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Isolation, Characterization and Effect of Ferrous Salts on Growth of Iron Bacteria

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Abstract*: A study investigating bacterial isolates having iron oxidising ability was attempted. The isolates were from iron rich soil samples from kanjamalai mountain range of Salem region. A total of 92 isolates were successfully isolated from 312 morphologically distinct colonies and evaluated for their iron oxidizing ability. Among the 92 isolates, 12 strains (IB-7, IB-16, IB-22, IB-29, IB-34, IB-39, IB-47, IB-53, IB-56, IB-62, IB-71 AND IB-83) had superior (dark spot) iron oxidizing ability. Among these 12 superior isolates for $FeSo_4$, 25% (IB-16, IB-39, IB-83) 0f the isolate showed inhibition, 58% (IB-7, IB-22, IB-29, IB-39,IB-56,IB-71, IB-83) of the isolates showed no effect with $Fe_2(So_4)_3$. Most of the isolates showed stimulation with $C_6H_8FeO_7NH_3$ except the isolates IB-22, IB-39 and IB-83.

Key words: neutrophilic iron bacteria, ferrous salts, heavy metal, micronutrients, Leptothrix sps

I. INTRODUCTION

According to a survey conducted by US EPA showed that heavy metals were the most common contaminants in the 395 remedial action sites in the US (13) causing human hazards. Anthropogenic activities such as mining, smelting operation and agriculture have locally increased levels of heavy metals such as Cd, Co, Cr, Pb, As and Ni in soil up to dangerous levels. Metal contamination has led to different types of medical problems like birth defects, cancer, skin lesions, growth retardation leading to disabilities, heptorenal and other maladies. Heavy metals are persistent in nature, therefore get accumulated in soils and plants. Dietary intake of many heavy metals through consumption of plants has long term detrimental effects of human health (8). All metals are toxic at higher concentrations, but some of these are useful in low concentration (Fe, Zn, Cu, Co, Cr, Mn, Ni) for human metabolism. These metal toxicity cause serious morbidity and mortality (11). Others like lead, mercury, cadmium ad arsenic etc. have no beneficial role and are positively toxic. Small amounts of fluoride help to prevent dental caries, but excess is harmful. Toxicity of these is of considerable concern in India because of their environmental burden. A number of heavy metals are required as micronutrients to plants. They act as cofactors as part of prosthetic groups of enzymes which are involved in a wide variety of metabolic pathways. However, when they are present in high levels, most heavy metals are toxic to plants (9). Various methods are available for removal and management of heavy metals, which involve technical inputs. Physico-chemical methods such a chemical precipitation, oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion-exchange and membrane technologies are widely used to remove heavy metal ions. These process may be ineffective or expensive, especially when then heavy metal ion concentrations in solutions are 1-100mgL^{-1}(4).

Microbiological processes are of significance in determining metal mobility and have actual and potential application in metal pollution. The microbiological aspect involves biostimulation(stimulating viable native microbial population), Bioaugmentation(artificial introduction of viable population), bioaccumulation(sequestration and accumulation of heavy metals by microbes) and biosorption (adsorption by living or dead microbes). Numerous studies have identified a number of potential bacterial sps capable of accumulating metals from the environment. Bacillus sps, Pseudomonas sps, Zoogloea ramigera, Streptomyces sps, are capable of absorbing most heavy metals. Considering the above perspectives, the present study was designed to cultivate iron oxidizing bacteria under laboratory conditions from iron rich soil samples. The isolated strains were further characterized and optimized for the effect of different pH and temperatures on their growth and iron sorption with application of optimal dose of Fe^{2+}.

II. MATERIAL AND METHODS

A. Collection of soil sample

The soils samples used for bacterial isolation were collected from the surrounding of Kanjamalai mountain range (Latitude 11^0 36' 58.57", Longitude 780 3'23.41"), a reserve forest located in Salem district, Tamilnadu, India.

The top 3 Cm soil was taken out and soil samples were collected at a depth of 1-10 Cm from randomly selected sites in each area. The collected samples were then transferred to a sterile polythene bags and were preserved at 4°C. The collected soil samples were passed through a sieve (2mm) to remove large pieces of debris.

