metals. Morpholine is among these 1500 chemicals which are released each day into environment and commonly found in the industrial effluents of textile, rubber, plate, pharmaceutical, personal care, fungicide industry.

Morpholine is a versatile xenobiotic compound with a variety of applications and uses in different industrial setup. Earlier literature supports the un-degradability nature of morpholine due to its chemical nature (presence of ether group C=O) but later Many authors found that majority of morpholine degraders are mainly *Mycobacteria* sp. (Cech *et al.,* 1988, Brown *et al.,* 1990) which has its own limitation like slow growth rate and pathogenicity. Therefore, it becomes important to explore other microbes from diverse background for their biodegradation capability. So, this study was undertaken to screen natural microbes from different geographical area and their

acclimatization to degrade morpholine through the route of biodegradation. Many microbes were isolated and uses morpholine as sole source of carbon and energy to degrade the morpholine.

Moreno-Andrade, I. and G. Buitrón 2004 analysed that the acclimation was performed using two strategies, the first one fixing the reaction time, independent of the degradation removal efficiency (fixed time) and the second one fixing a removal efficiency/complete removal (variable time), independent of the reaction time. The second strategy (variable time) results a microbial community with higher specific activity compared with that obtained for the fixed time strategy. This second strategy was implemented in our study for near complete removal of morpholine in left out spent media. Initially low concentration of 0.10% morpholine was used to adapt the isolates against morpholine stress and later slowly at an interval of 2-3 weeks the morpholine concentration was increased nearby ~ 0.30% to select the acclimatized isolate. This was well experimented and SK-5, BAC-1, BAC-2, RK-1, RK-2 and RK-11 was found to be potential morpholine degraders. These isolates have shown complete removal of initial morpholine concentration of 0.21%, 0.28%, 0.2%, 0.2%, 0.2% and 0.15% respectively when result was validated with gas chromatography analysis of bacterial culture supernatant with appropriate dilution of solvent in comparison with positive control and media blank.

Further study was carried out to find out the degradation pathway of morpholine as there were two hypothetical pathways (either the ethanolamine or diglycollic route of degradation) reported by Mazure and Truffaut 1994, Combourieu, B., *et al.*, 1998. Author noticed that depending on the morpholine concentration in the medium, one pathway could be used while the other was inhibited suggest that one pathway exhibits the other pathway to be succeed or not? Different analytical approach was used to find out the degradation intermediate compounds to establish the degradation pathway by isolate. It was found the RK-11 (closely related to *Halobacillus blutaparonensis*, based on 16SRNA sequence analysis) bacteria undergoes the glycolic route of degradation instead of ethanolamine route as it inhibits the bacterial growths. The illustrated degradative pathway might be starts with the first step of the cleavage of the C-N bond; this leads formation of an intermediary amino acid, which is further followed by deamination and oxidation of this amino acid to a diacid.

More ever, presence of other intermediate compounds also supports this finding with a conclusion that diglycolic route of biodegradation might be common degradation mechanism shown by other groups of bacteria too. This was well reported by other author Poupin *et al.*, 1998, Combourie *et al.*, 1998, 2000 to prove our finding. Whatever the degradation pathway was exhibited by bacteria, the end product was ammonia which would biochemically use by bacteria. Our studies confirm the presence of ammonia as end product and molar ratio of morpholine to ammonia was found to be 1:0.014. Due to low amount of ammonia produced, the pH of the media did not change throughout the experiment. However higher molar ratio of morpholine to ammonia results the inhibitory effect on the growth of bacteria because it increases the pH of media to shift it toward alkalinity. The ratio of morpholine to ammonia was found different for different set of bacteria as reported by Magda M.Aly 2011, Schrader *et al* 2000, S. Chandrasekaran and D. Lalithakumari 1998 , N.Mazure and N.Truffat 1994, Swain *et al.*, 1991 and Knapp *et al.*, 1982 in Table 12.

We end with examining how to take these potential isolate action as individuals or consortium, to address the biological treatment of waste water by immobilization techniques. There are few reports about the degradation efficacy of immobilized morpholine degraders in comparison with free cells. Mazure, N. and Truffaut 1994 reported 2.67-fold of higher morpholine degradation efficacy in comparison with free cells by *Mycobacterium aurum* M01. However, other *Mycobacterium* sp. results 2.09-fold of morpholine degradation when immobilized in 2% sodium alginate solution (Magda M. and Aly 2011). Our finding showed that with respect to complete degradation by acclimatized free RK-1 isolate, it showed only 70% degradation when immobilized it with 2% sodium alginate solution. One important finding was noticed that within 48 hours, beads itself decompose which might be due to fact that the either isolate itself produce alginase or other possibility is that the available amine in culture media itself reacts with alginate and undergoes reductive amination. Dalheim, M. Ø et al., 2016 found that alginate undergoes reductive amination by coupling of amine to form Schiff base which further subsequently reduced. Due to this limitation, we are looking for other suitable hydrophillic polymeric matrix to evaluate the degradation studies for industrial purpose, which is future scope of the present study.

CONCLUSION

The workhorses of biological treatment or bioremediation are microbes, more precisely bacteria and today they seem to provide many solutions to industrial effluents treatment. Microorganisms especially bacteria, are capable of mediating biodegradation (in a specific set of environmental and culture conditions) of a wide variety of simple to complex and recalcitrant organic compounds owing to their ability to adaptation and genetic manipulation under the prevailing environmental conditions. From the present series of experiments, it can be concluded that laboratory trials show the effective and efficient removal of morpholine since it is technically feasible, ecofriendly and sustainable solution for industry such as viscose (rayon) fiber-based ones which uses NMMO as a cellulose solvent in recent years.

The study was aimed to isolate and acclimatize indigenous microbes from different geographical location (from natural sources or industrial sites) for their capacity to degrade morpholine. Due to chemical structure of morpholine, it is not easy for microbes to attack on this xenobiotic. Hence acclimatization strategies were used to make it resistant to the presence of morpholine stress itself. The study went further in evaluating the bio-remedial efficacy of morpholine in a spent culture media. After estimating the left out morpholine in culture supernatant, it was subjected to increase the concentration of morpholine to improve the efficacy properties to make these isolates more suitable for higher morpholine degradation. Six bacterial isolates namely SK-5, BAC 1, BAC-2, RK-1, RK-2 and RK-11 that grew on morpholine were isolated from different environmental samples. One of potential isolate was identified as a *Hallobacter* sp.by 16S rRNA sequence characterization. The operational % degradation was found to be over 99% in a 2/3 weeks old adapted culture supernatant of all potential isolates with different initial concentration of morpholine. The % morpholine degradation (confirmed by die away test, and GC and MS analysis) by potential isolates follows descending order of BAC-1 > SK-5/ BAC-2/ RK-1/ RK-2 > RK-11. In this study, different approaches were used to elucidate the metabolic degradation pathway either follow the ethanolamine or diglycollic route of degradation. It was proved that certain potential isolate (RK-11, closely related to *Halobacillus blutaparonensis*, based on 16SRNA sequence analysis) underdoes morpholine degradation via glycolic route of degradation and supports the fact that in presence of morpholine, one of two branches of morpholine biodegradation pathway was induced while the

other branch was inhibited. The illustrated degradative pathway might be starts with the first step of the cleavage of the C-N bond; this leads formation of an intermediary amino acid, which is further followed by deamination and oxidation of this amino acid to a diacid. Whatever the degradation pathway is, appearance of ammonia will be the product of the degradation. The release of ammonia was correlated with morpholine degradation, confirming that morpholine is entirely mineralized by isolates and used as sole source of carbon, nitrogen and energy, however the molar ratio of morpholine to NH_4^+ produced was 1/0.014 indicating that ammonia produced was assimilated by these isolates. This ratio was found to be different for different set of bacteria as reported by many authors. Further, effect of immobilization on degradation studies of morpholine was carried out and result showed that with respect to complete degradation of morpholine by acclimatized free RK-1 isolate, immobilized cells showed only 70% degradation when immobilized with 2% sodium alginate solution. One important finding was noticed that within 48 hours, beads itself decompose which might be due to fact that the either isolate itself produce alginase or other possibility is that the available amine in culture media itself reacts with alginate and undergoes reductive amination. Due to this limitation, we are looking for other suitable hydrophillic polymeric matrix to evaluate the degradation studies for industrial purpose, which is future scope of the present study.

Thus, this biological approach uses the utilization of nature's own technology for pollutants abatement through efficient and cost-effective methods. In conclusion, biological treatment is one of the largest and most important controlled applications of microorganisms that can be easily deployed for contaminants removal in waste water.

LIMITATIONS AND FUTURE SCOPE OF THE STUDY

1. In this study, isolated microbes utilized morpholine (up to 0.28%) as a source of carbon, nitrogen and energy to remove morpholine from waste water. However, at higher concentration of morpholine, there was no growth of bacteria even after longer time of incubation indicating that morpholine is bactericidal in nature. Therefore, this study could have achieved complete removal of morpholine and its derivatives from industrial effluents when the pollutant concentration is adjusted to around 0.28% in the effluent. This study can be extended with use of genetically modified microbes tailored to sustain at higher morpholine concentration in the effluents and get a better sustainable environmental solution for this xenobiotic.
2. The study is purely in vitro, using simulated waste water, though an in vivo study (using actual effluents), it would have given more information of the capability of the microbes in presence of other chemicals or waste materials along with morpholine and its derivatives in the industrial effluents samples. Thus, the study can be extended to evaluate the response of indigenous microbes towards co-existence of other chemicals in biological treatment of industrial effluents. Further reduce the effect of other chemicals on microbes in waste water where effluent discharges.
3. The potent acclimatized strains obtained in this study may be further harnessed with different immobilization matrix for in situ treatment of industrial effluents. Other immobilization techniques can further be studied to check the functional efficacy of the microbes for degradation of more xenobiotics.
4. Microbial strain or consortia in a serial culture may be used to increase the biodegradation of morpholine or its derivatives. Some microbial strain was tried on lyocell plant effluents and showed reduction in the carbon oxygen demand-COD (data not shown). Hence, this study should be extended further to evaluate other potential microbes to understand the underlying mechanism for degradation of morpholine and explore the degradation pathway of morpholine too.
5. Extend this present approach to other industrial discharges. However, microbial extracellular enzymatic based treatment of morpholine or other pollutants should be applied in future as a new domain of green solution of waste water treatment.

