CHAPTER FOUR

RESULTS

4.1 Siderophore production in *Aeromonas hydrophila.*

The siderophore activity of the *Aeromonas hydrophila* isolates was analyzed under iron-limiting and iron-rich cultural conditions at 28°C and 37°C. A comparison was made between the siderophore producing ability of the clinical and environmental isolates of *A. hydrophila.* The relationship between the presence or absence of plasmids and siderophore production of *A. hydrophila* isolates was also investigated by curing of the plasmids by treating the isolates with acridine orange. This latter experiment was undertaken in view of at least one report indicating that plasmids have a role in moderating the siderophore production in *A. hydrophila* (Borrego *et al.*, 1991). The detailed results of the siderophore activity of the *A. hydrophila* isolates are summarised in Appendix A. The findings of this study have been published recently (Naidu and Yadav, 1997) [Appendix G].

4.1.1 Siderophore assay on CAS plates

All the 30 *A. hydrophila* isolates tested gave positive results with an orange halo (2 - 3 mm) around the colonies on CAS agar plates after incubation for 24h at both 28°C and 37°C (Table 4.1).

Table 4.1

The number of clinical and environmental isolates of *Aeromonas hydrophila* exhibiting siderophore production on CAS plates.

Source of isolates	No. of isolates	No. (%) positive for siderophore production	
		28°C	37°C
Clinical isolates	19	19 (100 %)	19 (100 %)
Environmental isolates	11	11 (100 %)	11 (100 %)
Total	30	30 (100 %)	30 (100 %)

4.1.2 Siderophore assay with CAS assay solution

The CAS assay of Schwyn and Neilands (1987) was used to detect siderophores in the cell-free supernatant of the *A. hydrophila* isolates. All the clinical and environmental isolates produced siderophores under iron-limiting conditions. More high producers were detected under iron-limiting conditions compared to iron-rich conditions (Table 4.2).

Table 4.3 shows the mean siderophore activity of the clinical and environmental isolates under iron-limiting and iron-rich conditions. Under iron-limited conditions at 28°C, the mean production of siderophores increased almost 100% for the clinical isolates and 50% for the environmental isolates when compared to normal iron-rich culture conditions. Similar increases of 90% for the clinical isolates and 50% for the environmental isolates were noted for siderophore production at 37°C. These increases were significant ($p < 0.01$) by the Student's t-test (Table 4.3).

At 28°C. the clinical isolates were found to produce 73% more siderophores under iron-limiting conditions than the environmental isolates while at 37°C, the clinical isolates possessed 44% more siderophore activity than the environmental isolates (Table 4.4).

Table 4.2

The clinical and environmental isolates of *Aeromonas hydrophila* grouped into different categories of siderophore producers under iron-limiting and iron-rich conditions.

	No. of clinical isolates				No. of environmental isolates			
	28°C		37°C		28°C		37°C	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
Non-siderophore producer (%)	–	–	–	–	–	–	–	–
Low siderophore producer (%) ($A_{630} \geq 1.00$ to 1.50)	3 (15.8%)	11 (57.9%)	4 (21.1%)	15 (78.9%)	4 (36.4%)	10 (90.9%)	5 (45.5%)	10 (90.9%)
Medium siderophore producer (%) ($0.50 < A_{630} < 1.00$)	5 (26.3%)	6 (31.6%)	4 (21.1%)	3 (15.8%)	3 (27.3%)	1 (9.1%)	2 (18.2%)	1 (9.1%)
High siderophore producer (%) ($A_{630} \leq 0.50$)	11 (57.9%)	2 (10.5%)	11 (57.9%)	1 (5.3%)	4 (36.4%)	–	5 (45.5%)	–
Total	19 (100 %)	19 (100 %)	19 (100 %)	19 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)

Note: A low absorbance value indicates high production of siderophores and vice-versa.

Table 4.3

Comparison between the siderophore production of the *Aeromonas hydrophila* isolates under iron-limiting and iron-rich conditions.

	Clinical				Environmental			
	28°		37°C		28°C		37°C	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
No. of positive isolates (%)	19 (100 %)	19 (100 %)	19 (100 %)	19 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)
Mean ± SD*	0.47 ± 0.38	0.99 ± 0.31	0.57 ± 0.39	1.09 ± 0.28	0.81 ± 0.45	1.22 ± 0.13	0.81 ± 0.50	1.20 ± 0.19
Student's T-test	t = 4.50 p < 0.01		t = 4.60 p < 0.01		t = 4.07 p < 0.01		t = 3.26 p < 0.01	

* Mean ± Standard deviation absorbance value at 630nm of reactants with ferrous ion (Fe^{2-}) present (+Fe) or absent (-Fe) in culture conditions.

Table 4.4

Comparison between the siderophore production of clinical and environmental isolates of *Aeromonas hydrophila* at 28°C and 37°C under iron-limiting and iron-rich conditions.