B. Isolation of iron bacteria

Soil samples were serially diluted in sterile distilled water up to 10^{-5} dilutions then standard pour plate method were carried out, all the plates were incubated at 37^0C for 24 hours. The viable counts of bacteria were determined by counting visible colonies as colony forming unit per ml. Independently growing colonies were selected based on morphology, shape and colour. All the strains were purified by repeatedly streaking on citrate agar and stored at 4^0C for further studies (2). All the cultures were tested for cultivability by streaking on the various media Vinograski's medium, IM media (M622), R2A, CGYA, Citrate agar.

C. Characterization of the isolates

Standard macroscopic and microscopic morphological characteristics of all isolates were determined from plates incubated for 2 to 5 days. All isolates were examined microscopically (100x) for cellular and staining characteristics. Morphological features with respect to colony formation that were determined are shape, color, elevation of colonies and microscopic features like cell shape, Gram reaction, PHB staining for detection of polyhydroxybutyrate granules. Routine biochemical tests were conducted with all the isolates. Standard tests conducted are Indole test, MR (methyl red), VP (Voges- Proskauer), Nitrate reduction, Catalase, Urease, Oxidase, Citrate utilization and Nitrate tests. The isolates were tested for their ability to grow on various sugars and carbon substrate groups. The carbon sources tested were lactose, maltose, dextrose, fumarate, fructose, L-alanine, L- Aspargine, Butyrate, glucose, D-gluconate.

D. Iron Oxidation Test

Spent media was used. Cell lysates were prepared after collecting the cells from100ml early stationary phase culture by centrifugation. The cell pellets were resuspended in 5ml of 10mM HEPES(N-2 hydroxy-ethylpiperazine-N'-2-ethene sulfonic acid) and 5 µL of a saturated solution of phenylmethyl sulfonyl fluoride in isobutanol was added. The suspension were sonicated 10 times for 10 secs each under cooling in ice and then centrifuged at 15,000Xg for 10 mins. Samples of unknown protein content were spotted in decreasing amount on a nitrocellulose filter in a BIO-Dot microfiltration apparatus. The filter was removed from the apparatus and rinsed briefly in demineralized water. It was then incubated in a solution of 100µm $MnCl_2$ in 10mM HEPES, pH 7.5 (3).

E. Determination of Optimum Growth Temperature of The Isolates

In order to find out the optimum growth temperature of bacterial isolates, nutrient agar plates were prepared. Agar plates were inoculated with the isolates by a single straight streak method and were allowed to grow at different temperature such as 4, 10, 30, 37 and 45^0C for 48hrs. results were observed and recorded carefully.

F. Growth Response at Different pH

For testing the growth response of the isolates at different pH, buffered peptone water broth (4:1ratio) was adjusted to a wide range of pH i.e, 4.5, 6.5, 8.0. pH of the medium was adjusted with N/10 HCl or N/10 NaOH solution, as required. growth was recorded after 48hrs incubation by using spectrophotometer at 450nm absorbance.

G. Growth Response at Different Concentration of Nacl

To test the salt tolerance of the selected isolates peptone water with different concentration of NaCl *viz* 0, 2, 5, 7 and 10% were used. The inoculated test tubes were incubated at 37^0C. The growth was recorded after 48hrs.

H. Comparison of Different Iron Compounds on The Growth of The Isolates

The selected isolates were tested for their growth responses in different iron salt containing media by the disc diffusion metho (1). Isolates were seeded by spread plate method and then the paper disc impregnated with selected iron salt solutions ($FeCl_3$, $Fe_2(SO_4)_3$, $C_6H_8FeO_7.NH_3$) were placed on the surface of the agar plates. Each disc was 6mm in diameter and the concentration of salt was 50µg/disc. After 24hr of incubation, the plates were observed and the zones of inhibition and stimulation were recorded (12).

