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Full Length Research Paper

Degradation of morpholine by *Mycobacterium* sp. isolated from contaminated wastewater collected from Egypt

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The biodegradation of morpholine has attracted much interest because morpholine causes environmental pollution. Ten species belonging to nine genera were tested for their abilities to degrade morpholine in mineral salts medium containing morpholine (1 g/l). *Mycobacterium* sp. isolated from polluted water sample collected from Abu Za"baal lakes, effectively utilized morpholine as carbon, nitrogen and energy source. The tested *Mycobacterium* was able to grow in high concentrations of morpholine but the rapidly increase in pH of the growth medium and accumulation of ammonia inhibited bacterial growth and complete mineralization of morpholine. The molar conversion ratio of morpholine to ammonia was 1:0.89. Growing of the selected bacterium in liquid medium with 1 g/l morpholine at 37°C and pH 6.5, enhanced morpholine degradation. Addition of metyrapone to the growth medium inhibited morpholine degradation. Immobilization of *Mycobacterium* cells in sodium alginate increased morpholine degradation compared with free cells. At high concentrations of morpholine (4 to 6 g/l), there was a decrease in both cell viability and respiration of *Mycobacterium* but no genotoxicity was found.

Key words: Morpholine, *Mycobacterium*, biodegradation, pollution, ammonia, cytochrome P450, metyrapone, immobilization**.**

INTRODUCTION

The heterocyclic xenobiotic compound morpholine (1-oxa-4-azacyclohexane) is of great importance for different industrial purposes (Mijos, 1978). Morpholine (C4H9NO) is a colourless, oily, hygroscopic, volatile liquid with a characteristic amine smell and it is completely miscible with water. Because of its wide range of applications, morpholine occurs in the environment, detected in foods (Mohri, 1987) and water. Thus, the removal of morpholine from contaminated industrial waste waters is of environmental interest. Unfortunately, Calamari et al. (1980) and Tölgyessy et al. (1986) both reported the resistance of morpholine to biodegradation. In contrast, many authors have indicated that bacteria utilize morpholine as sole source of carbon, nitrogen and energy (Subrahmanyam et al., 1983). Knap et al. (1982) first discovered two strains of *Mycobacterium* (MorD and MorG) that were able to utilize morpholine as a sole source of carbon, nitrogen and energy. A few years later, Cech et al. (1988) found a strain of *Mycobacterium aurum* MO1 that had morpholine degradation properties. Knapp's group studied other *Mycobacterium* strains isolated from activated sludges (Knapp and Whytell, 1990). Aly (2004) attributed the decrease in morpholine degradation to ammonia accumulation in the growth medium. The aims of this study were selection of the most active bacterium in morpholine degradation and studying factors affecting the degradation process. Growths of the selected bacterium using either intermediate or heterocyclic compounds similar to morpholine were also studied.

MATERIALS AND METHODS

Chemicals used

Morpholine, glycolic acid, ethanolamine, piperidine, pyrrolidine,

thiomorpholine, metyrapone and other chemical compounds were obtained from Sigma-Aldrich.

Microorganisms used

Xanthomonas campestris ATCC33913, *Pseudomonas fluorescens* ATCC17483, *Escherichia coli* ATCC35218, *Mycobacterium butyricum* ATCC 19979, *Enterobacter aerogens* MTCC6804, *Salmonella choleraesuis* ATCC8759 and *Staphylococcus aureus* ATCC14154 were obtained from the culture collection of Dr. R. Bonally, Laboratoire de Biochimie Microbienne, Fac. De Pharmacie, Nancy, France. *Mycobacterium* sp, *Nocardia brasiliensis* and *Streptomyces albidovlavus* were isolated from contaminated water samples collected from Abu Za"baal lakes, Egypt (Rabah and Azab, 2006).