	28°C		37°C	
	-Fe	**+Fe**	**-Fe**	**+Fe**
Clinical (Mean ± SD)*	0.47 ± 0.38	0.99 ± 0.31	0.57 ± 0.39	1.09 ± 0.28
Environmental (Mean ± SD)*	0.81 ± 0.45	1.22 ± 0.13	0.81 ± 0.50	1.20 ± 0.19
Student's T-test	$t = 2.13$ $p < 0.025$	$t = 2.26$ $p < 0.025$	$t = 1.41$ $p < 0.10$	NS $p > 0.10$

* Mean ± standard deviation absorbance value at 630nm of reactants.
NS: Non-significant

4.1.3 Effect of growth temperature on siderophore production

Both under iron-limiting and iron-rich conditions, clinical isolates produced slightly lower amounts of siderophores at 37°C compared to 28°C. A non-significant 20% decrease was observed in the siderophore production of the clinical isolates grown under iron-limiting conditions at 37°C compared to 28°C. Under iron-rich conditions the production of siderophores decreased at 37°C compared to 28°C by 10%. However, this difference was not seen with the environmental isolates (Table 4.5).

4.1.4 Characteristics of plasmid profile and curing

Twenty-four of the 30 isolates of *A. hydrophila* tested contained plasmids (Table 4.6). Half of the isolates with plasmids harbours more than one plasmid. Four environmental isolates contained only large plasmids (>20 Mda) while 15 isolates contained only small plasmids (<20 Mda) and 5 isolates contained both large and small plasmids. The maximum number of plasmids found in clinical isolates was 4 and in environmental isolates was 7. The clinical isolates contained 18 plasmids in the range of 5-16 Mda but only one environmental isolate harboured just one plasmid in this range. The environmental isolates contained 16 plasmids below 5.0 Mda while the clinical isolates harboured 8 plasmids in the same range. The agarose gel electrophoresis profiles of the plasmids are shown in Figures 4.1 - 4.5.

Table 4.5

Comparison between the siderophore production of the *Aeromonas hydrophila* isolates at 28°C and 37°C.

	Clinical isolates				Environmental isolates			
	-Fe		+Fe		-Fe		+Fe	
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
No. of positive isolates (%)	19 (100 %)	19 (100 %)	19 (100 %)	19 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)
Mean ± SD*	0.47 ± 0.38	0.57 ± 0.39	0.99 ± 0.31	1.09 ± 0.28	0.81 ± 0.45	0.81 ± 0.50	1.22 ± 0.13	1.20 ± 0.19
Student's T-test	Non-significant p > 0.05		Non-significant p > 0.05		Non-significant p > 0.05		Non-significant p > 0.05	

* Mean ± standard deviation absorbance value at 630nm of reactants.

Table 4.6

Plasmid profile of *Aeromonas hydrophila* isolates compared to the siderophore production before and after curing.

Isolate No.	No. of plasmids	Molecular Weight (Mda)	Cultured at	
			28°C	37°C
SL2	1	11.1	N.C	N.C
SL3	3	37.0,12.5, 3.9	N.C	-
SL4	3	9.1, 5.5, 3.9	N.C	-
SL6	0		N.C	N.C
SL8	3	35.7, 10.3, 3.9	N.C	N.C
SL9	4	3.9, 3.4, 1.8, 1.2	N.C	N.C
SL10	0		+	+
SL11	1	10.6	N.C	N.C
SL12	1	11.1	N.C	N.C
SL13	1	11.1	+	+
SL14	1	12.7	N.C	N.C
SL15	1	12.1	N.C	+
SL16	4	9.9, 7.3, 5.9, 4.0	+	N.C
SL17	2	11.8, 9.6	N.C	-
SL18	1	15.3	N.C	N.C
SL19	3	15.3, 4.1, 3.6	N.C	N.C
SL20	0		-	+
SL21	2	39.0, 15.3	N.C	N.C
SL22	2	3.7, 3.2	N.C	N.C
X2	0		N.C	+
X8	7	33.8, 10.9, 4.3, 4.2, 3.8, 3.7, 3.3	+	N.C
X13	1	36.4	N.C	N.C
X14	1	37.1	-	-
X36	0		N.C	N.C
X38	0		N.C	N.C
X52	1	35.7	+	-
X53	5	3.7, 3.6, 3.4, 2.9, 2.2	N.C	N.C
X54	1	35.7	N.C	N.C
E29	6	31.0, 5.0, 4.7, 3.8, 3.7, 3.2	N.C	-
H10	1	2.7	N.C	N.C

When underlined it indicates that the plasmids were retained after curing.
N.C: No change in siderophore production after curing.
Increase (+) or decrease (-) in siderophore production after curing. A difference of at least 0.50 in the absorbance values before and after curing is considered significant.

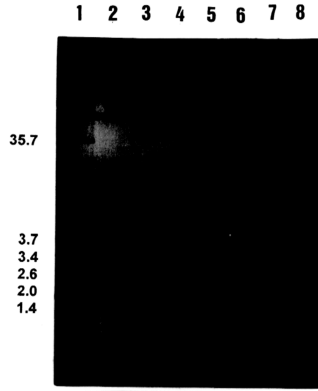

Figure 4.1

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL2, SL3, SL4, SL6, SL8, SL9 and SL10. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. SL2
3. SL3
4. SL4
5. SL6
6. SL8
7. SL9
8. SL10

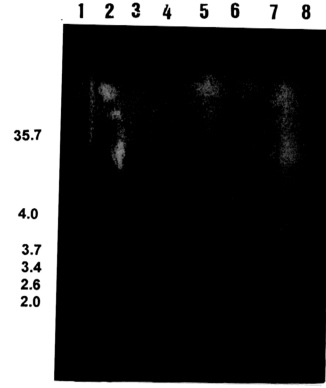

Figure 4.2

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL11, SL12, SL13, SL14, SL15, SL16 and SL17. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. SL11
3. SL12
4. SL13
5. SL14
6. SL15
7. SL16
8. SL17