III. RESULT

A. Isolation and Characterization of iron Oxidizing Bacteria

A total of 92 isolates were successfully isolated from 312 morphologically distinct colonies and evaluated for their iron oxidizing ability. The analysis based on cell morphology revealed the presence of bacteria, belonging to the genera Leptothrix, Sphaerotilus, Pedomicrobium, Crenothrix and Siderococcus. Among the 92 isolates, 12 strains (IB-7, IB-16, IB-22, IB-29,IB-34, IB-39, IB-47, IB-53, IB-56, IB-62, IB-71 AND IB-83) had superior (dark spot) iron oxidizing ability. Growth of the isolates was maintained on Citrate agar and stored at 4˚C. Growth of the selected isolates was observed on Citrate agar medium. Most of the colonies were circular, rhizoid, flat, elevated, entire, rough, gloosy, and dark brown in colour. With regard to staining properties, all the 12 isolates were found to be gram negative and PHB positive. Comparison of biochemical and physiological properties of the isolated strains revealed important fundamental differences among bacterial groups with respect to their overall biochemical activity, temperature and pH tolerance and ability to degrade a variety of carbon sources. Most of the strains were negative to IMViC test which mean that they do not belong to Enterobacteriaceae group. All the isolates were catalase, oxidase, nitrate positive. All the isolates were able to utilise glucose, fructose and maltose as carbon source. Out of the 12 strains, 7 strains showed negative for fumarate, fructose, L-Alanine, L-aspargine, Butyrate, D-gluconate whereas rest of the 5 isolates showed positive for the above mentioned carbon sources (Table I). The iron oxidising bacteria isolated from the iron rich soil samples around Kanjamalai hills showed very distinct growth characteristics which distinguish it from the other known iron oxidising bacteria's reported by other researches. Unlike most of the other iron oxidising bacteria, this strain is aerobic. The result of the present study suggested that these organisms are chemoautotrophic bacteria which use inorganic compounds (H_2, NO^{-2}, NH_3, $Fe^{2+,}$ H_2S) as energy source, is different from other bacteria (12). Other iron bacteria which have been isolated to date are Gallionella, Metallogenium and Leptospirillum among others, which are morphologically very distinct and different from the isolates of the present study.

B. Growth Response of The Isolates at Different Temperature

Selected isolates were tested for their growth response at different temperatures. The optimum temperature for growth was found in between 30-37^{0}C. All the strains were able to grow at 47˚C (Fig:I) which did not differ much with the work done by Zeng (14) where it was 48˚C. The optimum temperature for growth in the present study was found in between 30-37˚C which correlated much with the work by Seder-Colomina (6). According to 'The Prokaryotes', *L.cholodnii* and *L.mobilis* showed optimum temperature at 35˚C (10).

C. Growth Response of the ISOLATES at different pH

Selected isolates were tested for their growth response at different pH level (pH 4.5,6.5, 8.0). The optimum pH for the isolates were found to be 6.5. But there was growth in pH 8.0 also (Fig:II). The optimum pH range of iron oxidising bacteria is generally from 6.5-7.5 but they are active over a wide range of pH (5). But there was growth in pH 8 also as mentioned in 'The Prokaryotes' (10). The bacteria in the present study is capable of growing under oxic and microaerophilic conditions; it is mesophilic and neutrophilic as it grows at optimum pH ranging between 6.5 and 7.5 respectively.

D. Growth response at Different Concentrations of Nacl

Effects of salt concentrations on the selected isolates were tested. The result showed that the organisms had a wide range of tolerance towards NaCl concentration. The maximum growth of the organisms were in between 0-2% NaCl. Growth reduced drastically at higher concentration of salt (Table II). Isolates IB-53 and IB-56 showed profuse growth at 10% NaCl. This was really surprising.

E. Growth response of the selected isolates in Different Iron Salt Containing Media as Determined by The disc diffusion Method

All the 12 selected isolates were tested For their oligodynamic reaction to three different iron salts (*Viz* $FeS0_4.7H_2O$, $FeCl_3$, $C_6H_8O_7.FeNH_3$) concentrations. In case of $FeSO_4$, isolates IB-29, IB-56, and IB-71 showed no effect. Out of 12 isolates, 3 isolates showed inhibition. Only one strain Viz IB-47 showed stimulation $FeSo_4$, IB-16, IB-39, IB-83 were inhibited first, then showed stimulation at a certain distance. Remaining 5 isolates Viz, IB-7, IB-22, IB-34, IB-53, IB-62 were stimulated first then at a certain distance showed inhibition. Out of the 12 isolates, 3 isolates IB-16, IB-34, IB-62 showed inhibition then stimulation with $Fe_2(SO_4)_3$. Two isolates IB- 47, and IB-53 showed inhibition. 7 isolates showed no effect with $FeCl_3$. In case of $C_6H_8O_7.FeNH_3$ most of the strain showed stimulation except IB-22, IB-39 and IB-83 showed no effect with this salt. Results were shown in Table III. In case of $FeSo_4$, 25% 0f the isolate showed inhibition, 58% of the isolates showed no effect with $Fe_2(So_4)_3$. Most of the isolates showed