SPECIFIC CONTRIBUTIONS

1. Many authors have reported that morpholine degraders are mainly *Mycobacteria* sp. isolated from activated sludge, soils and water in most cases. However, using *Mycobacterium* for this purpose has its own limitation like slow growth rate and pathogenicity. Therefore, it becomes important to explore other microbes from diverse background from different geological sites for their capacity to degrade morpholine.
2. We here report a strategy for acclimatizing several fast-growing gram negative and positive bacteria for near complete removal of morpholine in culture supernatant. In this study, a cohort of bacterial strains has been used for in vitro degradation of morpholine. The cultures were adapted for 2-3 weeks' time in a step-wise manner in increasing concentration of morpholine.
3. Over twenty-two different screened bacteria, nearly 27% (6 isolates) of the exposed bacterial cohort was successfully able to use morpholine as the sole source of carbon and nitrogen and survive in presence of 0.1 to 0.28%. morpholine concentrations. Out of 6 isolates, all isolates i.e. 100% (n=6) showed successful growth till 0.15% of morpholine; whereas 83% (n=5) stopped growing at 0.21% of morpholine and only 17% (n=1) of the isolates could metabolize more than 0.21% morpholine.
4. The available morpholine concentration in the spent culture medium was estimated using spectrophotometer and gas chromatography. However, presence of intermediate or products of degradation in culture supernatant was chemically tested and analyzed with mass spectroscopy to establish the degradation pathway.
5. Microbes that are acclimatized to morpholine were also effective in reducing COD of the lyocell plant effluent having presence of morpholine derivatives named N-Methyl Morpholine N-oxide (NMMO). This would make the NMMO based regenerated cellulose fiber process more ecological than CS_2 (Carbon disulphide) and H_2SO_4 (Sulphuric acid) based process.
6. Immobilization of microbes with sodium alginate was found to be suitable for use in industrial effluents treatment, albeit with a few restrictions such as stability of the alginate beads.

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Annexure 1

Preparation of culture and selective media

- Luria Bertani Broth, Miller (LB): It was prepared by taking 25 gm in 1000 ml of water and sterilize by autoclaving at 15 psi pressure at 121ºC for 15 minutes. Cool the flasks before inoculation.
- Luria Bertani Agar, Miller: It was prepared by taking 40 gm in 1000 ml of water and sterilize by autoclaving at 15 psi pressure at 121ºC for 15 minutes. Mix well and pour into sterile Petri plates.
- MacConkey Broth W/Neutral red: It was prepared by taking 40 gm in 1000 ml of water and sterilize by autoclaving at 15 lbs/psig pressure at 121ºC for 15 minutes. Cool the tubes before inoculation.
- Sabouraud Dextrose Broth: It was prepared by taking 30 gm in 1000 ml of water and sterilize by autoclaving at 15 lbs/psig pressure at 121ºC for 15 minutes. Cool the tubes before inoculation.
- Sabouraud Dextrose Agar: It was prepared by taking 65 gm in 1000 ml of water and sterilize by autoclaving at 15 lbs/psig pressure at 121ºC for 15 minutes. Cool the tubes before inoculation.

Annexure 2

Preparation of enrichment media

I. **Mineral salt solution (MSS) broth:** It was prepared by measuring following

Components	Quantity/100ml
KH_2PO_4	100 mg
K_2HPO_4	100 mg
$(NH_4)_2SO_4$	100 mg
$MgSO_4.7H_2O$	4 mg
$FeCl_3$	0.2 mg

It was autoclaved at 15 lbs/psig pressure at 121ºC for 15 minutes. Cool the tubes before inoculation were seeded in this enrichment media for adaptation studies.

II. **Mineral salt solution (MSS) broth supplement with morpholine:** 100 ml of MSS broth was prepared and autoclaved. After cooling the flask, % v/v solution of Morpholine was prepared in MSS broth.

III. **Mineral salt solution (MSS) agar:** It was prepared as follow before autoclaved at 15 lbs/psig pressure at 121ºC for 15 minutes.

Components	**Quantity/100ml**
KH_2PO_4	100 mg
K_2HPO_4	100 mg
$(NH_4)_2SO_4$	100 mg
$MgSO_4.7H_2O$	4 mg
$FeCl_3$	0.2 mg
Agar	2 gm

IV. **Mineral salt solution (MSS) agar supplement with morpholine:** 100 ml of MSS Agar was prepared and autoclaved. After mild cooling of MSS Agar flask, respective % v/v solution of Morpholine was poured and mixed in this media. It was further poured in Petri plates to make MSS Agar morpholine plate.

Annexure 3 (1)

Biochemical characterization

[A] Gram staining

Primary stain: Crystal violet

Mordant: Gram's iodine

Decolourizing agent: Ethyl alcohol 95%

Counter Stain: Safranin

Procedure:

- Clean the glass slide with washing alcohol of 70% by holding them at the edge.
- By the means of sterile wire loop (redness and air cool) transfer a loopful of bacterial suspension to the center of the slide inside the laminar air flow.
- Spread it evenly to form a thin smear
- Fix the smear with heat (quickly passing it 2-3 times through a flame)
- Flood the smear with crystal violet for 2 minutes
- Rinse with tap water and dry off
- Flood the smear with gram iodine and leave it for 1 minute
- Gently rinse off iodine with tap water
- Add decolorizer drop by drop on the slide until all free color has been removed and leave it for 30 seconds
- Rinse the slide with tap water
- Counter strain with safranin and wait for 30 seconds
- Gently rinse off excess safranin with tap water. Drain slide and allow it to air dry
- Examine the stained slide under microscope to determine gram positive and gram-negative organism

Gram Positive	Gram Negative
Retain blue/purple color Cocci: purple Rods: blue	Decolorized and counterstained pink Cocci: pink Rods: pink

[B] Acid-fast staining

Primary stain: Carbol fuschsin, Decolourizing agent: Acid alchohol (3% HCl + 9.5% Ethanol)

Counter stain: Methylene Blue

Procedure:

- 3 glass slides were cleaned and prepared
- A thin uniform smear of each organism was prepared
- Smears were air dried and subsequently heat fixed
- Carbol fushsin was added onto the slides (slides were placed on warm hot plate; stain was replenished as it evaporated; stain was not allowed to boil was allowed to steam for 5 min)
- Slides were cooled and washed with cool water
- Decolourized with acid-alcohol adding the reagent drop by drop until carbol fuchsin failed to wash from smear
- Rinsed with tap water
- Counter stained with methylene blue for 2 min
- Smear washed with tap water and blotted dry with filter paper
- Examine the stained slide under microscope 100X to determine acid-fast positive or negative organism

[C] Growth on selective media (HI-CHROME M1353 Media)

Ingredient and colony color

Ingredient	Concentration (g/l)	Bacterial *sp.*	Colony color
Peptone special Chromogenic mixture Agar	15.00 2.45 15.00	*Enterococcus faecalis* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Kelbsiella pneumonia* *Escherichia coli*	Blue Colorless Golden yellow Blue-purple, slimy Pink-red
Final pH (at 25 ° C)	6.8 ± 0.2		

Procedure:

- Pure colonies of isolates were streak on HI-CHROME plate
- It was incubated at 37ºC for 24-48 hrs.
- Examine the colony color to identify the microorganism species.

[D] Methyl red test

Procedure:

- Approximately 1/3 of the culture from each tube was set aside in separate labelled tubes for the Voges-Proskauer test.

- Five drops of methyl red indicator were added to the remaining aliquot in each tube and the tube was shaken to mix well.
- The color of the cultures was examined.

. **[E] Voges-proskauer test**

Procedure:

- 10 drops of Barrit's reagent A were added to each of the aliquots of cultures transferred earlier into fresh tubes, and the tubes were shaken well to mix the contents. 10 drops of Barrit's reagent B were immediately added to each of the tubes and the tubes were shaken well. The cultures were mixed every 3 minutes by shaking.
- The colour of the cultures was examined 15 minutes after addition of Barrit's reagent.