Culture of morpholine degrading microorganisms

All the tested bacterial colonies grown on peptone-beef agar plates were picked and grown for 48 h at 30°C in peptone beef broth medium at 150 rpm. The medium consists of g l^{-1} (Casein peptone, 3; beef extract, 5 and agar, 15 when solid medium was needed). About 2ml of the resultant seeded medium were transferred to 500 ml Erlenmeyer flask containing 98 ml of the mineral salts growth medium containing 1 g/l morpholine as carbon and nitrogen sources. The mineral salts growth medium (Cech et al., 1988) composed of 220 mg K_2PO_4, 530 mg $NaHPO_4$ and 240 µl H_2SO_4 in 1000 ml dist. water. Each 100 ml were added to 900 ml of the salt solution containing 5g $CaCl_2$, 300 mg $FeCl_3$, 12 mg $ZnSO_4$, 11 mg $MnSO_4$, 15 mg $Co(NO_3)_2$, 8 mg $CuSO_4$, 2 mg $NaMoO_4$ in one liter of dist. water. The pH of the medium was adjusted to pH 7 and incubation was carried out at 30°C for 10 days and 150 rpm as described by Aly (2004). To confirm the bacterial growth using morpholine as carbon and nitrogen source, serial dilutions of the obtained cultures were plated on mineral agar plates (containing 2% agar) and incubated at 30°C for 10 days. The cultures of morpholine-degrading bacteria were maintained on the same medium at 4°C. Growth of bacteria was measured by the increase in OD at 550 nm and the change in the initial pH values of the medium.

Measurement of morpholine and ammonia concentrations

Morpholine concentration in the growth media was determined by measuring the chemical oxygen demand (COD) as described by American Public Health association (1976). The samples were heated in glass reagent vials with strong potassium dichromate as oxidizing agent. Oxidizable organic compound react with dichromate, reducing the orange dichromate ion (Cr_2O_7) to green chromic ion (Cr^{+3}). The amount of remaining dichromate was estimated colorimetrically with a spectrophotometer (UV/Vis Perkin-Elmer Lambda 4A) at 500 nm as described in Aly (2004). The amount of morpholine was determined from a standard curve of morpholine (100 to 2000 µg/ml). Ammonia was quantified by Nessler reagent. A yellow color was formed in proportion to the ammonium concentration and was measured at 425 nm using spectrophotometer (UV/Vis Perkin-Elmer Lambda 4A).

Optimization of the culture conditions for morpholine degradation

Optimization of culture conditions for morpholine degradation by *Mycobacterium* sp. was achieved at different incubation temperature (20 to 50°C), different agitation rates (50 to 300 rpm) and different initial pH values(5 to 8) using mineral salt medium and 1 g/l morpholine as carbon and nitrogen source (Aly, 2004). The growth (OD_{550}) and % of morpholine degradation (COD) were recorded after 10 days.

Immobilization

Entrapment of cells of *Mycobacterium* sp. was carried out according to the method of Eikmeier and Rehm (1987). Bacteria cells of a preculture were suspended in sodium alginate by stirring for 15 min to obtain a 2% alginate suspension. The mixture was extruded through a thin needle (0.6 mm) into a 1% $CaCl_2$ solution and left to harden for 1 h. The free Ca^{+2} was then washed out twice with a saline, followed by transferring the gel beads to growth medium containing different concentrations of morpholine

Effect of different concentrations of morpholine on *Mycobacterium* growth and morpholine degradation using free and immobilized cells

About 2 ml of *Mycobacterium* suspension (10^8 cfu/ml) or the immobilized cells (prepared gel beads) were added to the growth medium. Morpholine concentrations were ranged from 1 to 4 g/l. The OD was measured at 550 nm and % of morpholine degradation was determined by COD in addition to the quantity of NH_3 liberated.

Effect of morpholine on respiration, viability and genotoxicity of *Mycobacterium* sp.

Respiration of *Mycobacterium* sp. (Quntities of O_2 consumed µl.mg^{-1}.hr^{-1}) was measured under the effect of different concentration of morpholine using an Oxygraphe (MYSI 3500) as described by Mahmoud and Aly (2004). Cell genotoxicity was carried out using *E. coli* PQ315 and protocol described by Elepuru et al. (1979). Cell toxicity was recorded using *Artimia salina* as test organism as described in Aly (2004).