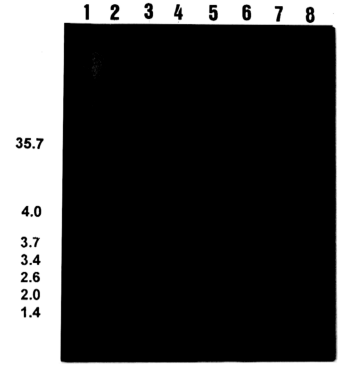

Figure 4.3

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL18, SL19, SL20, SL21, X2, X38 and X8. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. SL18
3. SL19
4. SL20
5. SL21
6. X2
7. X38
8. X8

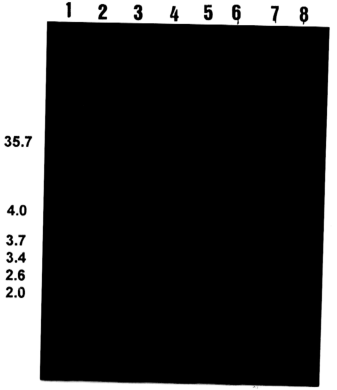

Figure 4.4

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates X13, X14, X36, X52, X53, X54 and E29. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. X13
3. X14
4. X36
5. X52
6. X53
7. X54
8. E29

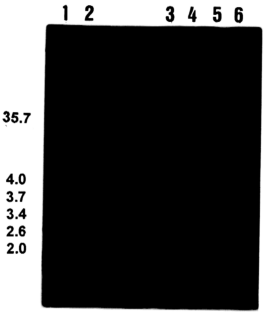

Figure 4.5

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates H10, SL2(cured), SL3(cured), SL49(cured) and SL6(cured). (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. H10
3. SL2(cured)
4. SL3(cured)
5. SL4(cured)
6. SL6(cured)

The isolates including those without plasmids were all subjected to acridine orange treatment. The plasmidless isolates were included as controls and to monitor the possible effect of the agent on chromosomal DNA. Large plasmids and small plasmids of greater than 5.0 Mda from both clinical and environmental isolates were lost after treatment for 24h but only 9 of 28 plasmids below 5.0 Mda were cured. As regards the smaller plasmids, the smaller plasmids of environmental isolates were more resistant towards curing than the clinical isolates because eight of the 12 plasmids less than 5.0 Mda of clinical isolates were lost after curing but only one of the 16 plasmids of same range in environmental isolates was lost (Table 4.6). Figures 4.5 - 4.9 show the agarose gel electrophoresis photographs of the plasmid profiles of the isolates after curing.

Among the 6 plasmidless isolates, 3 isolates did not exhibit any significant difference in the siderophore activity after acridine-orange treatment. One isolate (SL10) showed increased siderophore activity after treatment at both 28°C and 37°C. Two isolates (SL20 and X2) showed increased siderophore production at 37°C after acridine-orange treatment. At 28°C, a decrease in the siderophore activity was detected for SL20, while X2 exhibited no significant difference (Table 4.6).

4.1.5 Effect of plasmid curing on siderophore production

After treatment with acridine orange, the average production of siderophores under iron-limiting conditions at 28°C increased significantly by 150% for the clinical isolates and 55% for the environmental isolates compared to iron-rich conditions

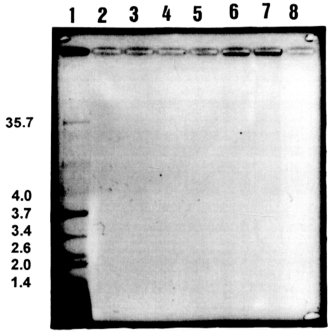

Figure 4.6

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL8, SL9, SL10, SL11, SL12, SL13 and SL14 after treatment with acridine-orange. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. SL8
3. SL9
4. SL10
5. SL11
6. SL12
7. SL13
8. SL14

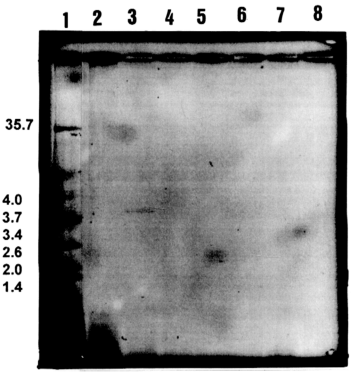

Figure 4.7

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL15, SL16, SL17, SL18, SL19, SL20 and SL21 after treatment with acridine-orange. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. *E. coli* V517 (marker)
2. SL15
3. SL16
4. SL17
5. SL18
6. SL19
7. SL20
8. SL21

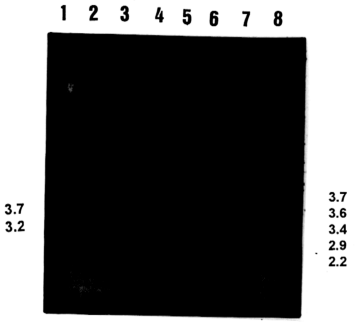

Figure 4.8

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates SL22, X2, X8, X13, X14, X52, X53 and X54 after treatment with acridine-orange. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. SL22
2. X2
3. X8
4. X13
5. X14
6. X52
7. X53
8. X54

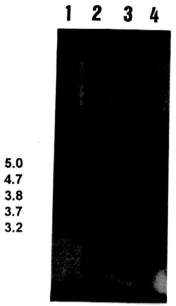

Figure 4.9

A 0.7% agarose gel showing the plasmid profile of *A. hydrophila* isolates E29, H10, SL2 and SL4 after treatment with acridine-orange. (Visualized and photographed by the Vilber-Lourmat Bio 1D computer-assisted imaging system in the negative mode).