. **[F] Indole production test**

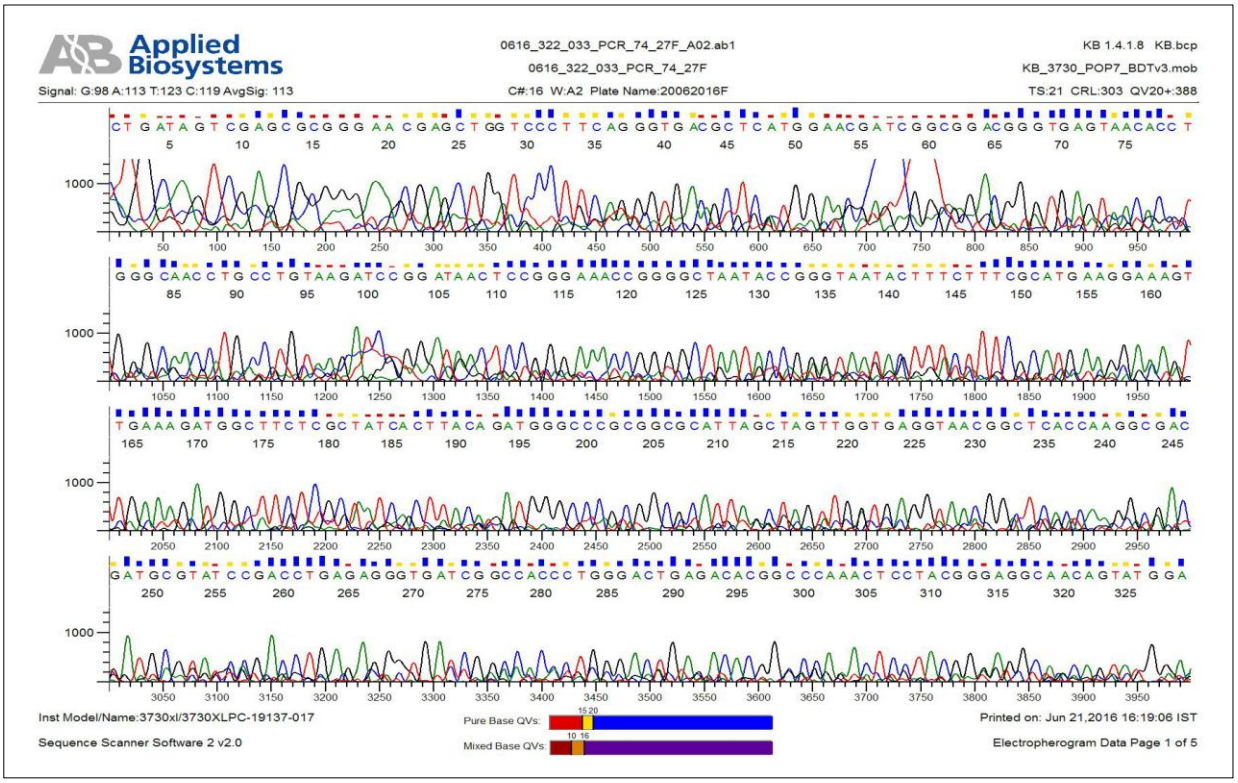
Procedure:

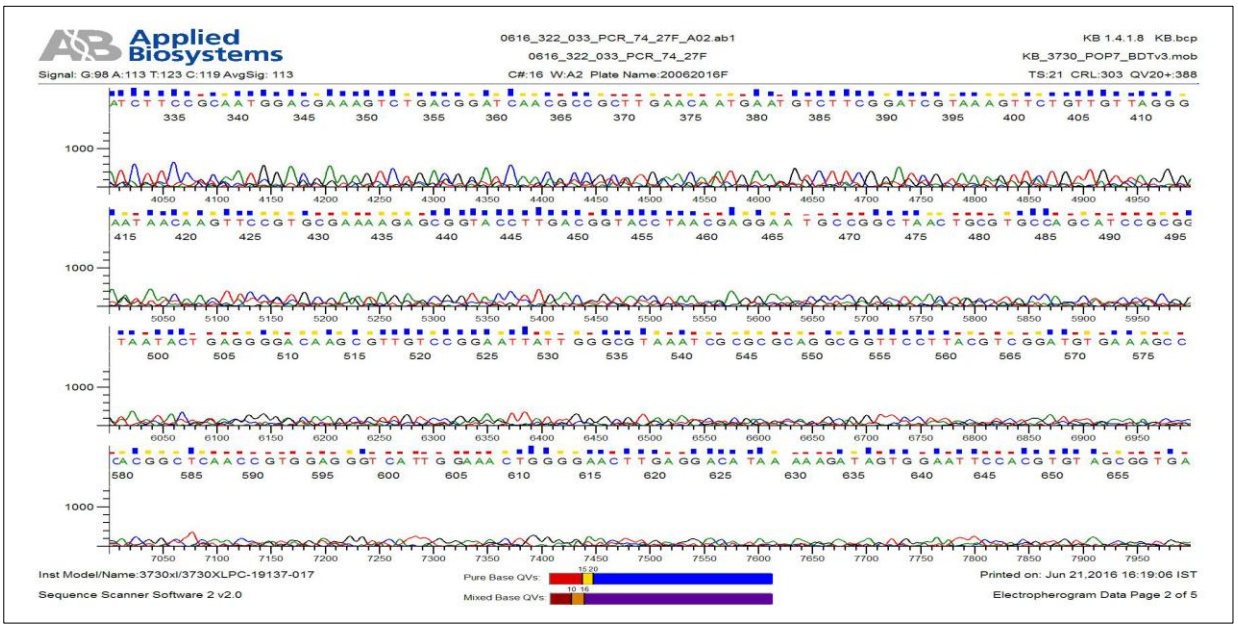
- 10 drops of Kovac's Reagent were added to all deep tube cultures and the cultures were gently agitated.
- The color of the reagent layer in each culture was examined. (Pink layer on top- positive/ no pink layer negative)

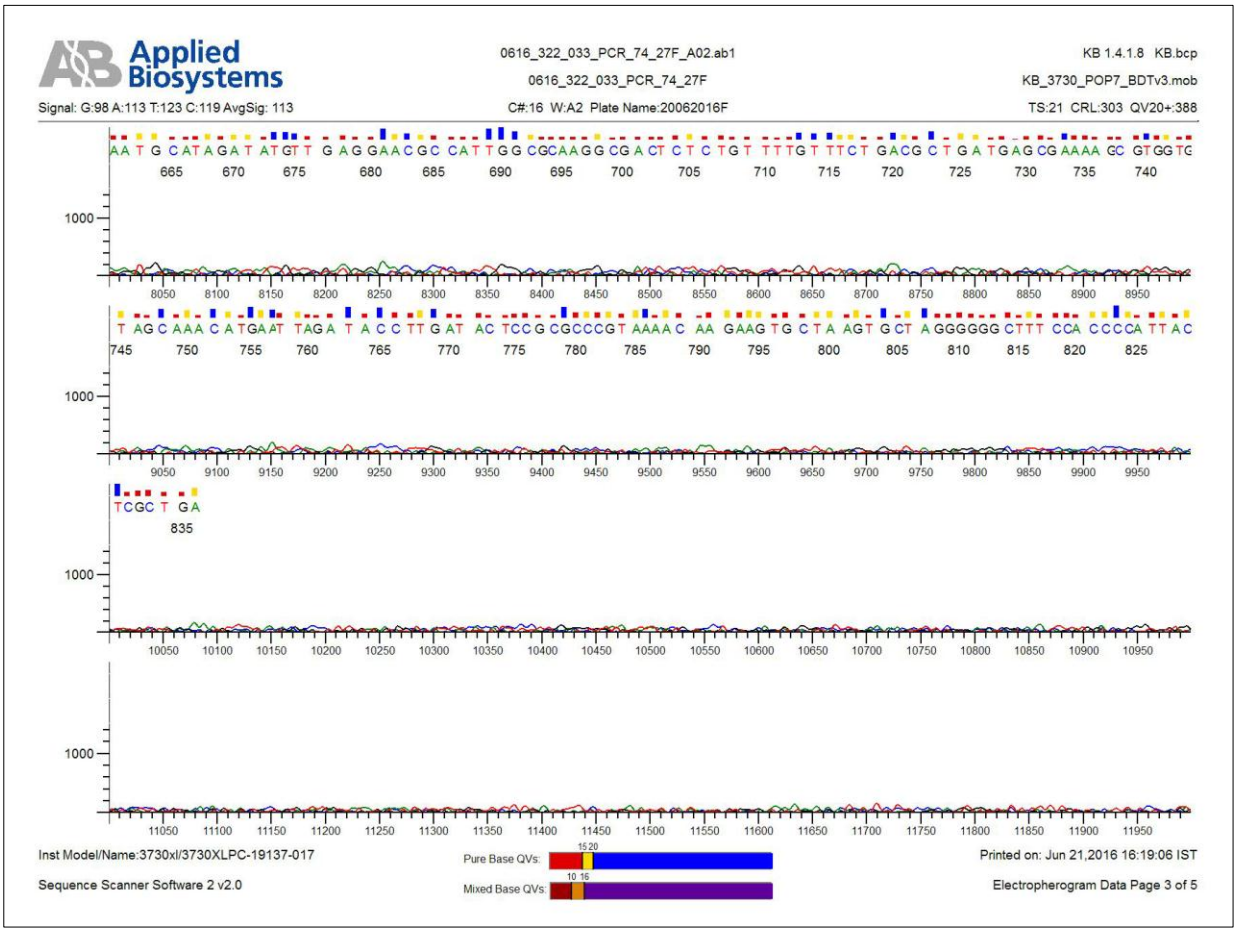
Annexure 3 (2)