Growth of *Mycobacterium* sp. using heterocyclic rings or intermediate

Growth of *Mycobacterium* sp. on different heterocyclic rings (pyrrolidine, piperidine, peperazine, thiazole, thiomorpholine, pyridine, quinoline, oxazole, imidazole, tetrahydroquinoline and tetrahydropyrane) was investigated by adding the corresponding compounds (1 g/l) to mineral salts medium. The pH of the medium was adjusting to pH 6.5 and the growth was carried out at 37°C and 180 rpm for ten days. On the other hand, growth of *Mycobacterium* sp. using intermediate compounds, detected during degradation by other bacterial strains using morpholine as carbon and nitrogen sources like aminoethoxyyethanol, ethanolamine, acetic acid, glyoxylic acid, glycolic acid and glyceric acid was tested in the presence and absence of 0.1 g/l morpholine as an inducer. The growth conditions were similar to that described before.

Spectrophotometric analysis of cytochrome P-450 in crude cell extract

Mycobacterium sp. was grown in mineral salts growth medium with either morpholine (5 mM) or sodium succinate (5 mM) and NH_4Cl (1 g/l) as a carbon and nitrogen sources. After 10 days, the cells were collected, washed with distilled Water and sonicated under cooling 3 successive times (3 min/time) using glass beads for preparation of cell free extracts. The cell debris and membranes

Table 1. The growth, final pH and % of morpholine degradation by different bacteria grown in mineral medium containing 1 g/l morpholine as carbon and nitrogen source.

Bacteria used	Final pH	Growth ($O.D._{550}$)	% of morpholine degradation
Xanthomonas campestris	7.0±0.07	0.090±0.05	0.0
Pseudomonas fluorescens	6.8±.0.6	0.140±0.03	11.0±0.6
Escherichia coli	7.0±0.11	0.090±0.03	0.0
Staphylococcus aureus	7.0±0.0	0.090±0.04	0.0
Nocardia brasiliensis	7.0±0.06	0.090±0.07	0.0
Streptomyces hygroscopicus	7.0±0.90	0.090±0.00	0.0
Mycobacterium butyricum	7.0±0.04	0.090±0.02	0.0
Mycobacterium sp	6.3±0.05	0.430±0.11	70± 4.0
Enterobacter aerogens	7.0±0.01	0.090±0.03	0.0
Salmonella choleraesuis	7.0±0.01	0.090±0.03	0.0

Table 2. Growth, final pH, % of morpholine degradation and the quantities of ammonia released by *Mycobacterium* sp.

Time (Day)	Growth (OD_{550})	Final pH	Quantity of NH_3 released (mg/l)	% of morpholine degradation
Control	0.01±0.00	7.0±0.07	0.0±0.01	0.0±0.01
2	0.12±0.00	6.5±0.04	27±0.31	11.0±0.71
4	0.14±0.01	6.3±0.01	50±1.01	18.0±0.91
6	0.25±0.01	6.3±0.11	100±0.00	35.0±0.11
8	0.33±0.11	6.3±0.11	180±2.01	50.2±0.01
10	0.44±0.00	6.3±0.04	180±1.01	70.8±0.00
12	0.42±0.01	6.9±0.08	188±0.91	71.0±0.11
14	0.40±0.08	7.5±0.00	200±0.41	71.0±0.41

were removed by centrifugation at 30000 *g* for 20 min two times. The obtained supernatant, designated crude extract, was applied to a Sepharose column (90 x 3 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 8.5 (Schrader et al., 2000). Unbound protein was washed off with the buffer three times and subsequently, the bound protein was eluted by 1 M NaCl. Protein was determined according to Bradford (1976) using bovine serum albumin as a standard. The cell free extract was reduced by the addition of dithionite (2 mM) and carbon monoxide was pushed through the cuvette and reduced extracts without carbon monoxide were used as control (Peterson and Lu, 1991). The spectra were recorded with a spectrophotometer (UV/Vis Perkin-Elmer Lambda 4A).

Statistical analysis

Each experiment was carried out in three replicates. Means of variable and standard deviation were recorded and *t*- student test was carried out to detect any significant differences between the results of control and the treated samples.