Lanes:

1. E29
2. H10
3. SL2
4. SL4

Table 4.7

Comparison between the siderophore production of the acridine orange treated/plasmid cured *Aeromonas hydrophila* isolates under iron-limiting and iron-rich conditions.

	Clinical isolates				Environmental isolates			
	28°C		37°C		28°C		37°C	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
No. of positive isolates (%)	19 (100 %)	19 (100 %)	19 (100 %)	19 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)
Mean ± SD*	0.34 ± 0.23	0.88 ± 0.37	0.50 ± 0.36	1.03 ± 0.36	0.81 ± 0.50	1.25 ± 0.12	0.77 ± 0.39	1.29 ± 0.07
Student's T-test	t = 5.26 p < 0.01		t = 4.42 p < 0.01		t = 2.71 p < 0.01		t = 4.15 p < 0.01	

* Mean ± standard deviation absorbance value at 630nm of reactants.

Table 4.8

Comparison between the siderophore production of the *Aeromonas hydrophila* isolates before and after curing/treatment with acridine orange under iron-limiting and iron-rich conditions.

	Clinical isolates				Environmental isolates			
	28°C		37°C		28°C		37°C	
	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe	+Fe
Before treatment (Mean ± SD)*	0.47 ± 0.38	0.99 ± 0.31	0.57 ± 0.39	1.09 ± 0.28	0.81 ± 0.45	1.22 ± 0.13	0.81 ± 0.50	1.20 ± 0.19
After treatment (Mean ± SD)*	0.34 ± 0.23	0.88 ± 0.37	0.50 ± 0.36	1.03 ± 0.36	0.81 ± 0.50	1.25 ± 0.12	0.77 ± 0.39	1.29 ± 0.07
Student's T-test	t = 1.24 NS	t = 1.11 NS	t = 0.56 NS	t = 0.56 NS	t = 0 NS	t = 0.54 NS	t = 0.20 NS	t = 1.41 NS

* Mean ± standard deviation absorbance value at 630nm of reactants.
NS: Non-significant

(Table 4.7). At 37°C, the increase was 92% and 68% for the clinical and environmental isolates, respectively. The cured clinical isolates were found to possess more siderophore activity than the environmental isolates under all cultural conditions tested. Under iron-limiting conditions, the acridine orange treated clinical isolates produced 140% and 55% more siderophores at 28°C and 37°C, respectively, than the environmental isolates (Table 4.8). As noted for the untreated clinical isolates a small non-significant reduction in the siderophore production of the treated clinical isolates was observed at 37°C compared to 28°C under both iron-limiting and iron-rich conditions (Table 4.9). For the environmental isolates, again there was no difference in the siderophore production of the plasmid-cured isolates at 28°C and 37°C under both iron-limited and iron-rich conditions (Table 4.9).

Under iron-limiting conditions, a 40% increase was obtained at 28°C in the siderophore production of the cured clinical isolates compared to the untreated isolates, but only 14% increase was observed at 37°C. Under iron-rich conditions the cured clinical isolates were found to produce 11% and 5% more siderophores at 28°C and 37°C, respectively, compared to the untreated isolates. The production of siderophores between the normal and cured isolates at 28°C and 37°C was not significantly altered under both iron-deficient and iron-rich conditions for the environmental isolates (Table 4.10).

Table 4.9

Comparison between the siderophore production of the acridine orange treated/plasmid cured *Aeromonas hydrophila* isolates at 28°C and 37°C.

	Clinical isolates				Environmental isolates			
	-Fe		+Fe		-Fe		+Fe	
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
No. of positive isolates (%)	19 (100 %)	19 (100 %)	19 (100 %)	19 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)	11 (100 %)
Mean ± SD*	0.34 ± 0.23	0.50 ± 0.36	0.88 ± 0.37	1.03 ± 0.36	0.81 ± 0.50	0.77 ± 0.39	1.25 ± 0.12	1.29 ± 0.07
Student's T-test	Non-significant		Non-significant		Non-significant		Non-significant	

* Mean ± standard deviation absorbance value at 630nm of reactants.

Table 4.10

Comparison between the siderophore production of the plasmid cured/acridine orange treated clinical and environmental isolates of *Aeromonas hydrophila* under iron-limiting and iron-rich conditions.

	28°C		37°C	
	-Fe	+Fe	-Fe	+Fe
Clinical isolates (Mean ± SD)*	0.34 ± 0.41	0.88 ± 0.37	0.50 ± 0.36	1.03 ± 0.36
Environmental isolates (Mean ± SD)*	0.81 ± 0.50	1.25 ± 0.12	0.77 ± 0.39	1.29 ± 0.07
Student's T-test	t = 3.07 p < 0.01	t = 3.11 p < 0.01	t = 1.85 p < 0.05	t = 2.29 p < 0.025

* Mean ± standard deviation absorbance value at 630nm of reactants.

4.2 Siderophore production in *Vibrio cholerae*

The amount of siderophore produced by the *V. cholerae* isolates was analyzed under iron-limiting and iron-rich cultural conditions at 28°C and 37°C. Appendix B summarizes the detailed results of the siderophore activity in the *V. cholerae* isolates.