Molecular characterization

[a] 16s rRNA sequence of 0616_322_033_PCR_74_27F_A02



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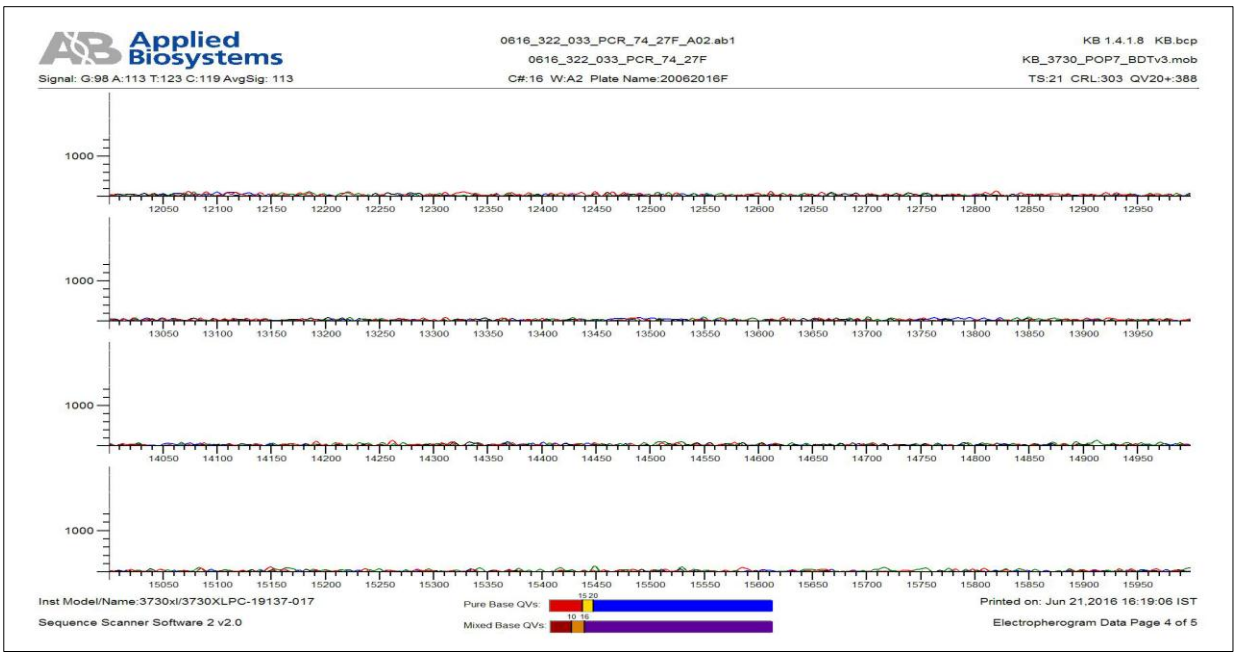
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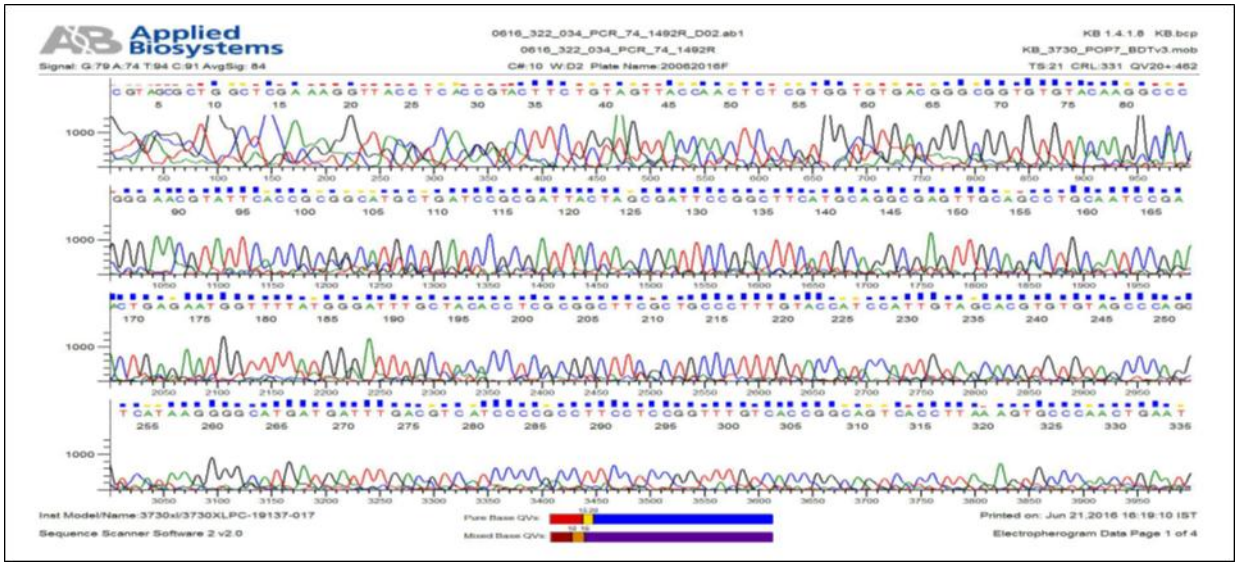
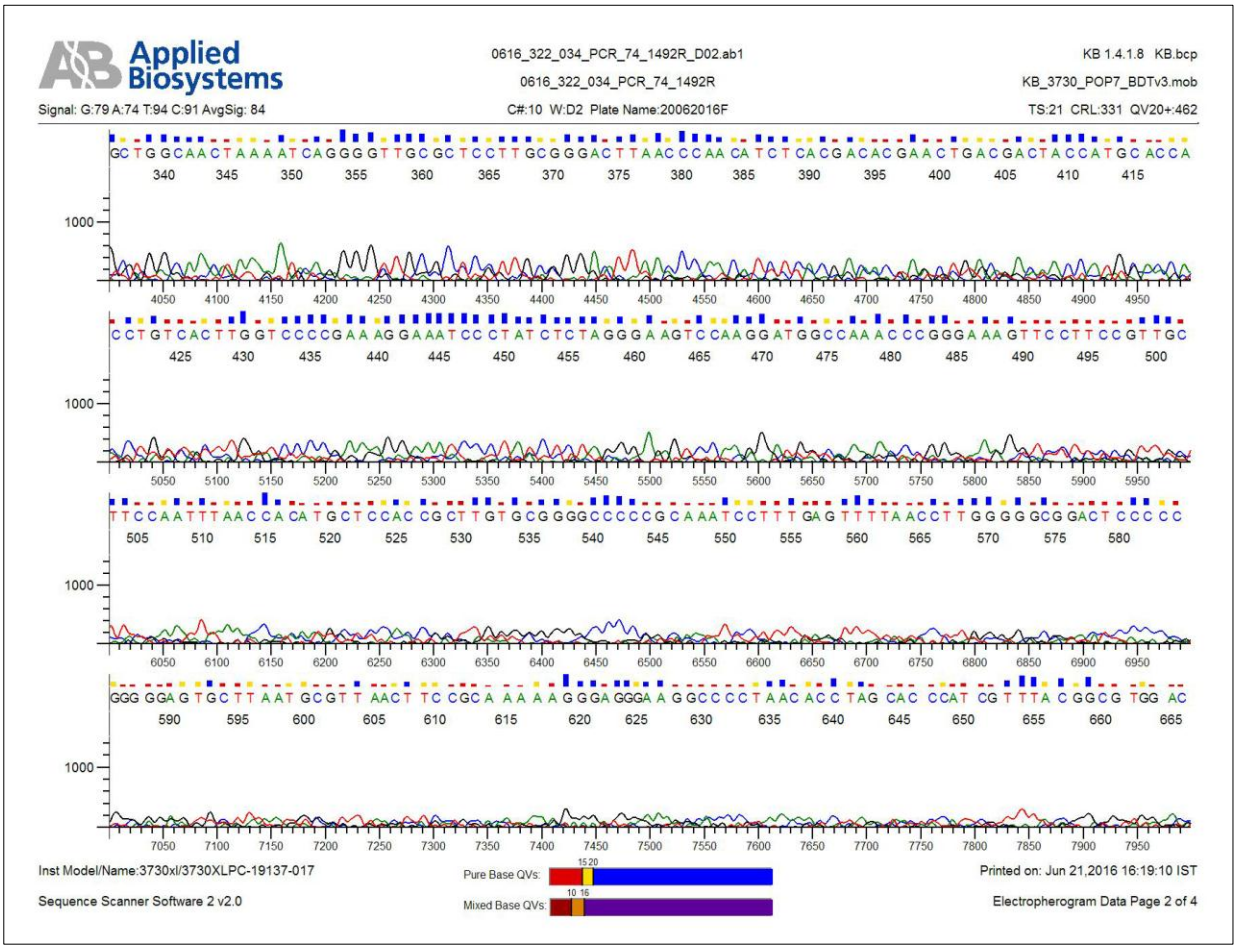
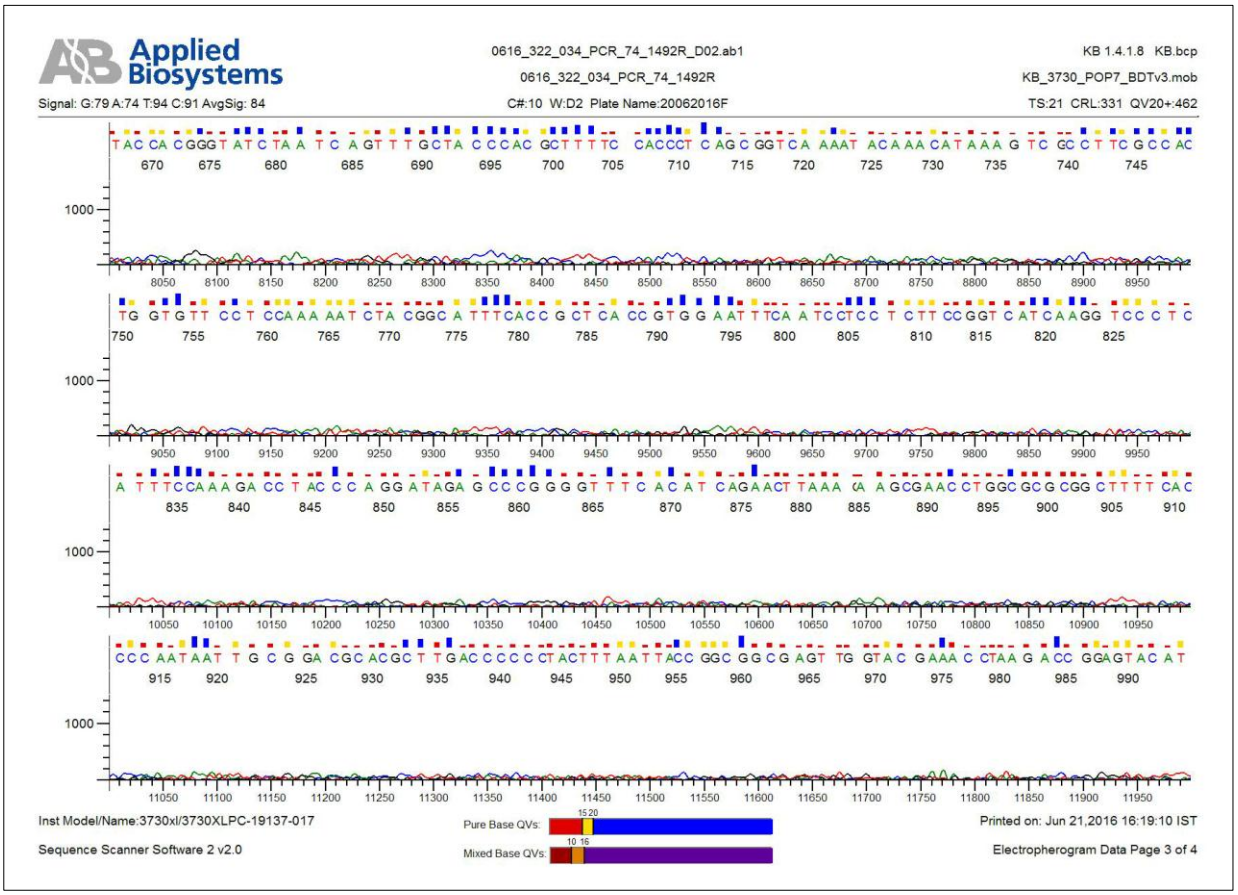
[b] 16s rRNA sequence of 0616_322_034_PCR_74_1492 R_D02