RESULTS

The abilities of ten tested bacteria to use morpholine as carbon and nitrogen source were studied (Table 1). The growth was detected by the decrease in the pH of the growth medium and was measured by the $O.D_{550}$. The growth was observed using *Mycobacterium* sp. and *P. fluorescens* only. *Mycobacterium* sp. effectively utilized morpholine as carbon, nitrogen and energy source. The pH was decreased by growth up to 6.3 and the $O.D_{550}$ was recorded to be 0.43. The percentage of morpholine degradation was reached to 70% after ten days of growth. Low growth ($O.D._{550}$ 0.14) was obtained for *P. fluorescens* and the % of morpholine degradation was 11%. No growth, no change in the initial pH or morpholine concentration was recorded for all the other tested bacterial genera.

Growth using morpholine as carbon and nitrogen sources by *Mycobacterium* sp. was studied for 14 days (Table 2). It was noticed that the highest growth (OD_{550}) and morpholine degradation was recorded after ten days. Effect of different incubation temperature and initial pH values on growth of *Mycobacterium* sp. and morpholine degradation was recorded (Figures 1 and 2). It was found that incubation at 37°C for ten days and initial pH 6.5 were the best conditions for both growth and utilization of morpholine (90% degradation).

The effect of different concentration of morpholine on

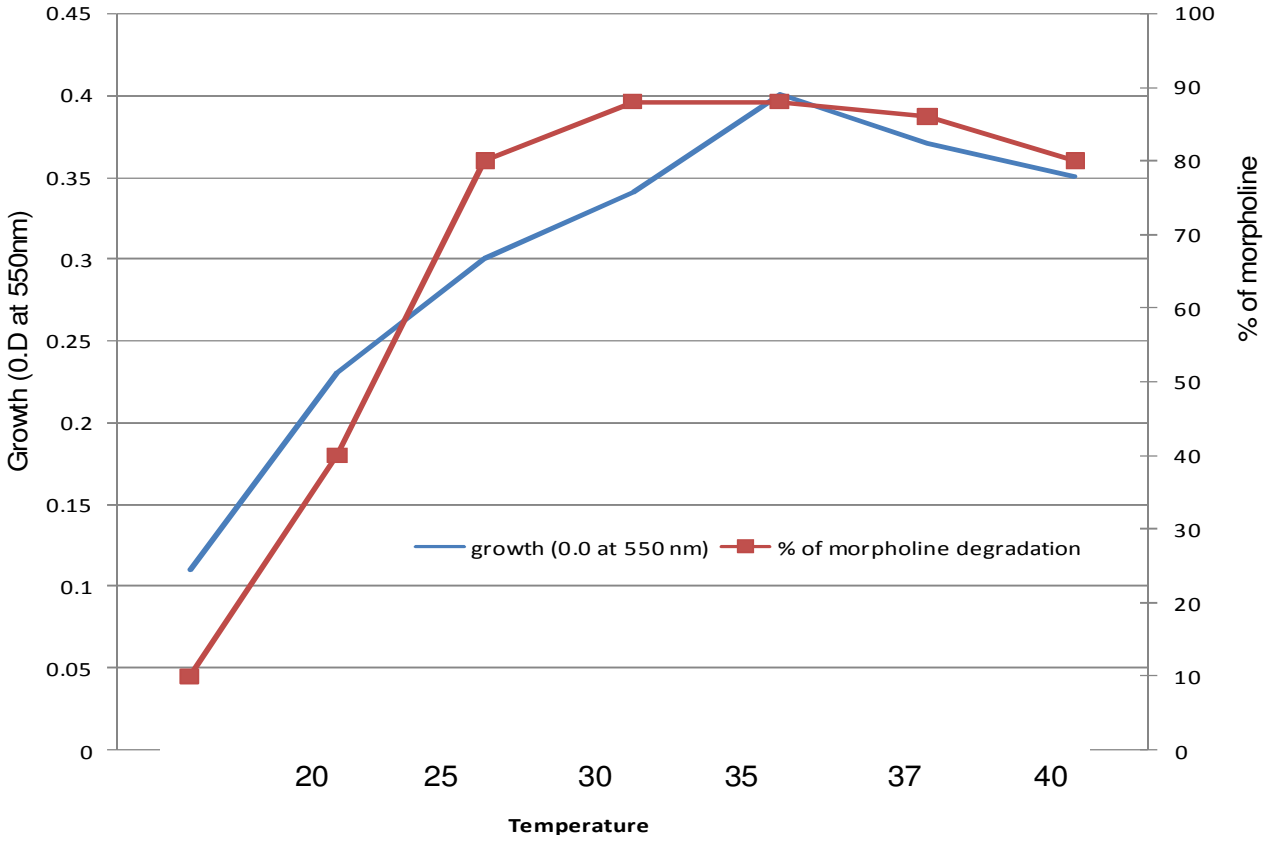

Figure 1. The effect of different temperature on growth of *Mycobacerium* sp. grown in mineral medium with 1 g/l morpholine at pH 7 and % of morpholine degradation after 10 days.

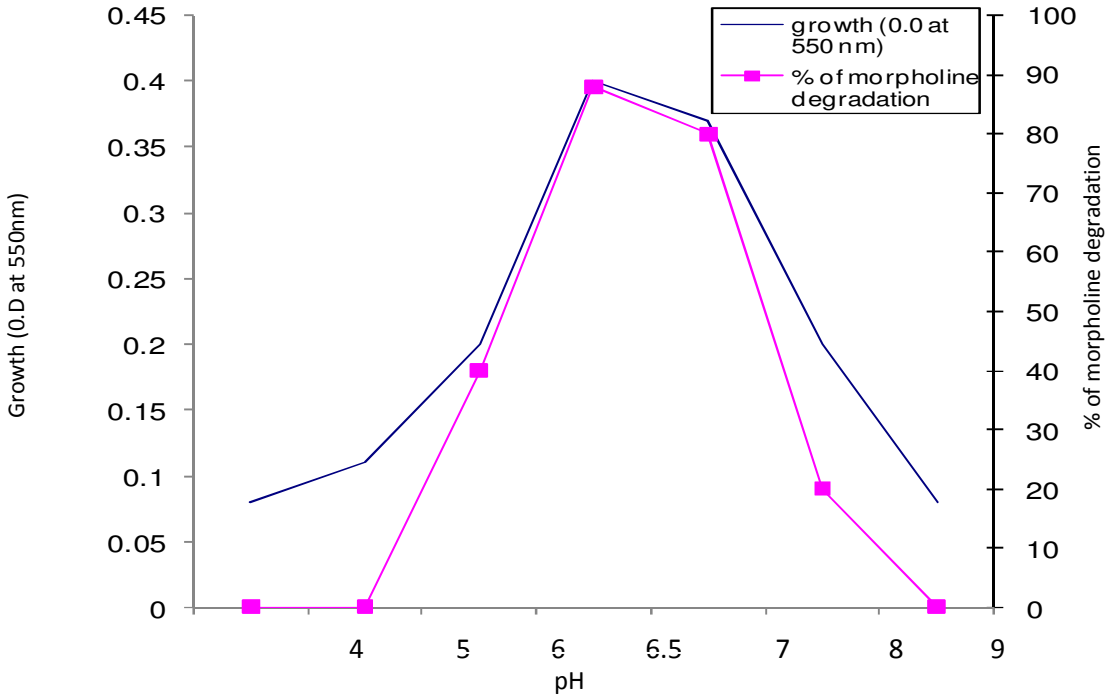

Figure 2. *Mycobacterium* sp. growth at 37°C for 10 days and % of morpholine degradation at different pH values using mineral medium with 1 g/l morpholine as carbon and nitrogen source.

Table 3. Effect of different concentration of morpholine on growth and morpholine degradation by free and immobilized cells of *Mycobacterium* sp.

