Isolation, Characterization and Identification of Mercury Resistant Bacteria from Heavy Metal Contaminated Sites of VAPI Industrial Area

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Abstract: A total of 25 bacterial cultures were isolated from the metal contaminated sites of Vapi industrial area. Out of total, culture coded as M16, M6 and M18 have shown high resistant towards increasing mercury concentration. All three cultures were studied for its biochemical properties and then identified. M16, M6 and M18 showed maximum mercury tolerance at pH 7, 10 and 6, respectively, otherwise active over broad pH range. However, optimum temperature for mercury tolerance for M16 and M6 was 30°C while it was 37°C for M18. All cultures were able to tolerate mercury even in presence of 2 to 4% of salt concentration. The results clearly show that the cultures would be used for the bioremediation of metal contaminated sites of Gujarat region in near future.

Keywords: Heavy metals; Bioremediation; Mercury resistant bacteria; Isolation; Characterization.

I. INTRODUCTION

Accumulation of heavy metals at any particular area poses severe threat to that environment and also its living systems. Mercury is one of the highly toxic as well as non-biodegradable metals. Thus, its constant accumulation in environment can show pronounced effect on higher nutrient level animals [1, 2]. Mercury is naturally present in environment, especially in volcanoes, soil erosion and oceans as cinnabar ores and also added due to anthropogenic activities [3]. The major anthropogenic activities that release mercury pollutants are industrial wastes and effluents, mining processes, sewage treatment plants, agricultural fungicides etc [3, 4]. Due to such industrial activities atmosphere, agricultural areas and natural water bodies have been affected and destroyed continuously. These may lead to change in natural ecosystem over the years [5, 6].

For the effective removal of mercury several researchers have proposed the physic-chemical treatments such as adsorption and chemical oxidation processes. These treatments are effective but due to high treatment cost those cannot be viable at larger scale. On the other side, microorganisms based treatments are considered as low cost and environmentally safer processes. Bacterial populations present in mercury rich environment or mercury contaminated sites have already evolved mechanism to detoxify the mercury compounds [7-9]. Thus, isolation and identification of mercury resistant bacteria have gain interest in recent years [10].

In the vicinity of Vapi, Gujarat, many industries are working with heavy metals and their preparations for industrial manufacturing. Many of them release heavy metals like mercury in soil and water bodies which are directly added to three major rivers- the Damanganga, Kalok and Balitha. In order to reduce the mercury contamination from those areas first it is necessary to identify the potent mercury resistant bacteria from such environments. Thus, the present research is focused on isolation, characterization and identification of mercury resistant bacterial cultures from the highly metal contaminated sites of Vapi region.

II. MATERIAL AND METHODS

Collection and storage of soil samples: In the vicinity of Vapi, there are number of industries found working with heavy metals and their preparations. So, in order to isolate potent mercury resistant bacteria, three samples of soil were collected in sterile plastic bags from mercury contaminated sites near to the river- Damanganga, Vapi. The samples were immediately transported in our laboratory and used for the isolation.

Sample processing and bacterial enumeration by viable cell count: 2g of each soil sample suspended in 10 of sterile distilled water was first passed through a sterile membrane filter of 0.45 µm pore size to remove the solid particulate matters. Next, from the filtered samples, the heterotrophic viable cell count of mercury resistant bacteria was performed using nutrient agar plates containing 5.0 and 10 ppm of mercury. The nutrient agar plate without mercury was served as a control set. After spreading 0.1 mL aliquot of sample, the plates were incubated at 30°C for 24h. After incubation, the colonies developed on control and test plates were enumerated (Prescott and Harley, 2002) and expressed as colony forming units (CFU/g).

Isolation of mercury resistant bacteria: For the isolation of mercury resistant bacteria LB agar plate containing 10 ppm

$HgCl_2$ was used. The pure bacterial cultures were maintained and preserved on the same medium at 4°C.

Microscopic characterization of mercury resistant bacteria: The potent bacterial culture was first characterized through microscopic observation after performing Gram's staining. Apart from that the cultures were also characterized for their motile nature and endospore formation ability.

Cultural characterization of mercury resistant bacteria: The well isolated colonies of potent bacterial culture on LB agar plates were observed for their size, shape, margin, opacity, texture, consistency and pigmentation. Growth curve of selected mercury resistant bacteria were prepared to understand the effect of $HgCl_2$ and $HgSO_4$ on their growth pattern.