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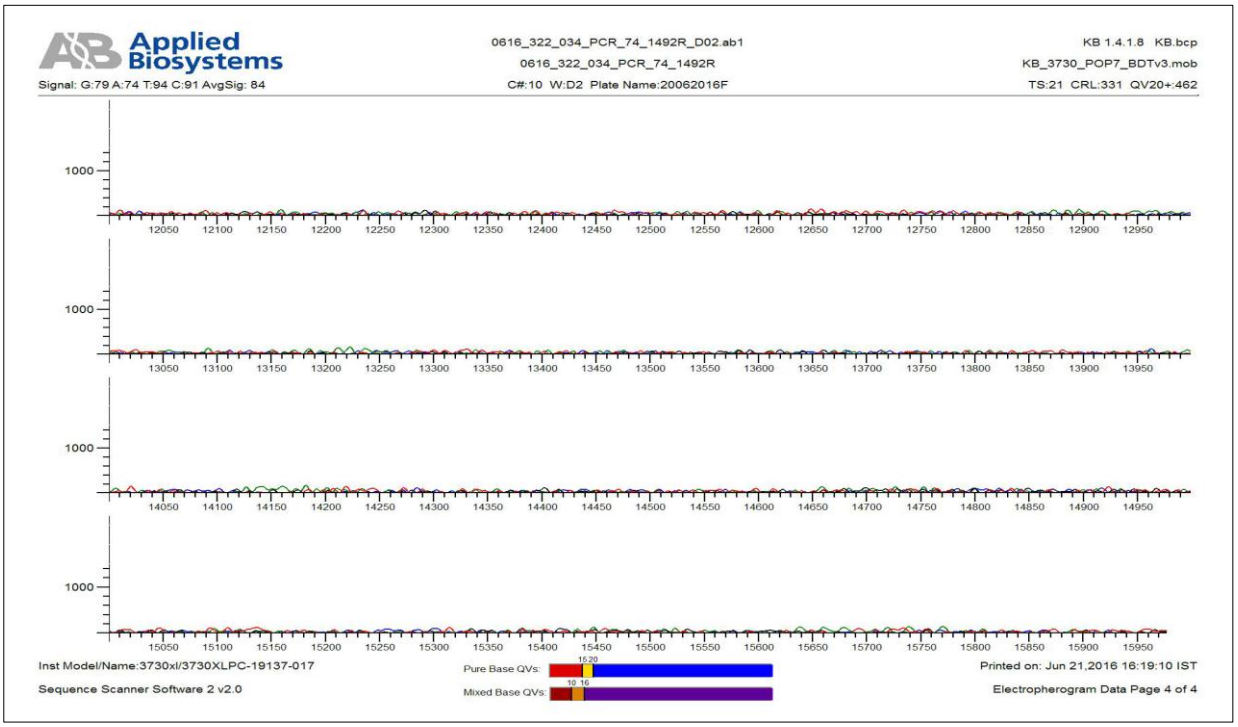
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Electropherogram Data Page 4 of 4

Contig Summary

Contig

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ACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTT
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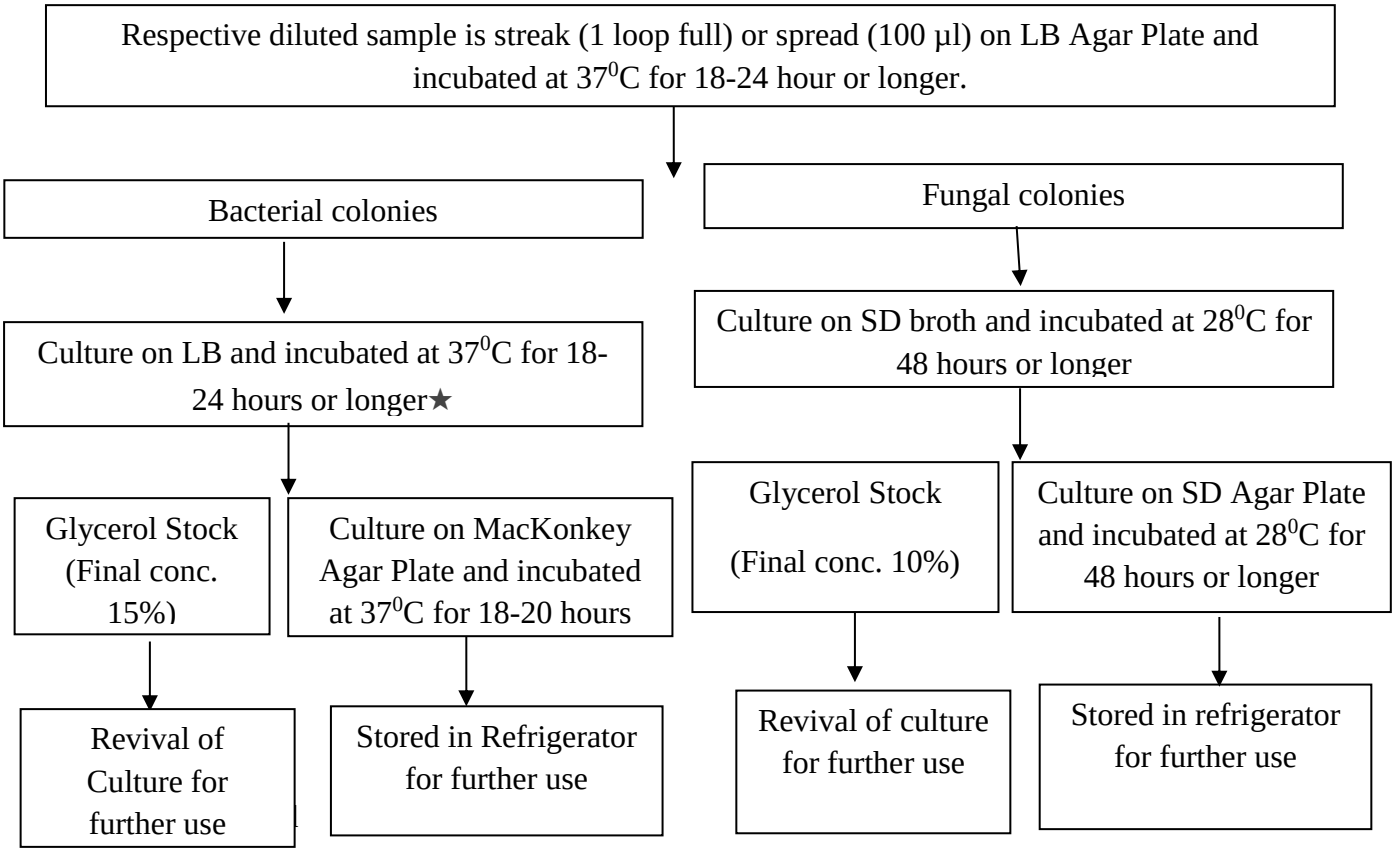
Annexure 4