Morpholine concentration (g/l)	Time (day)	Free cell			Immobilized cell		
		Final pH	Growth (OD_{550})	% of morpholine degradation (COD)	Final pH	Growth (OD_{550})	% of morpholine degradation (COD)
0.5	10	7.0	0.22	100.0	6.3	ND	100.0
1.0	10	7.0	0.63	90.0	7.3	ND	100.0
	10	7.3	0.60	45.0	7.0	ND	53.0
2.0	20	7.5	0.64	45.0	7.0	ND	53.0
	10	7.5	0.66	17.0	7.0	ND	27.0
3.0	20	7.6	0.65	15.0	7.0	ND	25.0
	10	7.0	0.64	10.0	7.0	ND	20.0
4.0	20	7.0	0.60	11.0	7.0	ND	23.0

ND: not detected.

Table 4. Growth of *Mycobacterium* sp. using different hypothetical intermediary compounds in the presence or absence of 0.1 g/l morpholine as an inducer.

Parameter	Without morpholine		With 0.1 g/l morpholine	
	Growth	Final pH	Growth	Final pH
Control (without carbon source)	0.08±0.00	6.5	0.11±0.01	6.5
Aminoethoxyethanol	0.08±0.03	6.5	0.11±0.04	6.5
Ethanolamine	0.08±0.00	6.5	0.11±0.01	6.5
Acetic acid	0.22±0.07*	7.0	0.25±0.00	6.7
Glyoxylic acid	0.08±0.01	6.5	0.24±0.05*	6.6
Glycolic acid	0.08±0.02	6.5	0.29±0.06*	6.8
Glyceric acid	0.08±0.04	6.5	0.21±0.08*	6.8

*: significant results at $p< 0.05$.

bacterial growth and degradation process was carried out at 37°C and initial the pH 6.5 (Table 3) using either free or immobilized cells. At 0.5 g/l morpholine, it was rapidly degraded (100% degradation) by free cells. During this time, pH of the medium was increased and the OD_{550} increased to 0.22 (Table 3). Increasing morpholine concentration to 1 g/l increased bacterial growth (OD_{550} 0.63) and morpholine degradation, but at 2 g/l morpholine, the bacteria need 10 days to use about 50% of the quantity of morpholine present (1 g) and increasing incubation period to 20 days did not increase growth or morpholine degradation. At 3 and 4 g/l morpholine, little growth was observed and % of morpholine degradation was at the lowest (11%). Comparing free and immobilized cells, it was clear that immobilization enhanced morpholine degradation where the % of morpholine degradation was 11 to 100% and 23 to 100% in case of using free and immobilized cells, respectively.

Growth of *Mycobacterium* was detected using acetic acid, glyoxylic acid, glycolic acid and glyceric acid as carbon sources in the presence or absence of 0.1 g/l morpholine as an inducer (Table 4). In the absence of morpholine, no growth was recorded for all tested carbon sources except for acetic acid.

The growth of *Mycobacterium* sp. occurred by using only peperazine, pyrrolidine and thiomorpholine as a carbon source (Table 5). The respiration of bacterial cells, measured by the quantities of oxygen consumed ($\mu l/mg.hr^{-1}$), was the same up to 4 g/l morpholine. More than 4 g/l morpholine, the respiration of the cells decreased to 0.0 $\mu l/mg.hr^{-1}$ (Table 6). No genotoxicity was recorded for all tested concentrations of morpholine, but the percentage of cell viability decreased by increasing morpholine concentration and acute toxicity (viability less than 50%) was recorded at concentrations more than 4 g/l.

The bacterial growth was highly affected by the presence of metyrapone which considered was as cytochrome P-450 inhibitors (Table 7). Reduced cell extracts of morpholine-grown cultures gave rise to a carbon monoxide with a peak near 450 nm, which indicated the presence of a soluble cytochrome P-450 (Figure 3). No peak was observed in the case of extract of cell grown on succinate as carbon source.

Table 5. Growth of *Mycobacterium sp*. on different heterocyclic compounds similar to morpholine in mineral medium, at 37°C and pH 6.5 for ten days.

