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RESEARCH ARTICLE

ANTIBIOTIC RESISTANCE AND BIOFILM DEVELOPMENT OF BACTERIAL ISOLATES IN CONTAMINATED WATER BODIES

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ABSTRACT

Studies suggest that the autolysis of bacterial strains also enhances the biofilm formation and the increase in incidence of pathogenicity. When the strains are subjected to adverse conditions either acidic or alkaline or to stress tolerance or to heat pressure, the parameters described above are all affected adversely. During this period, as a matter of escapism or to gain resistance power, the bacteria undergo autolysis. The pathogenecity of the organism tries to increase and also makes sure that its genomic DNA is released into the external surroundings. Pure river bodies are being contaminated by anthropogenic methods. Their contamination makes the water switch to acidic or alkaline environment. Depending on the source of contaminants the water becomes either highly acidic or highly alkaline. This fluctuation not only kills the flora and fauna of the ecosystem, but also adversely affects the pathogenicity of the bacterial species. When bacteria are subjected to adverse condition (pH), it undergoes autolysis as a mode of protection, survival and tolerance. Studies also suggest that the autolysis of bacterial strains also enhances the biofilm formation and the increase in incidence of pathogenicity. For the first time we try to find out the possible cooperative role of autolysis and biofilm formation and its antibiotic resistance power. The results confirmed of the presence of autolysis during adverse conditions. Our hypothesis of the pathogenic strains escaping from the unfavourable conditions by increasing its antibiotic resistance is a well marked figure in our thesis. The results also clearly showed that, the strains showed an enhancement in the formation of the biofilms.

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INTRODUCTION

Water contamination occurs when pollutants are discharged directly or indirectly into water bodies. Water contamination affects plants and organisms living in these water bodies. In almost all cases the effect is damaging not only to individual species and populations, but also to the natural biological communities (Auler, 2009). Water is typically referred to as contaminated when it is impaired by anthropogenic contaminants and either does not support a human use, such as drinking water, and/or undergoes a marked shift in its ability to support its constituent biotic communities, such as fish (Gupta et al., 2009). The specific contaminants leading to pollution in water include a wide spectrum of chemicals, pathogens, and physical or sensory changes such as elevated temperature and discoloration. While many of the chemicals and substances that are regulated may be naturally occurring (calcium, sodium, iron, manganese, etc.) the concentration is often the key in determining what is a natural component of water, and what is a contaminant (Blackman, 1998). High concentrations of naturally occurring substances can have negative impacts on aquatic flora and fauna. Pathogens can produce waterborne diseases in either human or animal hosts (Mitchell, 1957). Alteration of water's physical chemistry includes change in pH (acidic and alkaline), electrical conductivity, temperature, and eutrophication. Eutrophication is an increase in the concentration of chemical nutrients in an ecosystem to an extent that increases in the primary productivity of the ecosystem. Depending on the degree of eutrophication, subsequent negative environmental effects such as anoxia (oxygen depletion) and severe reductions in water quality (Chambers, 1985). Autolysis of bacterial strains enhances the biofilm formation and the increase in incidence of pathogenicity.

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When the strains are subjected to adverse conditions either acidic or alkaline or to stress tolerance or to heat pressure, the parameters described above are all affected adversely (Handwerger, 1985). During this period, as a matter of escapism or to gain resistance power, the bacteria undergoes autolysis. The pathogenecity of the organism tries to increase and also makes sure that its genomic DNA is released into the external surroundings (Ito, 1999). Bacterial autolysis is the result of the action of endogenous peptidoglycan hydrolases (autolysins) that hydrolyse specific bonds in the protective and shape-maintaining cell wall peptidoglycan (Pooley, 1970). On the basis of their cleavage specificity, autolysins are classified as: N-acetylmuramidase, N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase and endopeptidase (Valence and Lortal, 1995; Cibik and Chapot- Chartier, 2000). Bacteria need a physiological pH inside their cells, just like all other living organisms. Their ability to survive in extreme pH (either high or low) depends on their ability to correct for the difference between inside and out pH affects bacteria the same way it affects all living things. Extremes of pH affect the function of enzyme systems by denaturing them (Sheehan, A, 2006). However, bacteria become adapted over time to their surroundings. Microbial growth in alkaline conditions presents several complications to normal biochemical activity and reproduction, as high pH is detrimental to normal cellular processes. For example, alkalinity can lead to denaturation of DNA, instability of the plasma membrane and inactivation of cytosolic enzymes, as well as other unfavorable physiological changes (Stabb, 1994). Thus, to adequately circumvent these obstacles, alkaliphiles must either possess specific cellular machinery that works best in the alkaline range, or they must have methods of acidifying the cytosol in relation to the extracellular environment. To determine which of the above possibilities an alkaliphile uses, experimentation has demonstrated that alkaliphilic enzymes possess relatively normal pH optimums (Stoodley, 1994). The determination that these enzymes function most efficiently near physiologically neutral pH ranges (about 7.5-8.5) was one of the primary steps in elucidating how alkaliphiles survive intensely basic environments. Because the cytosolic pH must remain nearly neutral, alkaliphiles must have one or more mechanisms of acidifying the cytosol when in the presence of a highly alkaline environment (Kotiranta, 2000).