Isolation and culture of microbes

Methodology



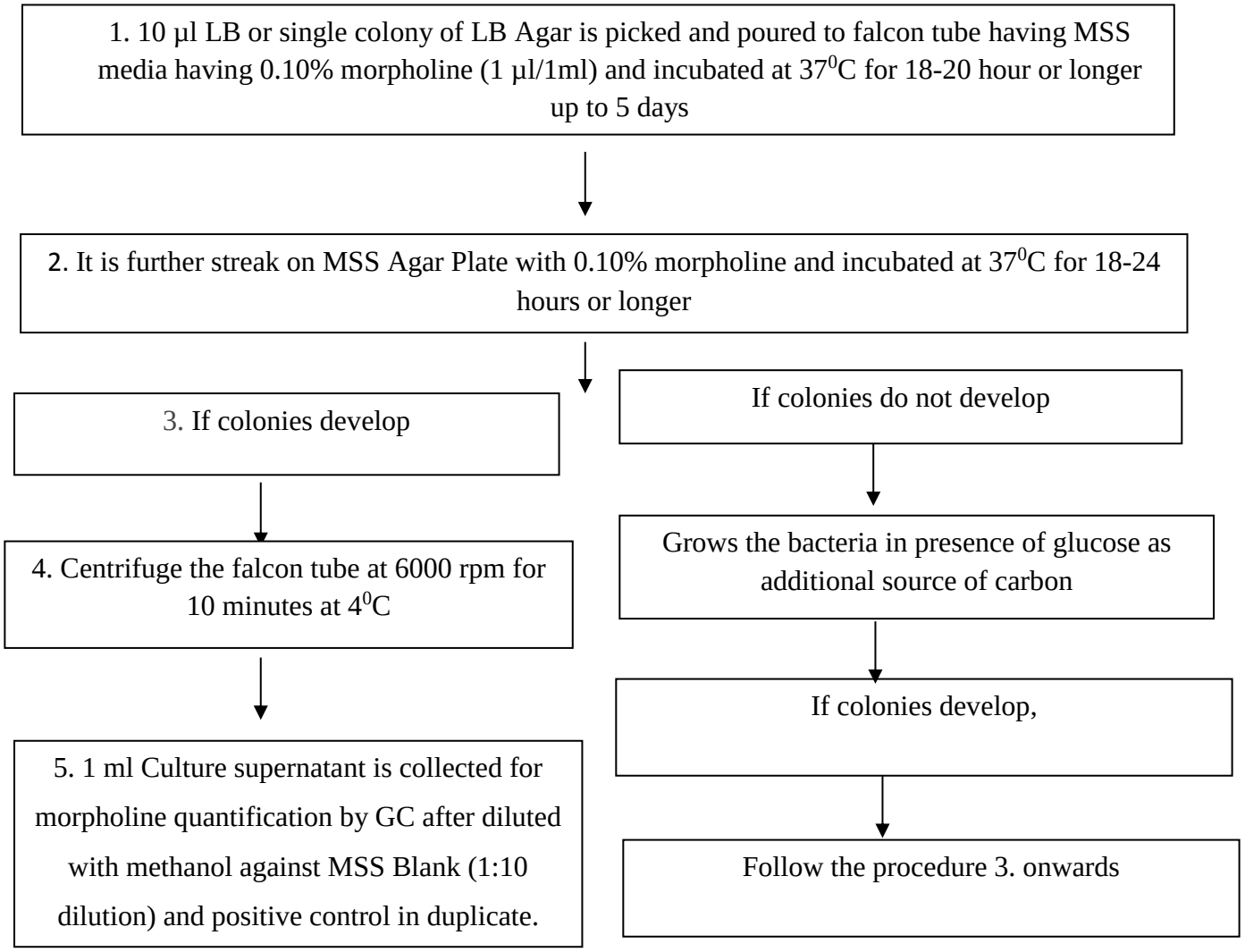
★: It is processed for morpholine degradation by acclimatized the isolates in presence of increases concentration of morpholine.

Annexure 5

Culture of morpholine degrading microorganisms and measurement of morpholine

Methodology:

Mineral Salt Solution (MSS) media is used as enrichment media for adaptation studies of morpholine degrading microorganism in presence of 0.5% morpholine. Cultures are incubated at 37^0 C, 18-24 hrs. for bacterial cultures and 28^oC, 48 hrs. for fungal cultures.

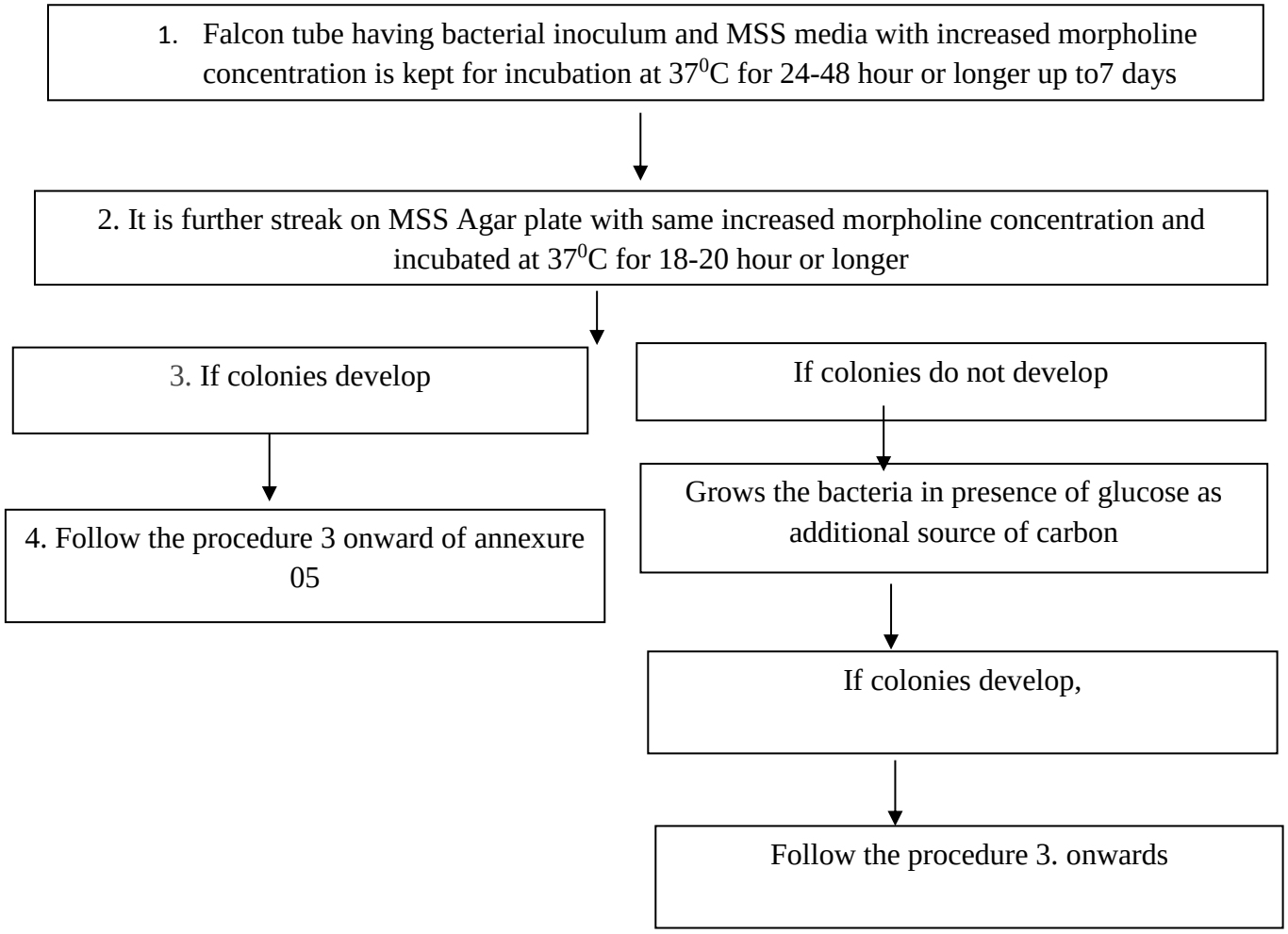


Annexure 6

Acclimatization of bacteria in MSS with increasing concentration of morpholine

Methodology:

Remaining culture supernatant is discarded after centrifugation and fresh MSS media with increased morpholine concentration (0.15% - 0.3% with an interval of 0.1%) is added in same falcon tube with same bacterial inoculum.



Annexure 7

Glycerol stock preparation

Glycerol is used as a cryoprotectant which allows for vitrification to occur, which is basically the lowering of the freezing temperature of the solution as well as the increasing of its viscosity. This process prevents crystallization (i.e. the ice crystals which are harmful to the cells). Glycerol is cheap, non-toxic to the cells having polyol that provides some nutrient value to the cells.

- Stock 60%V/V: 3 ml of neat glycerol mixed in 2ml of distilled water so that final volume should be 5 ml. Final glycerol stocks should be 15% for bacterial and 10% for fungal culture.
- Take LB bacterium inoculum for bacteria to make the final volume.
- Add 0.975 ml of LB with bacteria +0.325 ml of 60% glycerol stock= it gives 15% final glycerol stock.