4.2.1 Siderophore production under iron-limiting and iron-rich conditions

All the clinical *V. cholerae* isolated exhibited siderophore production on CAS agar plates after incubation at 28°C and 37°C for 24 - 48 hrs (Figures 4.10a and 4.10b) and also when tested by the quantitative CAS assay solution method. Comparatively, more high producers of siderophores were detected under iron-limited conditions compared to iron-rich condition at both 28°C and also 37°C (Table 4.11). Under iron-limiting conditions the mean production of siderophores increased 70% at 28°C and 135% at 37°C compared to iron-rich conditions (Table 4.12). These increases were analyzed by Student's one-tailed t-test and deemed highly significant ($p < 0.01$).

4.2.2 Effect of growth temperature on siderophore production

Under iron-limiting conditions the *V. cholerae* isolates cultured at 37°C produced 45% more siderophores than those cultured at 28°C and the increase was found to be highly significant ($p < 0.01$). Under iron-rich conditions, growth

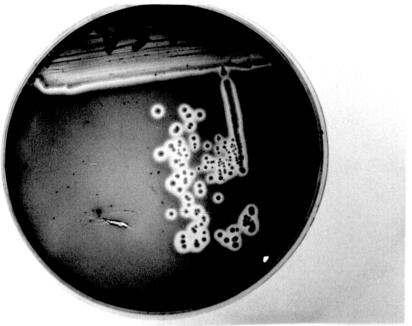

Figure 4.10a

V. cholerae O1 El Tor isolate V57 exhibiting siderophore production (indicated by orange halo around the colonies) on CAS agar plate.

Figure 4.10b

V. cholerae O1 El Tor isolate V138 exhibiting siderophore production (indicated by orange halo around the colonies) on CAS agar plate.

Table 4.11

The *Vibrio cholerae* isolates grouped into different categories of siderophore production under iron-limiting and iron-rich conditions

	28°C		37°C	
	-Fe	**+Fe**	**-Fe**	**+Fe**
Non-siderophore producer	–	–	–	–
Low siderophore producer (%) ($A_{630} \geq 1.00$ to 1.50)	2 (5.3 %)	8 (21.1 %)	–	6 (15.8 %)
Medium siderophore producer (%) ($0.50 < A_{630} < 1.00$)	21 (55.3 %)	30 (78.9 %)	11 (28.9 %)	31 (81.6 %)
High siderophore producer (%) ($A_{630} \leq 0.50$)	15 (39.5 %)	–	27 (71.1 %)	1 (2.6 %)
Total	38 (100 %)	38 (100 %)	38 (100 %)	38 (100 %)

Note: A low absorbance value indicates high production of siderophores.

Table 4.12

Comparison between the siderophore production of the *Vibrio cholerae* isolates under iron-limiting and iron-rich conditions.

	28°C		37°C	
	-Fe	+Fe	-Fe	+Fe
No. of positive isolates (%)	38 (100 %)	38 (100 %)	38 (100 %)	38 (100 %)
Mean ± SD*	0.56 ± 0.23	0.95 ± 0.15	0.39 ± 0.16	0.91 ± 0.13
Student's T-test	t = 5.63 p < 0.01		t = 15.34 p < 0.01	

* Mean ± standard deviation absorbance value at 630nm of reactants.

Table 4.13

Comparison between the siderophore production of the *Vibrio cholerae* isolates at 28°C and 37°C.

	-Fe (Iron-limiting)		+Fe (Iron-rich)	
	28°C	37°C	28°C	37°C
No. of positive isolates (%)	38 (100 %)	38 (100 %)	38 (100 %)	38 (100 %)
Mean ± SD*	0.56 ± 0.28	0.39 ± 0.16	0.95 ± 0.15	0.91 ± 0.13
Student's T-test	t = 3.70 p < 0.01		Non-significant	

* Mean ± standard deviation absorbance value at 630nm of reactants.

temperature, either at 28°C or 37°C does not exert an effect on the siderophore production (Table 4.13).

4.3 Hemolysin production in *Aeromonas hydrophila*

In this study, the hemolysin production by *Aeromonas hydrophila* isolates was analyzed under iron-limiting and iron-rich cultural conditions at 28°C and 37°C. The effect of growth temperature at 28°C and 37°C on hemolysin production of the isolates was also investigated. The results of the hemolytic activity of the *A. hydrophila* isolates analyzed by the microtiter plate method are listed in Appendix C.

4.3.1 Hemolysin assay with microtiter plate method

For comparison purposes the hemolytic activity of the strains was separated into low, medium and high hemolysin production (Table 4.14). Table 5.2 summarizes the *A. hydrophila* isolates into different categories of hemolysin production under the various conditions tested. About half of the isolates were non-hemolytic under all conditions tested (Table 4.15).

4.3.2 Hemolysin production under iron-limited conditions

Hemolysin production increased significantly under iron-limiting conditions compared with iron-rich conditions for both clinical and environmental isolates of *A. hydrophila* (Table 4.16). At 28°C, a two-fold increase in the hemolysin production

Table 4.14

The grouping of hemolytic activity.

Interpretation	Hemolysin titre*
Low hemolytic strain	1 2 4 8 16
Medium hemolytic strain	32 64 128
High hemolytic strain	256 512 1024

* Numbers indicate the reciprocal of dilution. Number 1 refers to the well in which the supernatant fluid was used undiluted.