Many bacteria lyse when growth of the cultures ceases, irrespective of whether growth has stopped because of the exhaustion of the growth medium or the addition of toxic substances such as the penicillins to the cultures. When bacteria are placed in unsuitable conditions that drastically disturb their metabolism, spontaneous lysis may occur by their own intarcellular glycanase peptidase which are found in any bacteria synthesizing peptidoglycan, this phenomenon is called "autolysis". These autolytic enzymes may cause lysis of other bacteria in close proximity from outside (Tomasz, 1970). Bacterial cells at the exponential phase are most sensitive to autolysis but those in stationary phase are tolerant. This may originate in perfectly balanced and well controlled system of peptideglycan-hydrolyzing and peptidoglycan-synthesizing enzymes occurring in the growth period. In such growth phase autolysis may be induced by the environmental factors (Brooun, 2000).

The present study is mainly focussed to find out the possible cooperative role of autolysis and biofilm formation and its antibiotic resistance power. The bacterial isolates are cultured and subjected to various treatments of pH. Thereafter the bacteria are screened for their growth and autolytic potential. Mainly the experiment is designed to confirm of its resistance power against the antibiotics in fluctuating environments.

MATERIALS AND METHODS

Collection of sample: water sample was collected from the lake in sterile containers. The samples were further taken to the lab for further studies. There was a time when the Benniganahalli Lake water was used to irrigate paddy fields in the surroundings of Old Madras Road. When the agricultural lands became residential layouts, the lake became a place for dumping garbage and for other activities. At present sewage water from surrounding areas flows into the lake. The lake also serves as garbage dumping place for people around the vicinity.

Isolation of bacteria from sample: The samples were serially diluted with sterile distilled water and the tubes labelled as 10^{-9} and 10^{-10} are plated onto the nutrient agar and then incubated at 37˚c for 24 hours. The colonies obtained were further subcultured to obtain pure colonies. The pure colonies obtained were used for the experimental study.

Growth in varied pH conditions: The bacteria isolated were used for the culturing in different pH conditions. The study is used to screen the growth and establishment of the isolate to varying environmental conditions. Briefly the assay was performed using different tubes maintained at different pH (control, and alkaline). The pH was adjusted in each tube in the range of 7.0-8.0 using a pH meter HANNAH instruments, H1 2215 pH/ORP meter. The samples were inoculated into each tube and grown overnight at 37˚C. The O/N grown cultures were then inoculated onto respective petriplates and incubated overnight at 37˚C. The colonies obtained were then checked for the colony morphology and other experimental objectives.

16s rRNA sequencing: The treated colonies along with the control were then checked for the three parameters like, colony morphology, colony edges/margins and colony elevation. The colonies with peculiar variations in the morphology were selected and sent 16s rRNA sequencing (Macrogen, Seoul, Korea).

Bacterial growth curve: The colonies selected were further used for the bacterial growth assay. To check for the limitations in their growth the cultures grown at different Ph were studied. 6 tubes of nutrient broth labelled and maintained in the range of 7.0-8.0 are used. The isolate was inoculated into the broths and were incubated overnight at 37˚c for 24 hrs. The overnight cultures were then checked for growth by reading the absorbance values at 670nm using a UV1800 spectrophotometer, Shimadzu. The growth was observed subsequently over the next three days at different time intervals.