Biochemical characterization of mercury resistant bacteria: In order to understand the metabolic characteristics of the selected mercury resistant bacteria, various biochemical tests were performed.

Optimization of environmental factors affecting mercury resistant bacteria:

(1) Effect of pH on mercury resistant bacteria: Due to the presence of various enzymatic systems bacteria are highly sensitive towards the changing pH. In order to find the optimum pH for the growth of mercury resistant bacteria, pH of the medium amended with either $HgCl_2$ (100 µg/mL) was varied in range of pH 6.0 to 10. After inoculation of 1 mL bacterial culture, all flasks were incubated at 30°C for 24h and then O.D. was measured at 540 nm. The control set without $HgCl_2$ was kept as a control. The growth of all cultures was also recorded at various time intervals up to 72 h.
(2) Effect of temperature on mercury resistant bacteria: The temperature is another important parameter to which bacteria show a wide growth pattern. To find out the optimum temperature for the growth of mercury resistant bacteria all inoculated flasks were incubated at 25 to 45°C. After incubation, the O.D. was measured as shown above.
(3) Effect of salinity on mercury resistant bacteria: A minimum level of salt is necessary for the growth of any bacteria. To check the growth efficiency of mercury resistant bacteria, sets were run in medium having 100 ppm $HgCl_2$ with varying salt concentration (2.0 to 4.0% of NaCl). The flasks were incubated and finally contents were checked spectrophotometrically at 540 nm as described above.

III. RESULTS AND DISCUSSION

Bacterial enumeration by viable cell count: Total three samples (i.e. M1, M2 and M3) were collected from area near to the river- Damanganga, Vapi. The results of viable count suggested that the bacterial count was ranged from 20×10^4 (CFU/g) in M1 to 4.5×10^5 (CFU/g) in M3. The frequencies of resistance to mercury varied from 26.32% in M1 to 83.93% in M3 as shown in Table 1. Percentage of mercury resistant bacteria in sample M1, M2 and M3 are 26.32%, 67.56% and 83.93% respectively.

Isolation of mercury resistant bacteria: From three different samples as depicted above, a total of 25 bacterial cultures were isolated showing tolerance towards varying concentrations of mercury. All cultures were tested against different concentrations of $HgCl_2$ to know the minimum inhibitory concentration (MIC). MIC of $HgCl_2$ for different cultures is shown in Fig. 1. On the basis of results, it can be conclude that the culture coded as M16, M6 and M18 have shown high resistant towards inhibitory concentration of $HgCl_2$. However, isolate number M5, M7, M11, M13, M15 and M19 showed highest sensitivity to $HgCl_2$. The proportionate amount of different mercury resistant bacteria is shown in Table 2. According to the results of this study it is suggested that mercury resistant bacteria are being isolated with primary enrichment method in the presence of Hg.

Primary identification of potent mercury resistant bacteria: On the basis of results of $HgCl_2$ MIC, the potent bacterial cultures, M16, M6 and M18 were further studied for their colony characteristics and Gram's reaction. Staining results indicated that M16, M6 and M18 are Gram's negative short rods, Gram's negative short rods and Gram's positive, respectively. The Gram's staining and colonial characteristics of M16 are depicted in Table 3.

Biochemical identification of potent mercury resistant bacteria: After primary identification, potent cultures M16, M6 and M18 were further studied for their biochemical properties in presence of varying concentrations of mercury (Table 4). From the results of staining and biochemical reactions it is assumed that the mercury resistant cultures, M16, M6 and M18 are belong to genus *Pseudomonas*, *Pseudomonas*, and *Streptomyces* respectively.

Effect of pH on mercury resistant bacteria: Effect of pH was observed by growing the bacterial strains on LB broth. Optical density (Absorbance) was taken at 540 nm wavelength. The pH of the media affects bacterial growth. It is clear from Fig. 2 that M16 showed most appropriate growth at pH 7. Hence, it preferred neutral pH more than the alkaline pH where growth was less. M6 showed the same trend as in case of M16 but the growth was drastically reduced at alkaline pH (pH 10). M18 grew the best at acidic pH (pH 6). Overall, mercury resistant bacteria (M16 and M18) can tolerate acidic pH as well as alkaline pH while growth of M6 was reduced as pH goes on becoming alkaline. Fig. 3 shows the growth pattern of

M16 and M6 between pH 6.5 to 8.5 at different time intervals.