Table 4.15

The clinical and environmental isolates of *Aeromonas hydrophila* grouped into different categories of hemolysin production under iron-limiting and iron-rich conditions.

	Clinical isolates				Environmental isolates			
	28°C		37°C		28°C		37°C	
	TSB + EDDA	TSB	TSB + EDDA	TSB	TSB + EDDA	TSB	TSB + EDDA	TSB
Non-hemolytic (%)	10 (45.5 %)	10 (45.5 %)	10 (45.5 %)	10 (45.5 %)	8 (44.4 %)	8 (44.4 %)	9 (50.0 %)	9 (50.0 %)
Low hemolytic (%)	1 (4.5 %)	6 (27.3 %)	2 (9.1 %)	3 (13.6 %)	1 (5.6 %)	1 (%.6 %)	–	–
Medium hemolytic (%)	9 (40.9 %)	5 (22.7 %)	7 (31.8 %)	8 (36.4 %)	5 (27.8 %)	5 (27.8 %)	5 (27.8 %)	5 (27.8 %)
High hemolytic (%)	2 (9.1 %)	1 (4.5 %)	3 (13.6 %)	1 (4.5 %)	4 (22.2 %)	4 (22.2 %)	4 (22.2 %)	4 (22.2 %)
Total (%)	22 (100 %)	22 (100 %)	22 (100 %)	22 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)

Note: Refer to Table 4.14 for the grouping of hemolytic activity.

Table 4.16

Comparison between the hemolysin production of the *Aeromonas hydrophila* isolates under iron-limiting and iron-rich conditions.

	Clinical isolates				Environmental isolates			
	28°C		37°C		28°		37°	
	TSB + EDDA	TSB	TSB + EDDA	TSB	TSB + EDDA	TSB	TSB + EDDA	TSB
No. of positive isolates (%)	12 (54.5 %)	12 (54.5 %)	12 (54.5 %)	12 (54.5 %)	10 (55.6 %)	10 (55.6 %)	9 (50.0 %)	9 (50.0 %)
Mean ± SD* (Hemolytic titre)	54 ± 3	28 ± 4	80 ± 4	54 ± 4	147 ± 4	104 ± 5	149 ± 3	110 ± 3
Student's T-test	t = 17.2 p < 0.01		t = 15.2 p < 0.01		t = 22.3 p < 0.01		t = 26.0 p < 0.01	

* Mean ± standard deviation

Table 4.17

Comparison between the hemolysin production of the *Aeromonas hydrophila* isolates at 28°C and 37°C.

	Clinical isolates				Environmental isolates			
	TSB + EDDA		TSB		TSB + EDDA		TSB	
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
No. of positive isolates (%)	12 (54.5 %)	12 (54.5 %)	12 (54.5 %)	12 (54.5 %)	10 (55.6 %)	9 (50.0 %)	10 (55.6 %)	9 (50.0 %)
Mean ± SD* (Hemolytic titre)	54 ± 3	80 ± 4	28 ± 4	54 ± 4	147 ± 4	149 ± 3	104 ± 5	110 ± 3
Student's T-test	t = 17.26 p < 0.01		t = 15.24 p < 0.01		Non-significant		Non-significant	

* Mean ± standard deviation

was obtained under iron-limiting conditions compared to iron-rich conditions while at 37°C the corresponding increase was 50%. Under iron-limiting conditions, the environmental isolates showed 42% and 35% more hemolytic activity at 28°C and 37°C, respectively, compared to iron-rich conditions. These increase were found to be highly significant when analyzed by the Student's t-test.

4.3.3 Effect of growth temperature on hemolysin production

For the clinical isolates, more hemolysins were produced at 37°C than 28°C (Table 4.17). Under iron-limiting conditions, the clinical isolates possessed 56% more hemolytic activity at 37°C than 28°C, while under iron-rich conditions 95% more hemolytic activity was observed at 37°C compared to 28°C. Hemolysin production by the environmental isolates was not affected by growth temperature at 28°C or 37°C.

4.3.4 Comparison between hemolysin production of clinical and environmental isolates.

It was also noticed that the environmental isolates of *A. hydrophila* produced more hemolysins than clinical isolates under all conditions tested (Table 4.18). At 28°C, the environmental isolates produced 170% more hemolysins than clinical isolates under iron-limited conditions, and 280% more hemolysins under iron-rich conditions. At 37°C, the environmental strains possessed 85% and 100% more hemolytic activity under iron-deficient and iron rich conditions, respectively, than the clinical isolates.

Table 4.18

Comparison between the hemolysin production of clinical and environmental isolates of *Aeromonas hydrophila*.

	28°C		37°C	
	TSB + EDDA	**TSB**	**TSB + EDDA**	**TSB**
Clinical isolates (Mean ± SD)*	54 ± 3	28 ± 4	80 ± 4	54 ± 4
Environmental isolates (Mean ± SD)*	147 ± 4	104 ± 5	149 ± 3	110 ± 3
Student's T-test	t = 59.34 p < 0.01	t = 37.77 p < 0.01	t = 41.28 p < 0.01	t = 33.50 p < 0.01

* Mean ± standard deviation of hemolytic titre

Table 4.19

Hemolytic activity (at 28°C) of selected *Aeromonas hydrophila* isolates measured 540 nm (A_{540})

Isolates	Hemolytic titer (A_{540})*		
	TSB	TSB + EDDA	TSB + $FeSO_4$
SL2 (Fig. 4.11)	85	135	85
SL4 (Fig. 4.12)	2400	6350	2400
SL17 (Fig.4.13)	400	920	480
X13 (Fig. 4.14)	150	240	165
X52 (Fig. 4.15)	130	195	160
X54 (Fig. 4.16)	1320	1780	1500
H10 (Fig. 4.17)	85	140	35
Mean	**653**	**1394**	**689**

* Absorbance value of the reactants (equal volumes of cell-free supernatant and human erythrocytes suspension) measured at 540nm.