LIST OF JOURNAL PUBLICATIONS

- Yeara Bar Oz, Hadas Mamane, Ofir Menashe, Vered Cohen Yaniv, **Rupak Kumar**, Lilach Iasur Kruh, Eyal Kurzbaum (2018) Treatment of Olive mill waste water using ozonation followed by an encapsulated acclimated biomass, Chemical Engineering Journal **(Under Review)**
- **Rupak Kumar,** Yasmin Raizner, Lilach Iasur Kruh, Ofir Menashe, Hassan Azaizeh, Suman Kapur, Eyal Kurzbaum (2018) Extracellular laccase production and phenolics degradation by an olive mill wastewater isolate, Grasas y Aceites, 69 (1), January–March 2018, e231, ISSN-L: 0017-3495 doi: http://dx.doi.org/10.3989/gya.0776171
- **Rupak Kumar** and Suman Kapur (2016) Miniaturization of a spectrophotometric assay for automated monitoring of industrial pollutant "Morpholine", International Journal of Biology, Pharmacy and Allied Sciences 5(12) 2016, ISSN 2277-4998
- **Rupak Kumar**, Vulichi Srinivasa R and Kapur Suman (2016) Emphasizing Morpholine and its derivatives (MAID): Typical candidates of pharmaceutical importance, Int. J. Chem. Sci.: 14(3), 2016, 1777-1788, ISSN 0972-768X
- **Rupak Kumar** and Suman Kapur (2016). Morpholine: A Glazing Agents for Fruits and Vegetables Coating/Waxing Int. J. Sci. Tech. Eng.,2(11): 694-697 ISSN (Online):2349-784X
- **Rupak Kumar** (2016) Health effect of morpholine based coating for fruits and vegetables. Int. J Med Res. Health Sci.,5(9):32-38, ISSN: 2319-5886 (Indexed in ESCI - Thomson Reuters)
- **Rupak Kumar** and Suman Kapur (2016). Cytochrome P450 Biocatalysts: A Route to Bioremediation, Int. J. Pharm. Res. Allied Sci., 5(3):113-123, ISSN: 2277-3657

BOOK CHAPTER (FULL MANUSCRIPT)

- **Rupak Kumar,** Punita Manga, Shivani Gupta, Suman Kapur. (2014). Biological approaches for treating industrial effluents containing Morpholine. Industrial and Environmental Biotechnology, edited by Krishna Pramanik and Jayant Kumar Patra. Studium Press India Pvt Ltd, New Delhi, PP-255-64, ISBN: 9789380012674

NATIONAL AND INTERNATIONAL CONFERENCE PROCEEDING (FULL PAPER)

- **Rupak Kumar**, Abhishek Dave, Suman Kapur (2016) A Sustainable Environmental Technology: Biological Treatment of Morpholine and Its Derivatives (MAID) in Industrial Effluents, National conference on large scale multidisciplinary systems of national significance-trends and challenges at Satish Dhawan Space Centre SHAR, Sriharikota, Andhra Pradesh, India, 24-25th June 2016
- **Rupak Kumar** (2015) Bacterial Biodegradation of Environmental Pollutant "Morpholine"-A Review, UGC Sponsored National Seminar on Socio-Economic Factors Influencing- Environmental Pollution, Biodiversity & Sustainable Development at Government Degree College, Nagari, Andhra Pradesh, India, 25-26th July 2015, (PP 263-71), under International E – Publication, International Science Congress Association, Indore, India. ISBN: 978-93-84659-06-6
- Manjyot Kaur, **Rupak Kumar**, DV Padmavathi, Shivani Gupta, Anuradha Pal and Suman Kapur (2015). Bacterial Strains for Morpholine Degradation: An adaptation and screening strategy, International Conference on Advances in Agricultural, Biological & Environmental Sciences (AABES-2015) under International Institute of Chemical, Biological and Environmental Engineering, London (UK), 22-23, July 2015, (PP 117-19), edited by Prof. Dr. Md. Aminur Rahman and Prof. Dr. Rahim Ahmadi. ISBN 978-93-84422-29-5, http://dx.doi.org/10.15242/IICBE.C0715
- **Rupak Kumar** and Suman Kapur (2014). The Biodegradation of Environmental pollutant – "Morpholine", National Conference on "Biodiversity, Environment Hazards – Therapeutic approaches and Drug Design" at Government Degree College for Women, Srikalahasthi, Andhra Pradesh, 26 -27th, July 2014, (PP 49-60) under International E – Publication, International Science Congress Association, Indore, India. ISBN:978-93-84648-32-9
- **Rupak Kumar** and Suman Kapur (2013) Quantitative Estimation of Morpholine by Gas Chromatography, National Conference on Innovations in Chemical Engineering, BITS Pilani, Hyderabad Campus, 15-16 Nov 2013, (PP 133-36) edited by D. Srikanta, V. Karthik C. and S. Vikranth K., BS Publication, Hyderabad, India.ISBN:978-81-7800-329-0

POSTER PRESENTATION (NATIONAL AND INTERNATIONAL)

- **Rupak Kumar**, Suman Kapur, Eyal Kurzbaum (2017). Will an Apple a Day Keep the Doctor Away: Effective Removal of Morpholine in ISM Annual Meeting – NGMS: Next Generation Microbiologists Scientists, Agricultural Research Organization, Volcani Center, Rishon LeZion, Israel on April 6, 2017
- **Rupak Kumar** (2016). Chemical Pollutant- morpholine: Contribution to Environment and Health presented in Young Researcher's South Asian Symposium on Sustainable Development Goals (SDGs)-Sustainable Production and Consumption under United Nations Sustainable Development Solution Network South Asia Region (UN-SDSN) and United Nations Environmental Programme (UNEP) at TERI University, New Delhi on 06-08 October 2016
- **Rupak Kumar**, Abhishek Dave, Shailendra K. Singh and Suman Kapur (2016). Biodegradation of NMMO-containing Industrial Effluents using Novel Bacterial Isolate in National Seminar on Science and Technology for Indigenous Development in India under Indian Science Congress-Hyderabad Chapter in association with RBVRR Women's College, Hyderabad on 22-24 February 2016 **(Best Poster-Second Prize)**
- Shailendra K. Singh, V.V.S.Gargeya, **Rupak Kumar** and Suman Kapur (2016). Biochemical Conversion, Life Cycle Assessment and Challenges of Algal Bioethanol for Sustainable Development in National Seminar on Science and Technology for Indigenous Development in India under Indian Science Congress-Hyderabad Chapter in association with RBVRR Women's College, Hyderabad on 22-24 February 2016
- **Rupak Kumar** and Suman Kapur (2015). A Sustainable Environmental Solution: Microbial degradation and Reduction of Morpholine in Industrial effluents presented in Young Researcher's South Asian Symposium on Sustainable Development under United Nations Sustainable Development Solution Network (UN-SDSN) and Delhi Sustainable Development Summit 2015 at TERI University, New Delhi on 05-07 February 2015 **(Best Poster Award).**
- **Rupak Kumar**, Shivani Gupta, Punita Manga and Suman Kapur (2013). Bioremediation of Morpholine containing Industrial waste water presented in 6th Science conclave at Indian Institute of Information Technology, Allahabad on 08-14 Dec 2013

LISTS OF AWARDS

- **Israel Govt. Fellowship, 2016-17** under Ministry of Human Resource Development, Govt. of India in the discipline of Environmental Studies
- **Indian Council of Medical Research (ICMR), Senior Research Fellowship** in the year 2011-13
- **Times of India, newspaper article** on "Toxic gloss on 'fresh' veggie plate" Hyderabad edition dated Dec 16, 2016.
- **Best Poster- Second Prize** in National Seminar on Science and Technology for Indigenous Development in India under Indian Science Congress-Hyderabad Chapter, 2016.
- **First Prize awarded** for the competition on concept proposal "How can we make our own environment better?" on the 153rd birth anniversary of Swami Vivekananda at BITS Pilani, Hyderabad Campus, 2015
- **Best Presentation Award** in Young Researcher's South Asian Symposium on Sustainable Development under United Nations Sustainable Development Solution Network (UN-SDSN) and Delhi Sustainable Development Summit (DSDS) New Delhi, 2015
- **Young Scientist Award** in National Conference on New Frontier in Biotechnology-Prospects and Challenges at UCW, Osmania University, Hyderabad, 2015
- **Best Oral Presentation Award** in National Conference on Emerging Challenges and Innovative Strategies in Freshwater Ecology, Biodiversity and Applied Toxicology at Nizam College, Osmania University, Hyderabad, 2014
- **Best Oral Presentation Award** in National Conference on Biodiversity, Environment Hazards – Therapeutic approaches and Drug Design at GDCW, Srikalahasthi, 2014.
- **Dope Sample Processing and Analysis** in **1st Summer Youth Olympics Game, Singapore (2010)** and **XIX Commonwealth Games, Delhi** (2010) under National Dope Testing Laboratory, Ministry of Youth affairs and Sports, Govt. of India.
- **Qualified the Graduate Aptitude Test in Engineering (GATE)** in the year of 2009 and 2011

INVITED RESOURCE LECTURE

- **Rupak Kumar** and Suman Kapur (2017) Waste to value: A solid and liquid waste management with reference to morpholine and its derivatives in National Seminar on Perspectives on Solid Waste Management at Krishna University, Machilipatnam, Andhra Pradesh on 12-13th October 2017

MEMBERSHIP OF PROFESSIONAL BODIES

- Membership of **Israel Society of Microbiology** (Registration Number: 562368), Tel Aviv, Israel
- Associate member (Membership No: LM02368) of **Indian Society of Systems for Science and Engineering**, Hyderabad Chapter, India
- Life member (Application No: 2015150616424) of **Global Initiative of Academic Networks (GIAN)** under Ministry of Human Resources Department, Govt. of India.
- Life member (Membership No:627) of **Vijnana Bharati** (VIBHA- a Guinness Book World Record Holder for the successful conduct of the 'Largest Practical Science Lesson), Delhi, India
- Life Member (Membership No: L29042) of **The Indian Science Congress Association, Kolkata**, India
- Life Member (Membership No: 3699) of **The Society of Biological Chemists, Bangalore**, India
- Member of **European Federation of Biotechnology (EFB), Barcelona,** Spain