Effect of temperature on mercury resistant bacteria: All strains were incubated at 20, 30, 37 and 45°C using LB for 24 hours. The temperature is an important factor to which bacteria show a wide pattern on growth behaviour. It is cleared from Fig.4 M16 and M6 showed maximum growth at 30°C and 37°C respectively and growth was largely reduced at 45°C and 20°C. M18 showed maximum growth at 37°C while minimum growth at 20°C. Over all, in all strains the optimum temperature was found to be 30°C and 37°C respectively. All cultures were also studied for growth at different time intervals between 30 to 37°C (Fig. 5).

Effect of salinity in mercury resistant bacteria: The salt concentration in an environment is the major contributor to the osmotic effect of ions on growth. Bacteria require ions that are provided by salts and typically moderate salt concentrations. High salt in the environment leads to loss of water from cells and ultimately, to the death. Some bacteria require an astonishingly high level of salt to begin growth, whereas other bacteria would be immediately killed in high levels of salt. The results are shown in Fig 6. Three strains viz. M16, M6 and M18 showed a moderate growth in different concentrations of NaCl.

Conclusion: The present study demonstrates the successful isolation, characterization and identification of mercury resistant bacterial cultures from the metal contaminated sites of Vapi region. A total of 25 mercury resistant bacterial cultures were isolated, among which culture M16, M6 and M18 had shown high level of mercury tolerance. All three cultures were able to tolerate high level of mercury under various environmental conditions indicating its usefulness even at larger scale at mercury contaminated site. In near future, all cultures will be evaluated for its tolerance towards heavy metals apart from mercury would open new door in biological remediation of heavy metal contaminated sites.

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Table 1 Total heterotrophic bacteria and mercury resistant bacteria present in all samples

Sample	Dilution Factor	THB (CFU/g)	MRB (CFU/g)		Percentage MRB
			5 ppm	10 ppm	
M1	10^{-2}	19×10^4	8.8×10^4	5.0×10^4	26.32%
M2	10^{-2}	4.5×10^5	3.4×10^4	3.04×10^4	67.56%,
M3	10^{-2}	11.2×10^4	9.8×10^4	9.4×10^4	83.93%

THB: Total heteroptophic bacteria; MRB: Mercury resistant bacteria

Table 2 Proportionate amount of different mercury resistant bacteria

MIC of $HgCl_2$	Number of mercury resistant bacteria
MIC less than 20 ppm	13
MIC more than 20 ppm but less than 50 ppm	08
MIC 50 ppm	03
MIC more than 50 ppm	01

Table 3 Cultural and microscopic characteristics of mercury resistant bacterial isolates

Colony characteristics	M16	M6	M18
Size	Medium	Medium	Large
Shape	Round	Round	Round
Texture	Smooth	Smooth	Smooth
Elevation	Convex	Raised	Convex
Margine	Regular	Regular	Regular
Opacity	Opaque	Opaque	Translucent
Consistency	Moist	Moist	Moist
Pigmentation	Yellowish	Dirty white	Yellowish

Table 4 Biochemical characteristics of mercury resistant bacterial isolates

Test	Medium	Biochemical characteristics		
		M16	M6	M18
Catalase Test	N-Agar slant	+	+	+
Citrate Utilization Test	Simmon Citrate Plate	+	+	+
Hydrogen Sulfide Test	TSI slant	+	+	+
Methyle Red Test	GPB	+	-	+
Vogus Proskuer Test	GPB	-	+	-
Gelatin liquification Test	1% Gelatin + N-broth	-	-	-
Urease Test	Urea broth	-	+	-
Growth at 37^0 C	N-broth	+	+	+
Starch Hydrolysis	Agar Plate	-	-	-
Nitrate Reduction	PNB	+	+	+
Hydrolysis of Gelatin	Gelatin Agar Plate	+	+	+
Sugar fermentation test	Xylose	+	+	+
	Mannitol	+	+	+
	Fructose	+	+	+
	Trehalose	-	+	-
	Glucose	+	+	+
	Lactose	-	+	-