Isolation of genomic DNA: Briefly 2ml of O/N culture was taken in a centrifuge tube and centrifuged at 5000rpm for 10 minutes. The supernatant obtained was discarded and the pellet

was resuspended in 300µl of lysis buffer (1M Tris HCl, 0.5M EDTA, 1% SDS) and gently vortexed. 500µl of TE buffer (1M Tris HCl, 0.5M EDTA) and 500µl of chloroform are further added to the contents and centrifuged at 9000rpm for 10 minutes. The upper aqueous layer is collected in a fresh tube and added with $1/10^{th}$ volume of 3M sodium acetate. The contents are mixed gently by tapping and the DNA is pelleted by adding 2 volumes of chilled ethanol. The DNA is further washed with 70% ethanol and finally resuspended in 50µl of TE buffer.

Extraction of extracellular DNA: Bacteria were grown to late exponential phase (OD=2.0) in 5 ml of nutrient broth at 37˚C. They were then centrifuged at 5000rpm for 10 minutes and the supernatant was collected in a fresh tube. The supernatant was now passed through sterile filters (0.2µm) and then the DNA is pelleted with chilled ethanol. The DNA thus obtained was further resuspended in TE buffer and stored at -20^0C.

Bacterial homotyping assay: This assay was performed to show the similarity of the strains used in the study. Hind III and EcoR I enzymes are used for the study. Briefly to 30µl of nuclease free water 4µl of assay buffer (EcoR1 and HIND III respectively) were added to each tube. To the respective tube 8µl of DNA was added. 1µl of enzyme (EcoR1 and HIND III respectively) were added and the tubes were incubated for 1-2 hours at 37ºC. The digested samples were analysed on 0.8% agarose gel. 1kb ladder is used as molecular marker.

Whole cell autolysis assay: Autolysis assays were performed accordingly to the protocol described by Mani *et al*. 1993. Briefly overnight culture was diluted to an OD of 0.1 with nutrient broth and incubated for 3hrs at 37^0 C on an orbital shaker (250 rpm). Following incubation the cultures were centrifuged at 4000rpm for about 10 minutes. The pellet obtained is washed twice with sterile cold water (4C) and resuspended in autolysis buffer ((50mM Tris HCl, 0.05% Triton X). 1ml of this suspension was used for the absorbance study 580nm. The readings were taken at an interval of 30 minutes.

Biofilm Cultivation: The overnight culture suspension of the treatment groups were diluted to a ratio of 1: 200 using Nutrient broth +Glucose solution. The diluted cultures were added to the microtitre plate and incubated at 37C for 24 hrs. The 1st well being the control (without treatment).The plate was incubated for overnight at 370C. After incubation the wells were washed with 200µl of PBS pH7.4 three times. The plates were then air dried and stained with 2% crystal violet for 15 minutes. The plates were then rinsed under running tap water, air dried and then crystal violet was solubilised in 200µl of ethanol:acetone 80:20. Absorbance was recorded at 590nm. The experiment was done for the different treatments separately.

Primary adherence assay: The cell suspension of the treatment groups were inoculated into respective flasks with Nutrient broth containing 0.5% glucose. One of the flask containing nutrient broth with glucose is labelled as control. The control is used separately for each species. The flasks were then incubated at 37C overnight. Following incubation, 200µl of the broth with the culture was diluted to an absorbance of 0.1 at 578 nanometres with nutrient Broth containing 0.5% glucose. 10µl of the suspension was added to slides and incubated for two hours at 37C. After incubation the slides was washed three times with PBS (pH7.4). The cells were then fixed with glycerine solution and then carried with the gram staining process. Adherent bacterial cells were observed under 40X and mean count was taken for 5 microscopic fields.

Antibiotic resistance: Briefly two sets of five tubes containing 10ml of nutrient broth in each of them was taken for six different ph in increasing order i.e. pH 7.0, pH 7.2, pH 7.4, pH 7.6, pH 7.8, pH 8.0. To the respective tube the antibiotic ampicilin (100mg/ml) was added in increasing order i.e. 1µl, 2µl, 3µl, 4µl and 5µl. The tubes with the respective pH were inoculated with the sample and incubated and their absorbance was read at 670nm at specific intervals of 12 hours, 24 hours, 36 hours 48 hours and 60 hours.