stimulation with $C_6H_8FeO_7NH_3$ except the isolates IB-22, IB-39 and IB-83. the present study in case of $FeSo_4$ and $C_6H_8FeO_7NH_3$ was in full agreement whereas in $Fe_2(So_4)_3$ there exist a contrast with the hypothesis (12).

IV. CONCLUSION

The isolates showed growth stimulation around the disc impregnated with the ferrous salts. This indicated its ability to use iron at a higher concentration. Bopp *et al*.,(1983) mentioned that there are several bacterial strains that contain genetic determinants of resistance to heavy metal. These degraded heavy metals become nontoxic and perhaps possibly be used for agricultural and horticultural purposes also. As micronutrients which are required in ppm level for most of the economically important plants. Since these non-toxic heavy metals are needed in ppm level for animals feed and human foods. This opens a novel avenue of food and feed supplement manufacturing industry as a by-product through bioremediation using these isolates after further studies.

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Table :1 Effect of Carbon sources on growth of the isolates

Sl.No	Lactose	Maltose	Dextrose	Fumarate	L- Alanine	L-Aspargine	Butyrate	Fructose	D-Gluconate	Glucose
IB-7	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	+	+	+	+	+	+
IB-	+	+	+	+	+	+	+	+	+	+

IB-	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	-	-	-	D	-	D
IB-	+	+	+	+	+	+	+	+	+	+
IB-	+	+	+	+	+	+	+	+	+	+
IB-	+	+	+	+	-	-	-	D	-	D
IB-83	+	+	+	+	+	+	+	+	+	+

+ = Positive, - = Negative, D = delayed

Table II : Growth response at different concentrations of NaCl

Isolates	Concentration of NaCl (%)				
	0	2	5	7	10
IB-7	+++	+++	++	+	+
IB-16	+++	+++	++	+	-
IB-22	+++	+++	++	++	++
IB-29	++++	+++	++	+++	+
IB-34	+++	++	++	++	+
IB-39	++++	++++	++	+	-
IB-47	++++	++++	+++	++	++
IB-53	++++	++++	+++	+++	+++
IB-56	++++	+++	+++	+++	+++
IB-62	+++	++	++	+	-
IB-71	++++	++++	+++	+++	+
IB-83	+	++++	+++	++	+

Table III: Growth response of the selected isolates in different iron salt containing media

Isolates	$FeSO_4$		$Fe_2(SO_4)_3$		$C_6H_8FeO_7NH_3$
	Inhibition (mm)	Stimulation (mm)	Inhibition (mm)	Stimulation (mm)	Stimulation (mm)
IB-7	6	10*	NE		8
IB-16	11	5	10	8	11
IB-22	10	12*	NE		NE
IB-29	NE		NE		7
IB-34	12	15*	9	1	4
IB-39	16	8	NE		NE
IB-47	-	15	7	-	14
IB-53	13	14*	8	-	9
IB-56	NE		NE		3
IB-62	11	13*	5	2	5
IB-71	NE		NE		8
IB-83	9	4	NE		NE