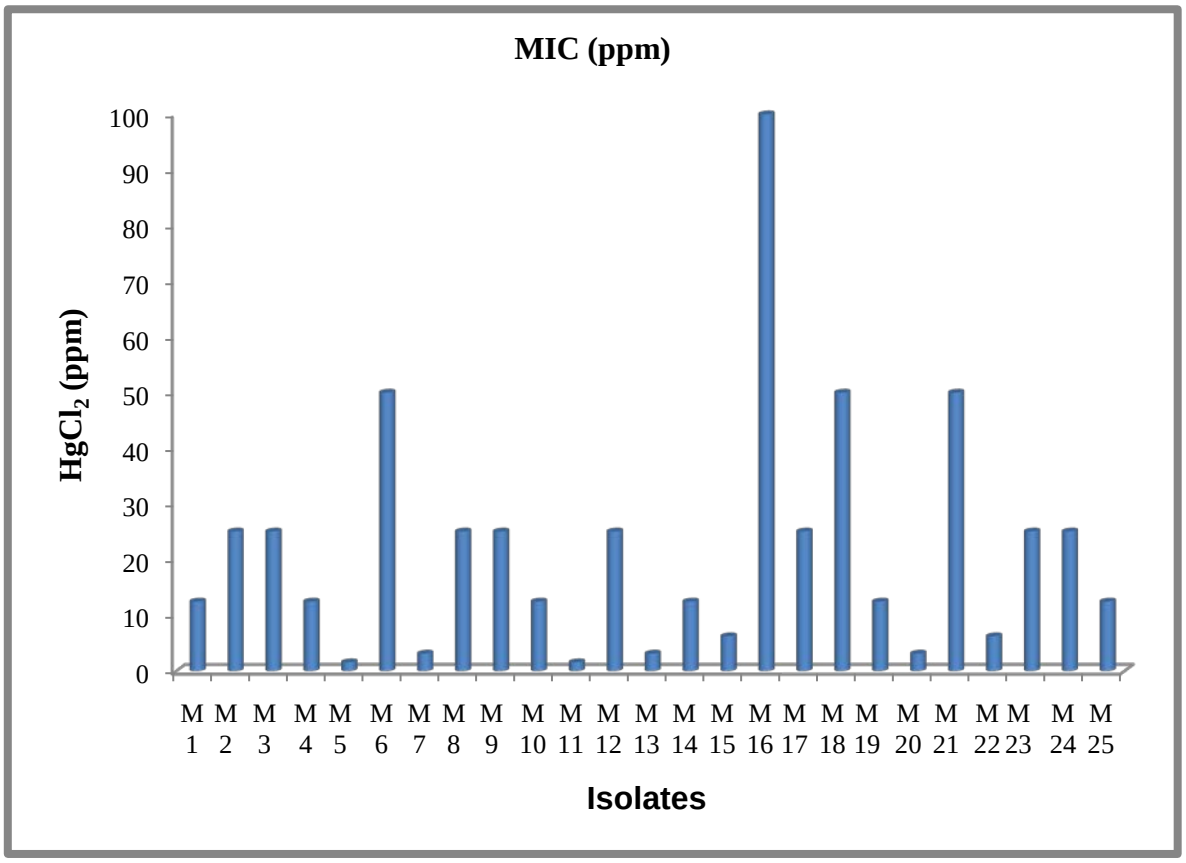

Fig. 1 MIC of $HgCl_2$ for different bacterial cultures

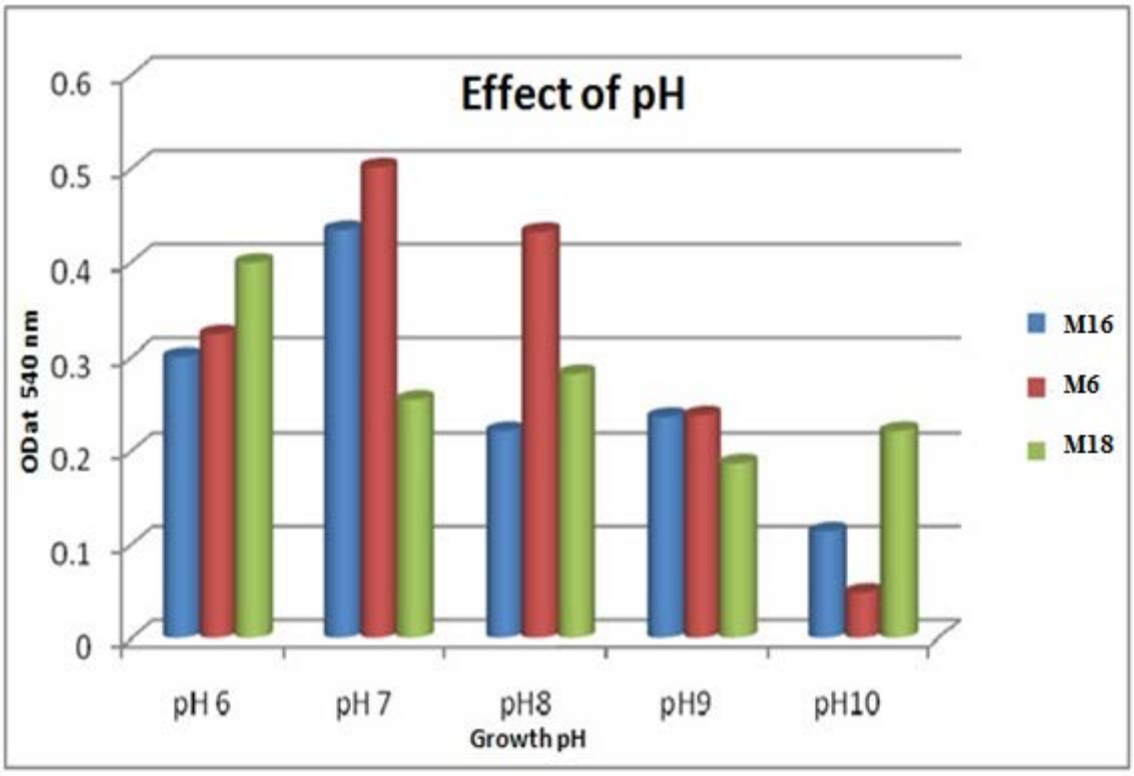