RESULTS AND DISCUSSION

Water sample: The water sample obtained from the lake was observed for a few physical parameters. The water is a bit of turbid with a pH of approximately 7.68. Colonies were enumerated on both the 10^{-9} and 10^{-10} plates. The colonies with distinct morphology were randomly picked up for the treatments. The colonies selected were labelled as X, Y, Z, A and B and then plated onto plates and maintained on slants.

Exposure to treatments: The cultures labelled as X, Y, Z, A and B were then treated with different pH levels. The treated colonies were then checked for the three parameters like, colony morphology, colony edges/margins and colony elevation. The colonies on control showed irregular colonies, undulate margins and raised elevation. All the colonies were similar in terms of colony morphology and elevation. But the pH treatment showed variation on the colony edges or margins. The two colonies viz., X and Y showed variations in the morphology of the edges on treatments. These two colonies were selected for the experiment.

16s rRNA sequencing: The colonies with peculiar variations in the morphology were made of pure culture and then sent for sequencing. Both X and Y showed colony morphology variations, and colony y was sent for 16sRNA sequencing. The sequencing protocol followed was to be capillary based (Macrogen, Seoul, Korea). The sequence was found to be around 982bp.

The sequence was then checked for sequence similarity among the organisms for identity. The sequence was blast using Blastn. The query was found to have 99% identity with Bacillus cereus, and a Bacillus sp.

Isolation of genomic DNA: Agarose gel electrophoresis: the DNA samples extracted were then run on 0.8% agarose gel together with a marker. 10µl of sample was loaded onto each well. The appearance of slender filament like structures confirms the presence of the DNA, which might have been released into the surroundings during the autolysis (Refer fig:).

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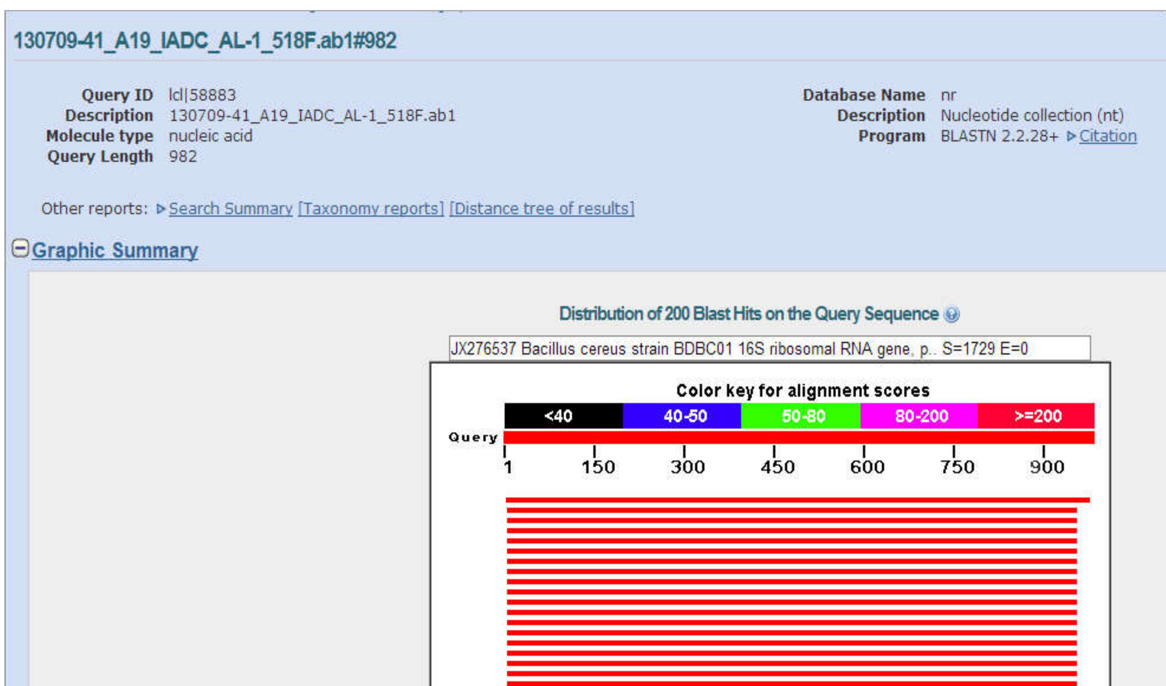