Heterocyclic compound (1 g/L)	Growth (OD_{550})
Control (without carbon source)	0.08±0.00
Peperazine	0.28±0.09*
Pyrrolidine	0.29±0.05*
Thiazole	0.08±0.03
Thiomorpholine	0.21±0.05*
Pyridine	0.08±0.01
Quinoline	0.08±0.03
Oxazole	0.08±0.01
Imidazole	0.08±0.00
Tetrahydroquinoline	0.08±0.01
Tetrahydropyrane	0.08±0.02

*: significant results at $p< 0.05$.

DISCUSSION

Morpholine as a heterocyclic amine can be degraded by specific genera of bacteria especially that belong to genera *Mycobacterium* and *Pseudomonas*. Similar results were obtained by Besse et al. (1998) and Chandrasekaran and Lalithakumari (1998). Growth studies on morpholine suggested that plasmid-encoded genes were involved in the heterocyclic ring cleavage and chromosomal genes mediated the remaining reactions (Chandrasekaran and Lalithakumari, 1998). Unfortunately, transformation studies showed that the morpholine degradative plasmid was expressed only in *Pseudomonas putida* and not in *E. coli*, which can grow rapidly and easily.

As observed for other morpholine-degrading organisms, *Mycobacterium* sp. released ammonia into the medium during growth on this substrate. Accumulation of ammonia in the growth medium, limited growth and degradation process. At 1 gl/l morpholine and after 10 days, the molar ratio of morpholine degraded to ammonia produced by the selected *Mycobacterium* was found to be 1:0.89. The ratio obtained by Knapp et al. (1982) and Swain et al. (1991) was 1:0.82 for *Mycobacterium* sp. MO1 and *Mycobacterium* sp. MorG, respectively and ratio was about 1: 0.5 for *Mycobacterium* sp. HE5 (Schrader et al., 2000).

Incubation temperature, initial pH and morpholine concentration influenced bacterial growth and % of morpholine degradation whereas the maximum growth and morpholine degradation by *Mycobacterium* sp. was observed at 37°C, initial pH 6.5 and at 1 g/l morpholine.

Similarly, the best growth conditions recorded for *Mycobacterium* LH60 were incubation at 30°C, initial pH 7.2 and morpholine concentration of 30 mM (Schrader et al., 2000). Immobilization of *Mycobacterium* in sodium alginate enhanced morpholine degradation that in these conditions began earlier and was faster than in free culture. Mazure and Truffaut (1994) and Aly, (2004), obtained similar results for *Mycobacterium* and *Pseudomonas,* respectively.

A few studies were carried out in order to understand the morpholine biodegradation process and its regulation (Shaikh et al., 2009). Swain et al. (1991) proposed a hypothetical pathway for the biodegradation of morpholine by *Mycobacterium chelonae* that could proceed via 2-(2-aminoethoxy) acetate and glycolate and/or ethanolamine. Our results confirmed the growth of *Mycobacterium* on some intermediary compounds and suggested that the degradation was via the ethanolamine and glycolate route.

The growth of *Mycobacterium* sp. using many heterocyclic compounds similar to morpholine was tested to determine the mechanisms of ring opening. *Mycobacterium* was naturally able to use pyrrolidine, peperazine and thiomorpholine and no growth was recorded using thiazole, pyridine, quinoline, oxazole, imidazole, tetrahydroquinoline or tetrahydropyrane. Emtiazib et al. (2001) found that pyrrolidine, piperidine and peperazine can be used as carbon sources by *Mycobacterium*; degradation occurred following the same pathways as morpholine and cytochrome P450 was involve in the process. At high concentration of morpholine, respiration and cell viability decreased but no genotoxicity was found. Similarly, morpholine showed no genotoxicity of *Salmonella typhimurium* (Texaco, 1979; Haworth et al., 1983) and acute toxicity of morpholine for *Pseudomonas* was 310 to 8700 mg/l (International Programme on Chemical Safety, 1996). The toxic concentration was calculated to be 10 g/l for *M. aurum* MO1 (Mazure and Truffaut, 1994) and more than 7 g/l for *P. putida* GR12.2 (Aly, 2004).