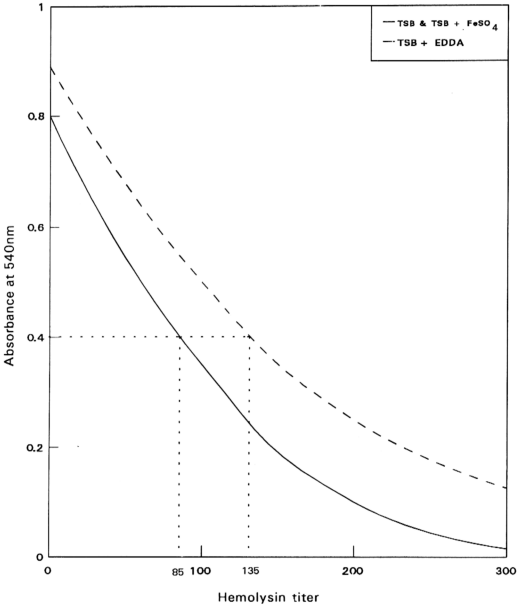

Figure 4.11: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** clinical isolate SL2

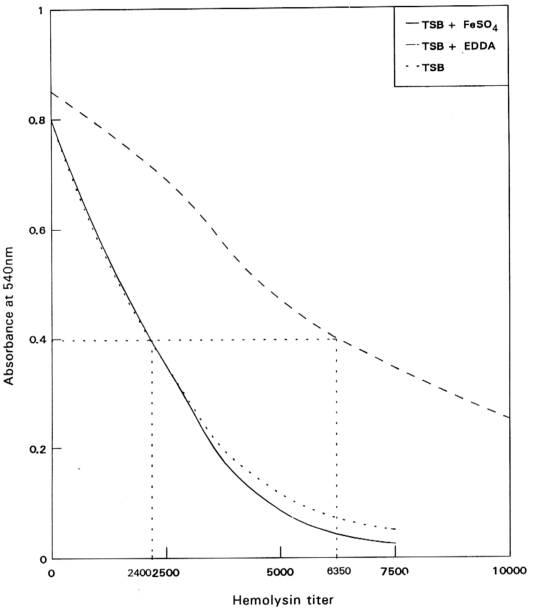

Figure 4.12: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** clinical isolate SL4.

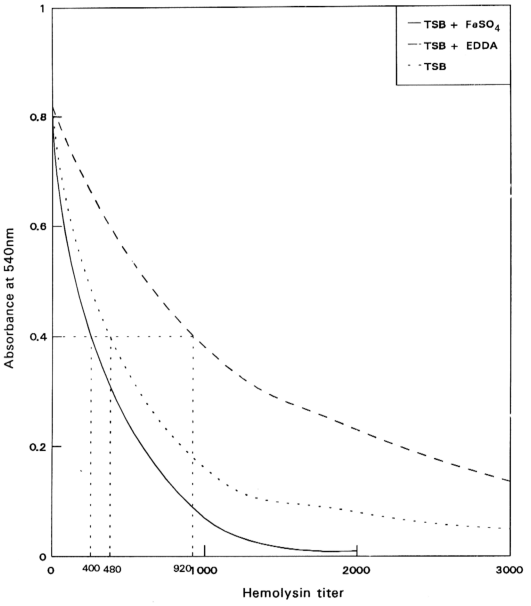

Figure 4.13: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** clinical isolate SL17.

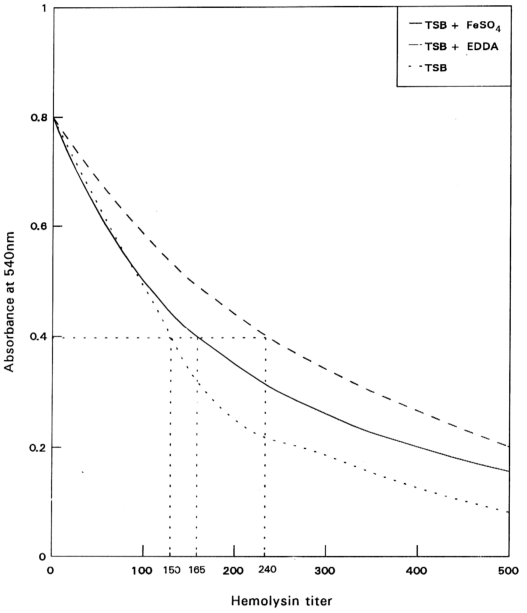

Figure 4.14: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** environmental isolate X13.