Sequences producing significant alignments:

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Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus cereus strain BDBC01 16S ribosomal RNA gene, partial sequence	1729	1729	98%	0.0	99%	JX276537.1
Bacillus sp. KS146 16S ribosomal RNA gene, partial sequence	1729	1729	96%	0.0	99%	JQ912679.1
Bacillus sp. C4-9 16S ribosomal RNA gene, partial sequence	1729	1729	96%	0.0	99%	JX134435.1
Bacterium CulalenE32 16S ribosomal RNA gene, partial sequence	1729	1729	96%	0.0	99%	KC484960.1
Bacterium CulaenoE10G 16S ribosomal RNA gene, partial sequence	1729	1729	96%	0.0	99%	KC484954.1
Bacillus anthracis strain G3-4-80 16S ribosomal RNA gene, partial sequence	1729	1729	96%	0.0	99%	KC494340.1
Bacillus cereus partial 16S rRNA gene, strain AntCr49	1729	1729	96%	0.0	99%	HF570083.1
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	1729	1729	96%	0.0	99%	NR_074453.1

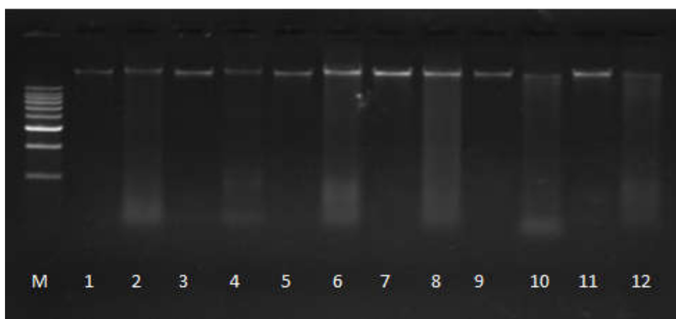

Fig. 1. 0.8% agaorse gel showing the genomic DNA and extracellular DNA bands. Lane M is 1kb marker, Genomic DNA: lanes 1,3,5,7,9,11; Extracellular DNA : lanes 2,4,6,8,10,12. Treatments: Lane 1 & 2: pH 7.0; Lane 3 & 4: pH 7.2; Lane 5 & 6: pH 7.4; Lane 7 & 8: pH 7.6; Lane 9 & 10: pH 7.8; Lane 11 & 12: pH 8.0

Homotyping: The cultures after treatments were then analysed by restriction digestion. The six isogenic types had identical banding patterns by electrophoresis (PFGE) using *Hind*III restriction enzyme digestion. The banding pattern showed similarity confirming of the homotypes or isogenic types.

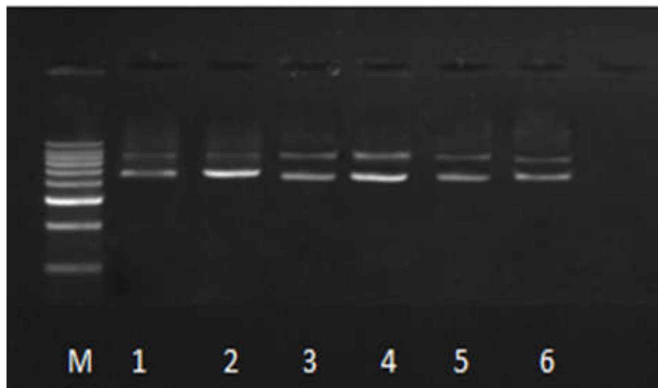

Fig. 2. 0.8% agaorse gel showing the restriction digestion . Lane M is 10kb marker, Lane 1:ph 7.0, Lane 2: ph 7.2, Lane 3: ph 7.4, Lane 4: ph 7.6, Lane 5: ph 7.8, Lane 6: ph 8

Bacterial growth curve: The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or Optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism.