Fig. 2 Effect of pH on growth of M16, M6 and M18 after 24 h

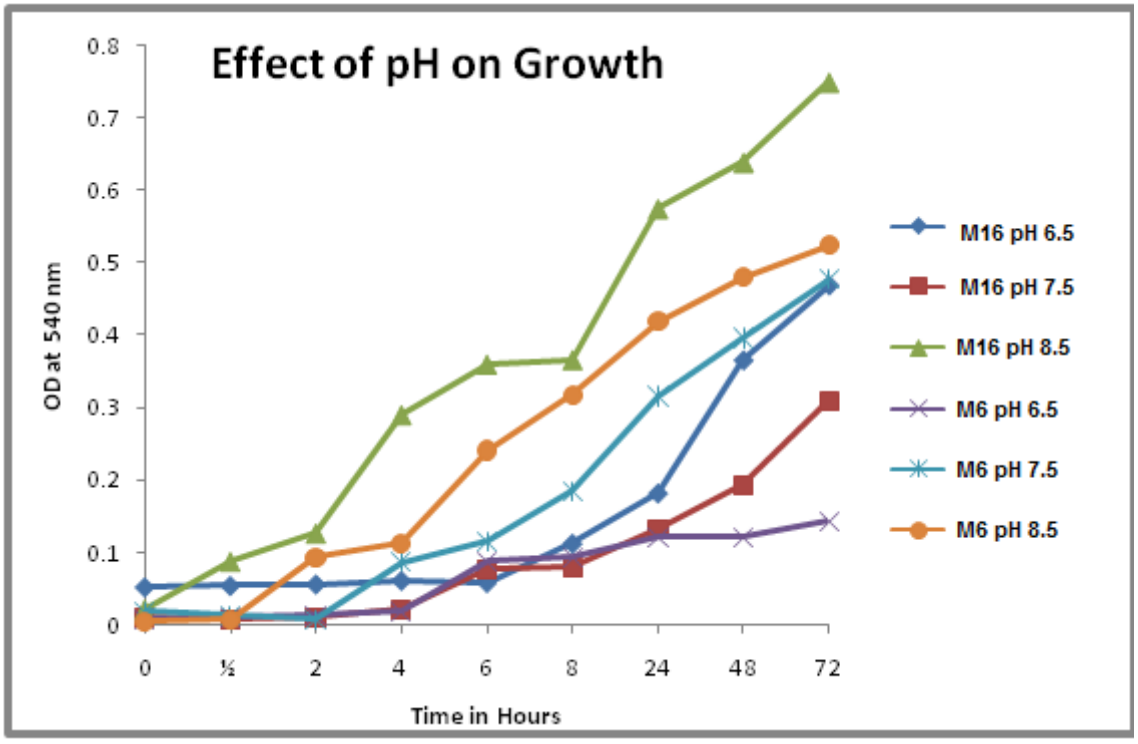

Fig. 3 Growth of M16 and M6 between pH range 6.5 to 8.5 at different time intervals