Fig 1: Growth response of the isolates at different temperature

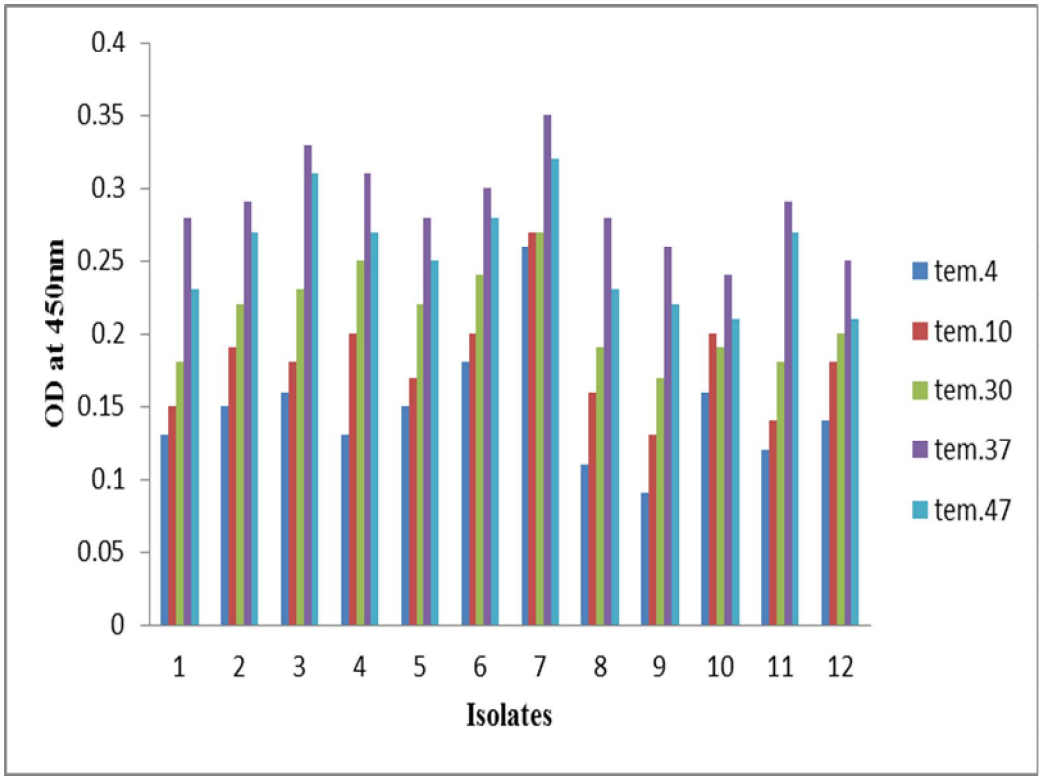
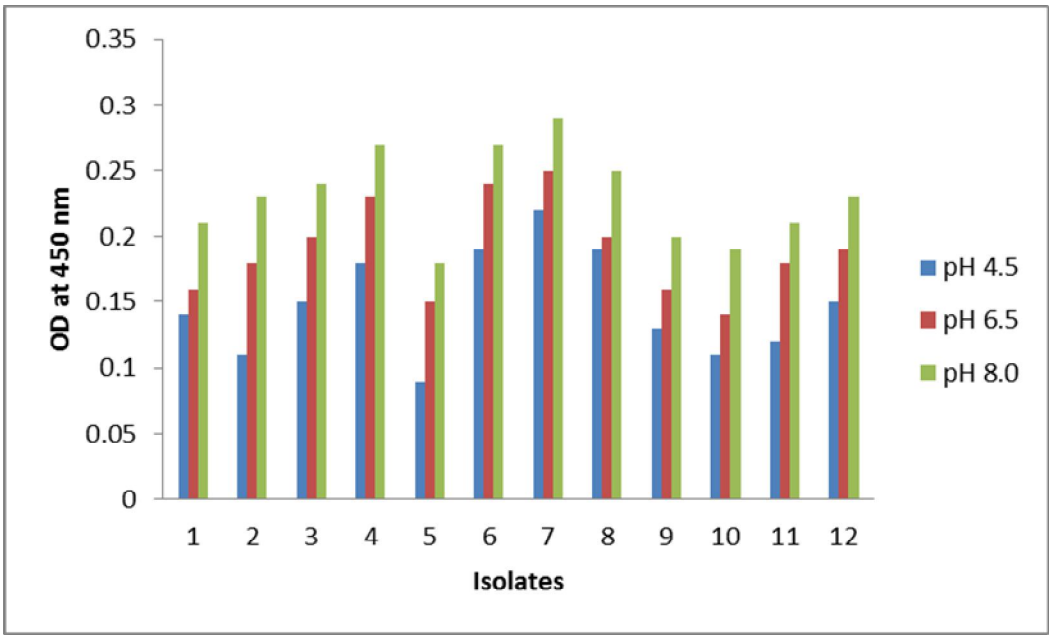