A two way ANOVA between the incubation time and pH treatments was conducted to compare bacterial growth of the culture Y. All effects were statistically significant at the 0.05 significance level. There was a significant effect in growth rate at different ph and incubation time remembered at the $p<0.05$ level. Culture Y showed significance to the incubation time ($F(3,15)$ =1803.93, p =2.10E-19) and to the pH treatments ($F(5,15)$= 0.56656, p=0.72442). Our results suggest that there was a significant effect on growth rate of cultures Y with increase in ph treatment.

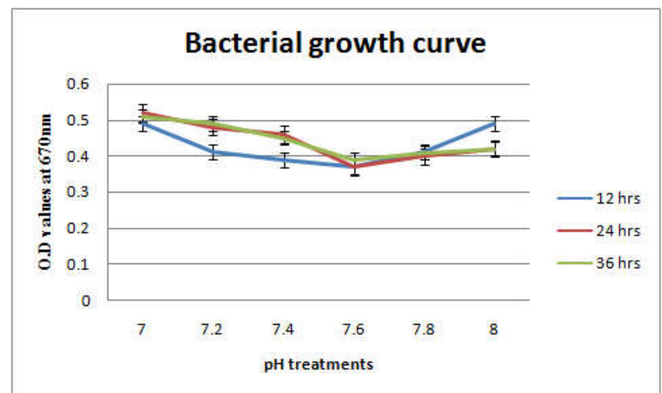

Fig. 3. Graph showing the O.D values of bacterial growth curve of bacterial culture X. All the values are the averages of triplicates. The pH treatments are at 7, 7.2, 7.4, 7.6, 7.8 and 8. The OD values were noted after incubation at different time intervals

Whole cell autolysis assay: Autolysis has been demonstrated by this protocol of autolysis assay. When bacteria are placed in unsuitable conditions that drastically disturb their metabolism, spontaneous lysis may occur by their own intercellular glycanase peptidase which were found in any bacteria synthesizing peptidoglycan (Boutrou, 1998)

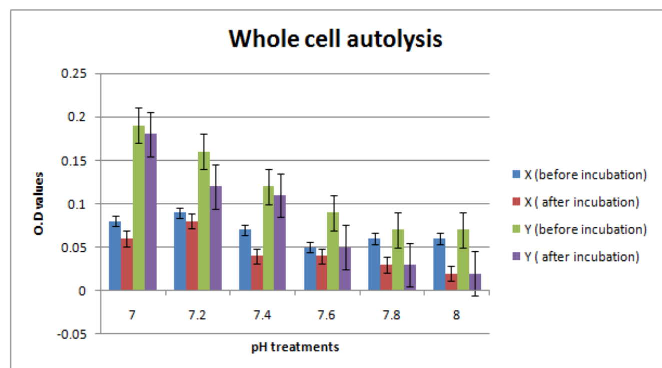

Fig. 4. Graph showing the O.D values of whole cell autolysis assay of bacterial culture X and Y. All the values are the averages of triplicates. The pH treatments are at 7, 7.2, 7.4, 7.6, 7.8 and 8. The OD values were noted after incubation with autolysis buffer for 3 hours

A two way ANOVA between the incubation time and pH treatments was conducted to compare the effect of pH on whole cell autolysis in both the cultures X and Y. All effects were statistically significant at the 0.05 significance level. There was a significant effect of alkalinity and the incubation time on whole cell autolysis in both the cultures X and Y remembered at the $p<0.05$ level. Culture X showed significance to the incubation time ($F(1,5)$ = 22.2727, p =5.25E-03) and to the pH treatments ($F(5,5)$= 8.36364, p=0.01798). Culture Y also showed a significance to the different Ph treatment ($F(1, 5)$ = 20.2809, p = 6.38E-03) and to the number of days ($F(5, 5)$ = 41.7191, p = 0.00044). Our results suggest that there was a significant increase in the rate of autolysis between control and other different pH.

Before incubation, cells showed normal growth, whereas after incubation with autolysis buffer, there was a significant increase in the rate of autolysis and reduced in cell growth.

Cultivation of biofilms: There was a significant increase in the rate of biofilm formation in both bacterial cultures X and Y at different pH. It was found from the results that the rate of biofilm formation increased with increase in pH.