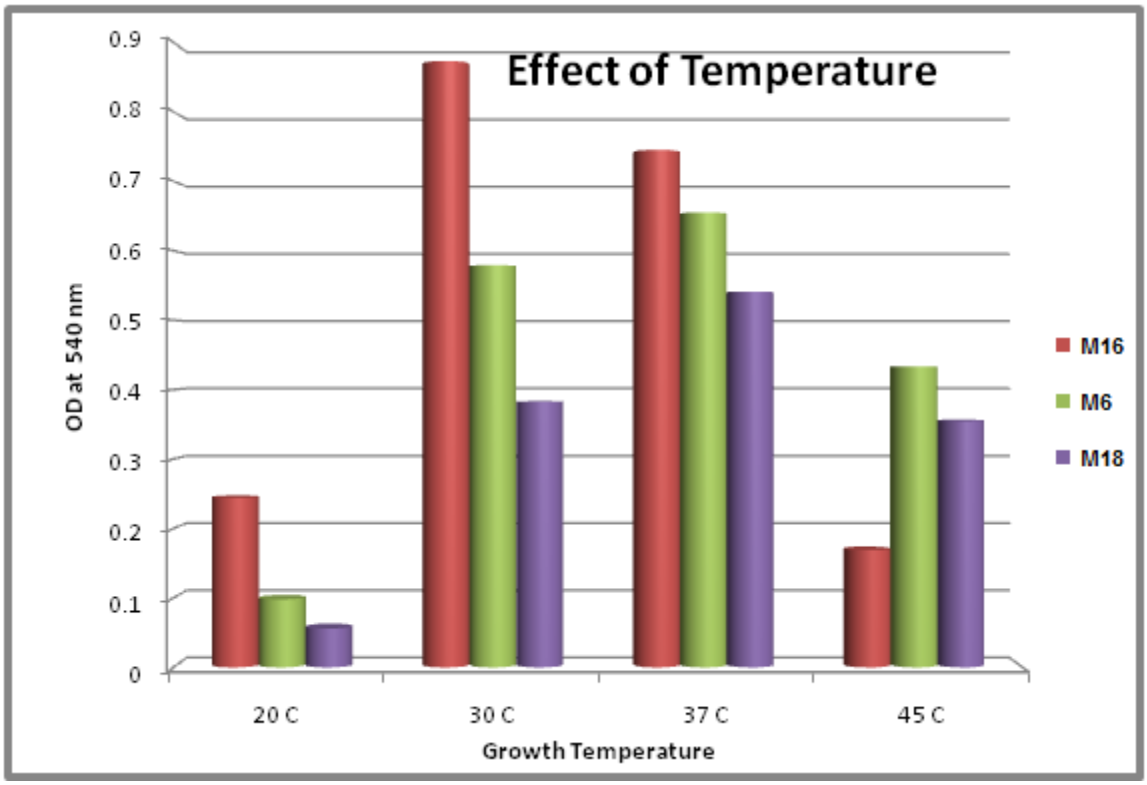

Fig. 4 Effect of temperature on growth of M16, M6 and M18 after 24 h

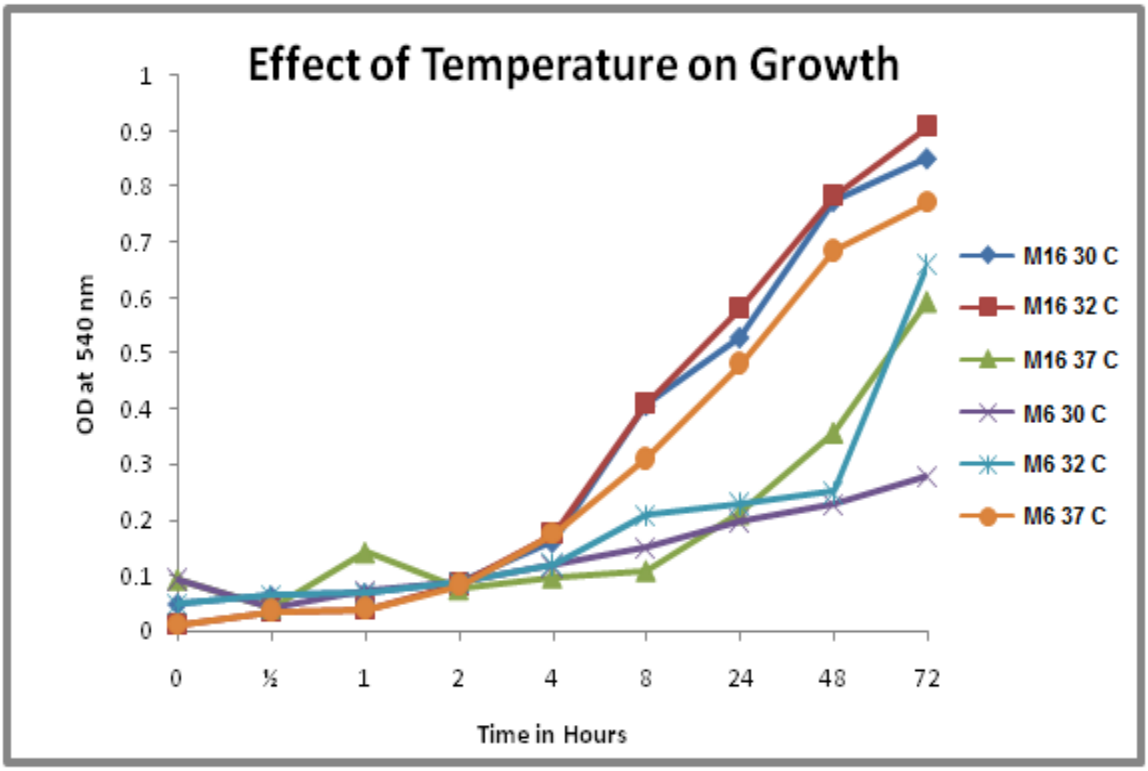