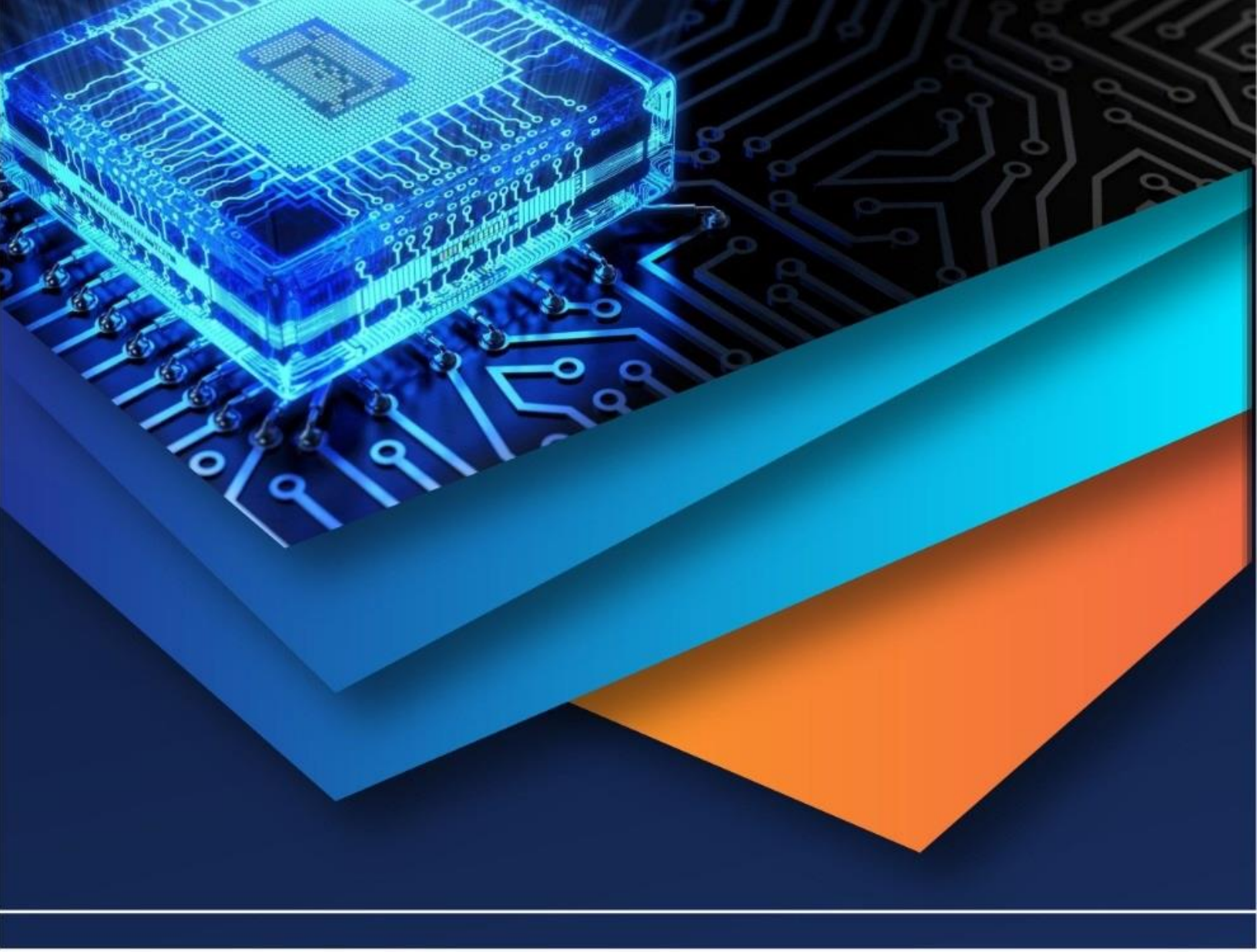


Fig 2: Growth response of the isolates at different pH





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