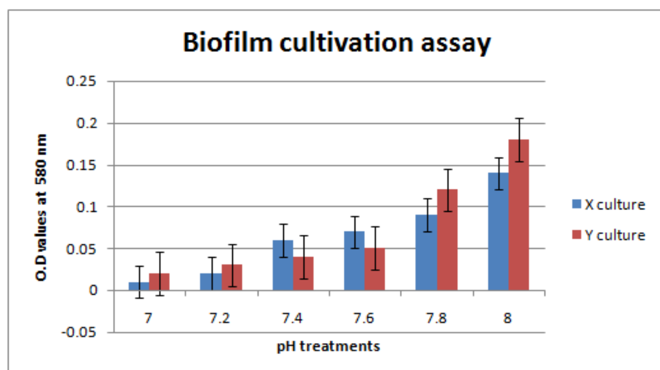

Fig. 5. Graph showing the O.D values of biofilm cultivation assay of bacterial culture X and Y. All the values are the averages of triplicates. The pH treatments are at 7, 7.2, 7.4, 7.6, 7.8 and 8. The O.D values were noted after solubilsation of the stained sample with ethanol solution following an incubation time of 24 hours

A two way ANOVA between the bacterial cultures X and Y and pH treatments was conducted to compare the effect of pH on biofilm formation in both the cultures X and Y. All effects were statistically significant at the 0.05 significance level. There was a significant effect of alkalinity on biofilm formation in both the cultures X and Y remembered at the $p<0.05$ level. Culture X and Y showed significance to the pH treatment ($F(5,5)$ =19.5745, p =2.68E-03) and the biofilm formation ($F(1,5)$= 0.42553, p=0.54299). Our results suggest that there was a significant increase in the rate of biofilm formation in both bacterial cultures X and Y at different pH. It was found from the results that the rate of biofilm formation increased with increase in pH.

Primary adherence assay: There was a significant increase in the adherence of the bacterial cells of both cultures X and Y at different pH. It was found that the adherence of cells was more at alkaline pH when compared to control.

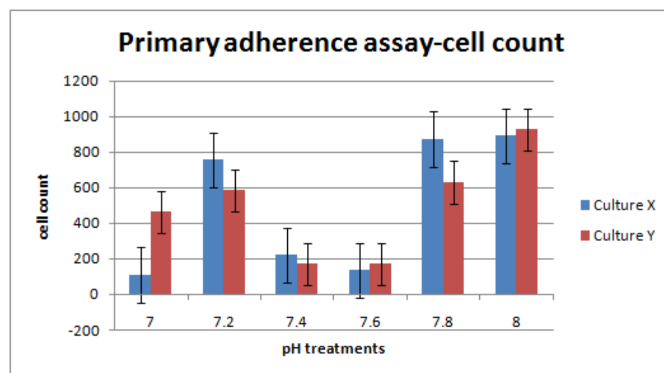

Fig. 6. Graph showing the cell count of primary adherence assay of bacterial culture X and Y. All the values are the averages of triplicates. The pH treatments are at 7, 7.2, 7.4, 7.6, 7.8 and 8. The cells were incubated for 2 hours, then counted after Gram staining

A two way ANOVA between the pH treatments and bacterial cultures was conducted to compare the adherence of bacterial cells at different pH in both the cultures X and Y. All effects were statistically significant at the 0.05 significance level. There was a significant effect adherence of bacterial cells of both culture X and Y at alkaline Ph remembered at the $p<0.05$ level. Culture X and Y showed significance to the pH treatment ($F(5,5)$ =9.45289, p =1.38E-02) and to the adherence of the bacterial cells ($F(1,5)$= 0.00607, p=0.94091). Our results suggest that there was a significant increase in the adherence of the bacterial cells of both cultures X and Y at different pH. It was found that the adherence of cells was more at alkaline pH when compared to control.

Antibiotic resistance: There was a significant effect of alkalinity and effect of concentration of ampicillin on the growth of the culture Y.