Fig. 5 Growth of M16 and M6 between 30 to 37°C at different time intervals

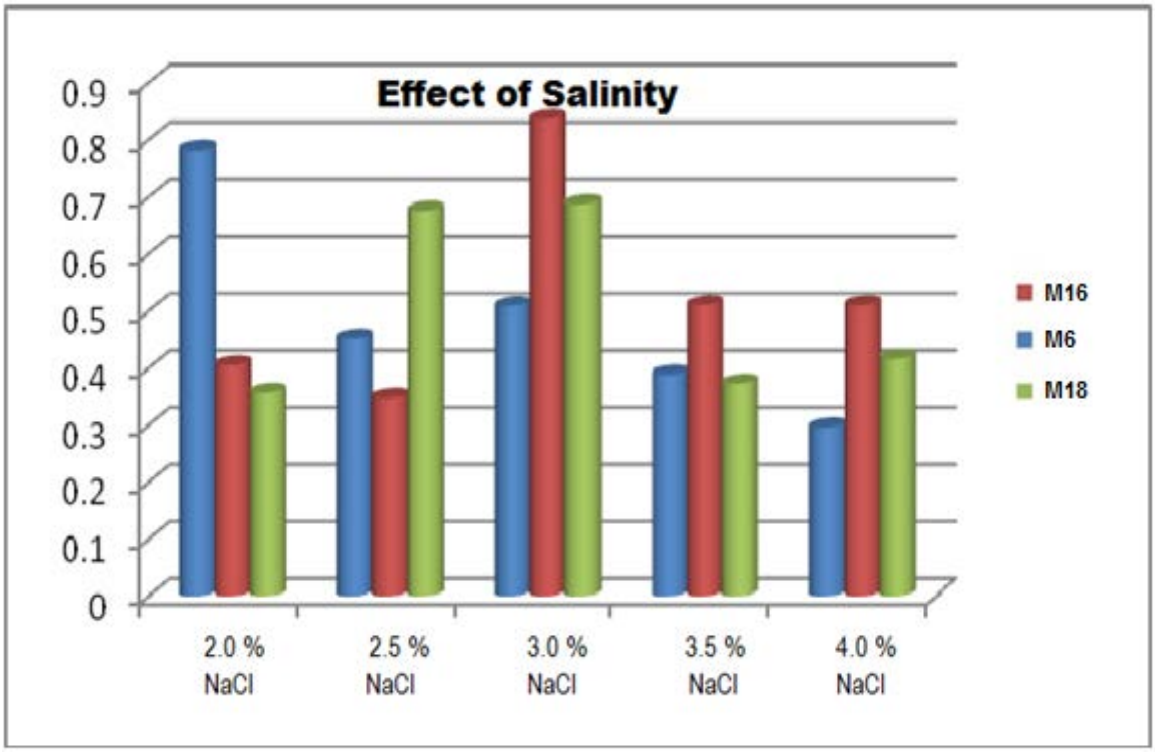

Fig. 6 Effect of different salt concentration