Degradation of morpholine is likely to begin by the breakage of a bond between the heteroatom and an adjacent carbon atom and the enzyme responsible for the ring cleavage was a monooxygenase. Morpholine bearing amine and ether functional groups 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). Metyrapone (cytochrome P-450 inhibitor) decreased degradation ability of *Mycobacterium* that mean involvement of cytochrome P-450 in degradation process. Poupin et al. (1998) attributed the inhibitory effects of metyrapone on the degradation ability of strain RP1 to the involve-ment of cytochrome P-450 in the bio-degradation of morpholine

In conclusion, data presented in this study could help technologists to remove morpholine effectively from

Table 6. Respiration of *Mycobacterium sp.* and toxicity under different concentration of morpholine.

Morpholine Concentration (g/l)	Cell respiration (quantity of O_2 consumed) µl/mg./h	Toxicity (% of cell viability)
0.0	27.0	100
0.5	27.9	100
1.0 (control)	28.7	100
2.0	27.9	100
3.0	27.0	80
4.0	29.0	50*
5.0	11.0**	40*
6.0	0.0**	40*

*: Toxic effect, ** : The result was significant at $p < 0.05$.

Table 7. Effect of metyrapone on growth and morpholine degradation by *Mycobacterium* sp isolated from contaminated water.

Tested material (100 mg/l)	Growth	% of morpholine degradation (COD)
Control	0.44 +0.10	70.0+4.0
Metyrapone	0.09*+0.10	0.0*

*: significant results at $p < 0.05$.

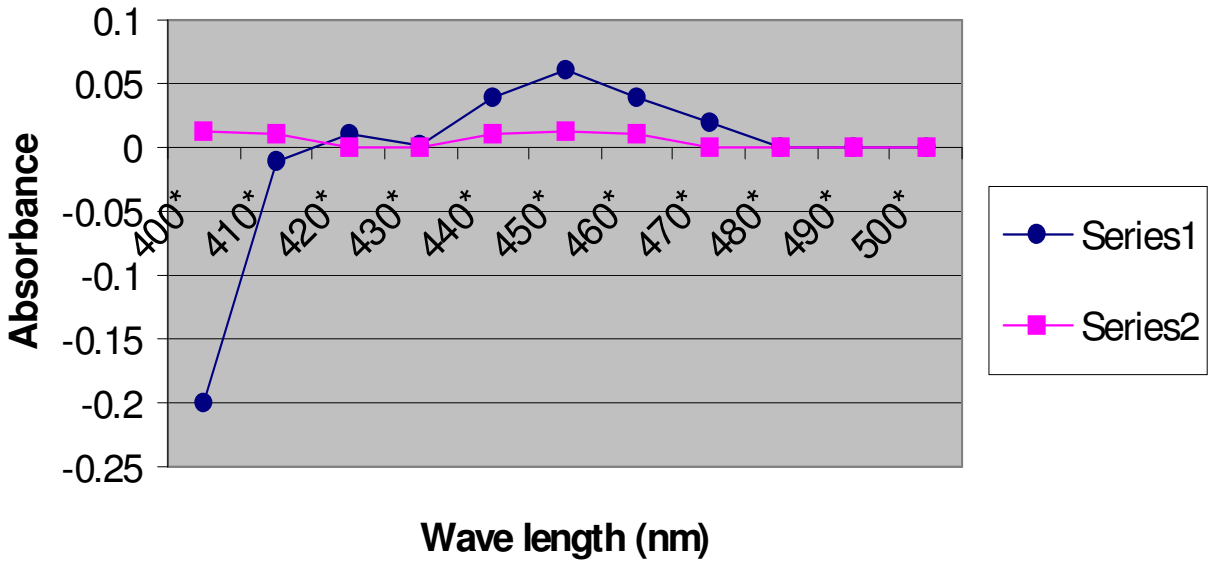

Figure 3. Spectra of cell extract 6 prepared from of *Mycobacterium* sp. grown in mineral medium with morpholine (series 1) and cell extract of cells grown on succinate as carbon source (series 2).

industrial wastewaters and contaminated soil during biological purification.

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