BIOGRAPHY OF THE CANDIDATE

Myself. Rupak Kumar has an umbilical cord relationship with Koshi region of Bihar and moved to different parts of India for higher education to explore the diversifying biological world. After graduating with an honor degree in Zoology at BNMU, I moved to North Orissa University to obtain Master of Biotechnology degree where I firstly expose myself towards research by doing a dissertation entitle Solid State Fermentation (SSF) of Oil-seed Cake for the production of Microbial Esterase under Dr. R.H. Balasubramanya, Head, CBPD, at Central Institute of Research on Cotton Technology, Mumbai under Indian Council of Agricultural Research (ICAR). I earned my M. Phil Life Science (Biochemistry) degree in 2008 under the mentorship of Dr. Saroj Mishra, Professor, Department of Biochemical Engineering & Biotechnology, Indian Institute of Technology (IIT), Delhi and Dr. Binata Nayak, Reader, School of Life Science, Sambalpur University on the thesis entitled Scale-up purification and characterization of β-Glucosidase from thermo tolerant yeast *Pichia etchellsii*. I am recipient of DBT, Govt. of India Studentship for pursuing Advance Diploma in Molecular Diagnostics at Bharathidasan University, Tiruchirappalli under the guidance of Dr. Rukmini Govekar, Scientific Officer -F, Advanced Centre for Treatment, Research & Education in Cancer (ACTREC), Tata Memorial Centre, Mumbai and Dr. K. Balakrishnan,, Associate Professor, School of Biological Sciences, Madurai Kamraj University, Madurai in which my project on Preliminary Investigations on the Proteomic profiles of Platelets from Patients with Chronic Myeloid Leukemia (CML) and Healthy Control. I gained industrial exposure by doing intern at Advanced Enzymes Technologies Ltd (AETL), Mumbai under DBT- Govt. of India, BCIL-BITP in 2008. During my internship, Industrial application of enzymes was explored in term of Efficacy and Potentiality Studies in different section of Industrial Application. I qualified GATE for MHRD, Govt. of India fellowship in 2009 and 2011. I have made original contributions to the Dope Sample Processing and analysis in 1st Summer Youth Olympics Games, Singapore and XIX Commonwealth Games, Delhi at National Dope Testing Laboratory under Ministry of Youth Affairs and Sports, Govt. of India in which I worked as research fellow for 1.6 years. Further after I joined the BITS Pilani Hyderabad Campus as a Doctoral Fellow in August 2011 under the mentorship of Professor Dr. Suman Kapur. I was awarded the senior research fellowship from the Indian Council of Medical Research (ICMR) to continue my doctoral work and have

been involved in projects dealing with development of Pint of Care (POC) device for the estimation of blood glucose. My scientific exposure is a conglomeration of not only microbiology but also biochemical analytical techniques.

Recently, I visited Shamir Research Institute, University of Haifa under Israel Govt. Fellowship, Ministry of Human Resource Development (MHRD), Govt. of India and worked on bacterial extracellular laccase for the treatment of Olive mill waste water. My work has been published in different peer reviewed journals, books and proceeding proceedings and timely presented in many national and international seminars or conferences in which I got two best presentation awards. I have published many research articles and chapter in peer-reviewed journals, books and proceeding and have given more than ten conference/seminar presentations and got best presentation awards in two national conference and Young Scientist Award in one. I got best presentation award given by Nobel Laureate Dr. R.K. Pachauri (Chair of the Inter-Governmental Panel on Climate Change) in Young Researcher's South Asian Symposium on Sustainable Development under United Nations Sustainable Development Solution Network (UN-SDSN) and Delhi Sustainable Development Summit 2015. I am very popular to teach Biology Laboratory courses and served many social responsibilities as a capacity of NSS Volunteer, Red Cross Society Volunteer, CAG Associate and others. As a PhD researcher, I believe that each scholar is highly trained in identifying problems and finding solutions to those problems. All the uncountable hours, days, weeks, months and years, I have spent trying to find answers to unknown questions, this gives me the value addition of uniqueness and patience.

Mistakes are painful when they happen. But years later a collection of mistakes called experience which leads us to success.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Suman Kapur, currently the university wide dean for International Programs and Collaboration division at BITS Pilani and as a Chief of IPCD, she is instrumental in orchestrating several student exchanges program, introduction of new fellowships and opportunities for both students and faculty of BITS Campuses. She has had a meritorious career as a topper in the master program at All India Institutes of Medical Sciences (AIIMS), New Delhi. She obtained her Ph.D. from AIIMS, New Delhi and Post-Doctoral training at university of Minneapolis, MN, USA. In 1990, she was awarded the prestigious Lalor Foundation Fellowship and moved to Kansas University Medical Center, Kansas USA, as a Lalor Fellow. In 1995 she becomes Group Leader, Biotechnology at Ranbaxy Research Laboratory, New Delhi. In 1999, Dr. Kapur becomes Director Arogyadham, DRI, Chitrakoot, M.P. She serves as Head of Department and Associate Professor in Biochemistry at IHBAS, Delhi in 2000. In 2004, Dr. Suman Kapur Joined BITS Pilani as Professor in the Center for Biotechnology, Biological Sciences Group. She is the unit chief community welfare and International relations (CWIRU) since January 2007. She has served as Dean for Research & Consultancy and Education Development Division at the Hyderabad Campus of BITS Pilani in 2010. Dr. Kapur is a popular teacher at BITS, Pilani and has been instrumental in introducing several new courses, curriculum for a new degree program Master of Public Health; incorporating learning through field visits and interdisciplinary teaching. She is the recipient of Shri B. K. Birla and Shrimati Sarala Birla Chair Professorship and BITSAA Faculty Honors in 2011 and 2014 respectively. She is key note speaker of many national and international seminar conference or workshop and successfully organized all edition of WIGH from 2013 onwards. Dr. Suman Kapur is a well-known academician in the field of Genomics and presently working as Senior Professor at Department of Biology at BITS Pilani, Hyderabad Campus. She has more than 30 years of teaching/research experience in Genetic markers for vulnerability to chronic human diseases, Screening strategies for low-cost settings, Medical devices for field deployment in low resource settings and Prevention of diseases through herbal/natural products and actively engaged in services to her profession. Dr. Kapur’s research interests lay in identifying biomarkers for unraveling the genetic basis of human diseases such as psychiatric disorders like depression,

schizophrenia, addiction and alzheimer disease and metabolic disorders such as diabetes (T2DM), obesity, cataract and metabolic syndrome. Early and specific diagnosis is the backbone of effective treatment and reduction of both disease and associated morbidity and mortality. Modern days, integration of electronics and biological possibilities on an integrated chip can be successfully used to develop Point of Care devices especially suited for low cost setting and her group has already developed two such devices and attempt for early noninvasive diagnosis of urinary bladder and prostate cancer are underway. Revival of research on traditional medicine/ herbal remedies with a locally relevant evidence-based disease-oriented approach is particularly relevant to India. Her group is also involved in developing clonal variants of Indian medicinal plants and screening natural products for anti-diabetic, anti-inflammatory and anti-obesity activities in specific animal models for these diseases. Several industries sponsored project are also underway for bioconversion, drug conjugation, remediation, and effluent treatment using consortia of microbial population. She has also taught several courses like Cell Biology, General Physiology, Biological Chemistry, Genetic Engineering, Instrumental Method of Analysis, General Biology, Molecular Mechanism of Gene Expression, Environmental Biotechnology, Cell and Tissue Culture, Stem Cell and Regenerative Biology, Public Health and Disease, Health Care Management and Epidemiology at Undergraduate, Postgraduate and Higher degree levels. She has supervised several interdisciplinary Graduate, Postgraduate and doctoral fellow. As a mentor, she has been able to motivate younger faculty and scholar to submit and execute independent grants in the form Women scientist (DST), Research Associate and Senior Research Fellows (ICMR & CSIR). She has to her credit publication of more than 80 research papers at National and International Journals and Proceedings. She edited two books. First one is “Immunosuppression - Role in Health and Diseases” by Intech Europe publishing house, and edited along with Maristela Barbosa Portela, Adjunct Professor Fluminense Federal University, Rio de Janeiro, Brazil and second one is “Empowering Women in Developing Countries through Information & Communication Technologies” published by NAM S &T Centre, India and edited along with Dr. FinaryaLegoh, Principal Engineer, The Agency for Assessment and Application of Technology (BPPT)]; Iraq. She is life member of several National and International Professional bodies like Life Member of several professional bodies ACBI, IIS, IANS, SBC,

IPS, RSSDI, SDF, RSA, ISBRA, ESBRA, SFN and ASBRA. She is Editor and reviewer for peer-reviewed international & National Journals like IJBB, IJCB, JTAS, IJFMT, Cell and Tissue Research, Eye (Nature Publications), BIOMARKERS (Taylor & Francis, USA). She has two patents for her credit for developing two indigenous technologies for testing antibiotic sensitivity of pathogens found in human urinary tract and sensing blood glucose. She promoted a company named Xcellence in Biological Innovations and Technologies and the company has a start-up grant of INR 50 lakhs from DBT under their SPARSH scheme. Her group recently won the first prize at ABLE (Association of Biotechnology Led Enterprises) an initiative of Department of Biotechnology, Govt. of India under BEST (Biotechnology Entrepreneurship Student Teams)-India 2014. In March 2015, under her mentorship, another achievement, when honorable President of India recognizes her innovation Rightbiotic-The faster antibiotic finder for GYTI (Gandhi Young Technological Innovation) Award 2015 under BIRAC-SRISTI Scheme at Festival of innovation program by NIF in president house. In continuation of achievement, she got National Technological Award 2018 for her Rightbiotic innovation by honorable president of India.

Our Mother Earth

Every part of this earth is sacred.

We are part of the earth and it is part of us.

The perfumed flowers are our sisters, the deer, the horse, the great eagle; these are our brothers……

The shining water that moves in the streams and rivers is not just water but the blood of our ancestors……

We must teach our children that the ground beneath their feet is the ashes of their ancestors, so that they will respect the land.

Let us tell our children that the earth is rich with the lives of our kin.

Let us teach our children that the earth is our mother……

Whatever befalls the earth befalls the sons of the earth.

If men spit upon the ground, they spit upon themselves……

This we know; the earth does not belong to man; man belongs to the earth….

This we know: all things are connected like the blood, which unite the family.

All things are connected.

Source: Environmental studies: by Rev.Fr. Slgnacimuthus.j, MJ Publishers.com (2012)

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