on growth of M16, M6 and M18 after 24 h

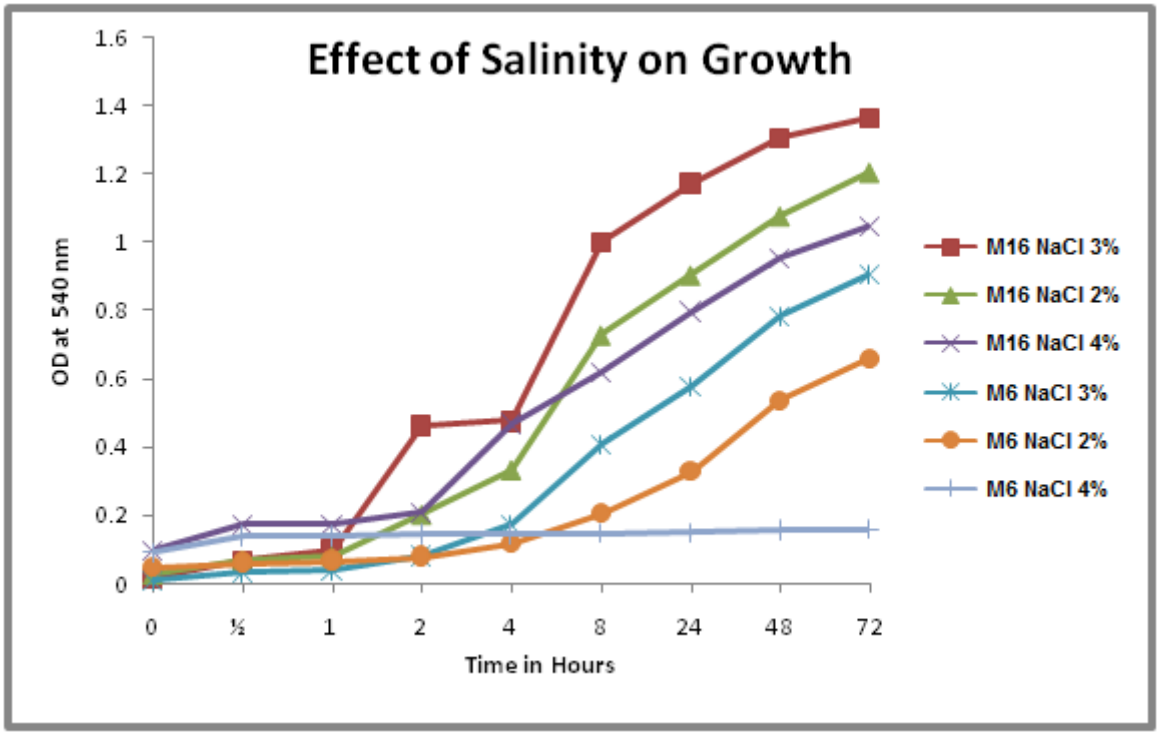

Fig. 7 Growth of M16 and M6 between 2 to 4% salt at different time intervals