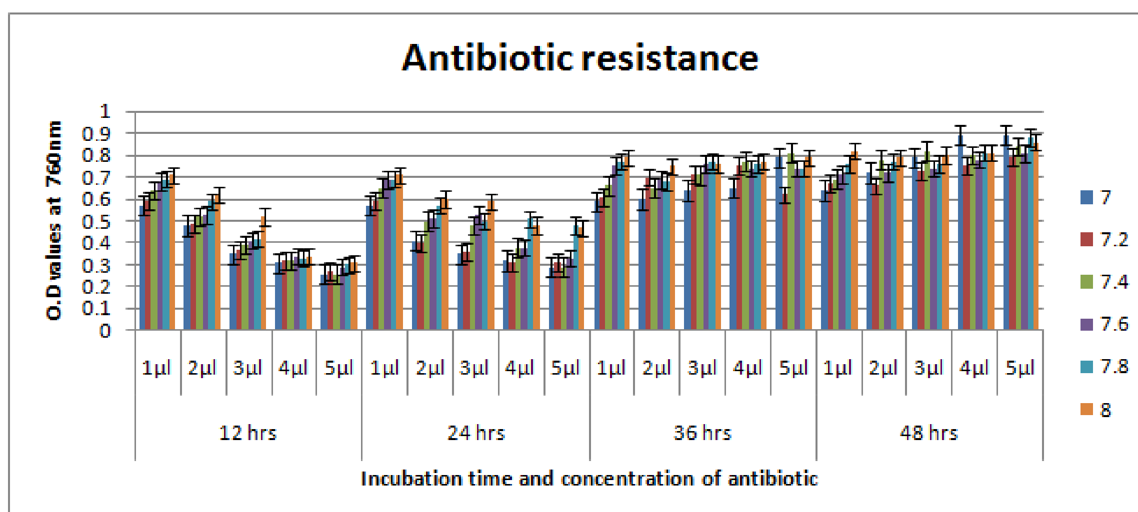

Fig. 7. Graph showing the O.D values of antibiotic resistance assay of bacterial culture Y. All the values are the averages of triplicates. The pH treatments are at 7, 7.2, 7.4, 7.6, 7.8 and 8. The O.D values were recorded at different time intervals after incubation

The culture Y showed a significant decrease in growth after 12 hours incubation with varying concentrations of ampicillin. After the incubation at 36 hours and onwards the bacterial culture Y showed increase in growth with increase in pH and concentration of ampicillin indicating that it might be developing resistance to ampicillin. A two way ANOVA of the bacterial culture Y with different pH treatments and concentrations of ampicillin at different time intervals was conducted to compare the effect of pH and the concentration of ampicillin on the growth of the bacterial culture Y. All effects were statistically significant at the 0.05 significance level. There was a significant effect of alkalinity and effect of concentration of ampicillin on the growth of the culture Y .The culture Y showed a significant decrease in growth after 12 hours incubation with varying concentrations of ampicillin. After the incubation at 36 hours and onwards the bacterial culture Y showed increase in growth with increase in ph and concentration of ampicillin indicating that it might be developing resistance to ampicillin.

Conclusion

Taking together, the results suggest the phenomenon of autolysis as a protective and escapism mechanism of the bacterial strains. When subjected to adverse conditions, the strains undergo autolysis and as a determined procedure, they try to form more biofilm levels and increase their antibiotic resistance. The results of the bacterial growth curve reveals that there was a significant effect on the growth rate of the culture with increase in pH treatment. As the pH increased the growth rate of the bacterial culture was increasing with the autolysis value also. The rate of autolysis in the bacterial culture showed evident increase between control and other different pH. Before incubation, the cells showed normal growth whereas after incubation with autolysis buffer there was a significant increase in autolysis and reduced cell growth. With the process of autolysis, the bacteria also showed an increase in the rate of biofilm formation at different ph treatments. It was found that the rate of biofilm formation increased with increase in pH. The adherence of the bacterial cells to surfaces was tested and the bacteria showed increased adherence when exposed to different ph treatments. The adherence of the bacterial cells was higher at alkaline pH. Antibiotic resistance in the bacteria showed a prominent effect of alkalinity and effect of concentration of ampicillin on the bacterial culture. The culture showed a decrease in growth after 12 hours incubation at different concentration of ampicillin. From 36 hours onwards, they showed increased growth indicating they might have developed resistance to the antibiotic.

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