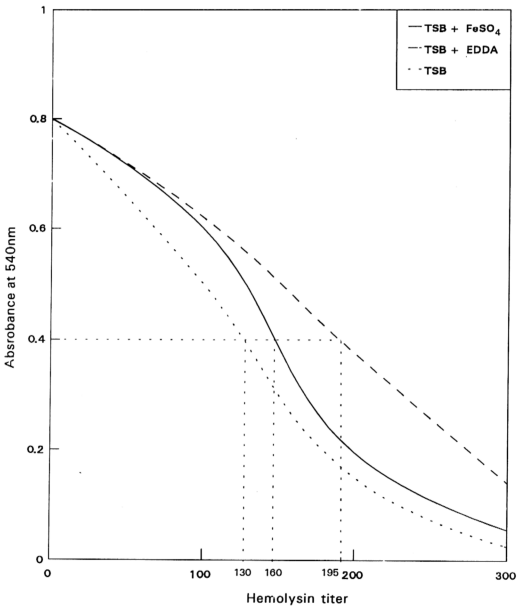

Figure 4.15: Effect of iron-limiting iron-rich conditions on the hemolysin production of the ***A. hydrophila*** environmental isolate X52.

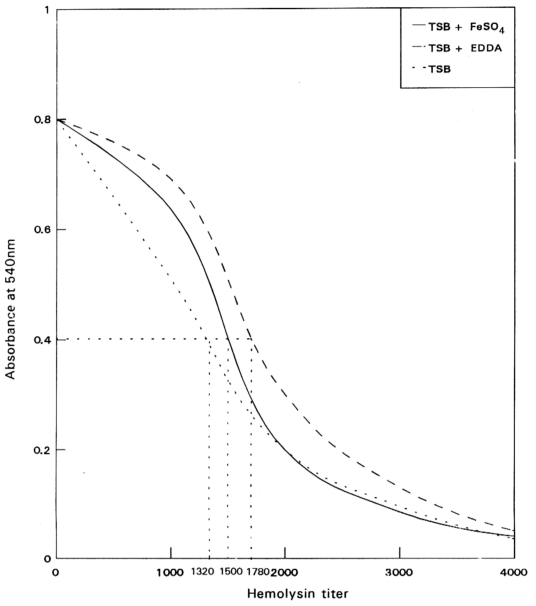

Figure 4.16: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** environmental isolate X54.

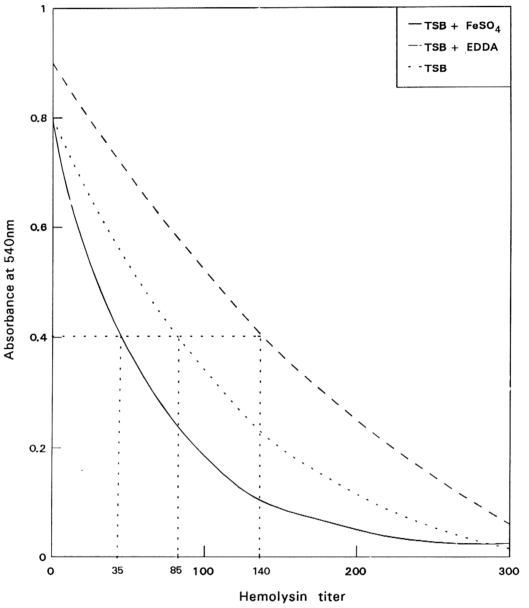

Figure 4.17: Effect of iron-limiting and iron-rich conditions on the hemolysin production of the ***A. hydrophila*** environmental isolate H10

4.3.5 Hemolytic activity of *A. hydrophila* isolates measured at 540nm (A_{540})

The hemolytic activity of selected *A. hydrophila* isolates (SL2, SL4, SL17, X13, X52, X54 and H10) at 28°C were assayed by measuring the absorbance at 540 nm (Table 4.19). The mean hemolytic titer of the strains increased more than two-fold under iron-deficient conditions compared to iron-rich conditions. The addition of 100 μM $FeSO_4.7H_2O$ to the growth media did not alter the hemolytic activity of the isolates tested. The hemolytic pattern of these isolates are shown in Figures 4.11 - 4.17.

4.4 Hemolysin production in *V. cholerae*

The hemolysin production by the *V. cholerae* isolates was analyzed under iron-limiting and iron-rich cultural conditions at 28°C and 37°C. Results of the hemolytic activity of the *V. cholerae* isolates tested by the microtiter plate assay are summarized in Appendix D.

4.4.1 Hemolysin assay with microtiter plate method

The 40 *V. cholerae* isolates did not exhibit any hemolytic activity when cultured at 37°C under both iron-limiting and iron-rich conditions. At 28°C, 17.5% and 32.5% of the isolates showed low hemolytic activity under iron-deficient and

Table 4.20

The *Vibrio cholerae* isolates grouped into different categories of hemolysin production under iron-limiting and iron-rich conditions.

	28°C		37°C	
	TSB + EDDA	TSB	TSB + EDDA	TSB
Non-hemolytic (%)	33 (82.5 %)	27 (67.5 %)	40 (100 %)	40 (100 %)
Low hemolytic (%)	7 (17.5 %)	13 (32.5 %)	–	–
Medium hemolytic (%)	–	–	–	–
High hemolytic (%)	–	–	–	–
Total (%)	40 (100 %)	40 (100 %)	40 (100 %)	40 (100 %)

Note: Refer to Table 4.14 for the grouping of hemolytic activity.

iron-rich conditions, respectively (Table 4.20). The hemolysin production of the strains at 28°C did not increase under iron-limiting conditions. In fact, the number of hemolytic isolates decreased under iron-limiting conditions. For instance, 6 isolates which were hemolytic under iron-rich conditions were non-hemolytic under iron-limiting conditions. Four isolates showed decreased hemolytic activity under iron-limiting conditions compared to iron-rich conditions. Only 2 isolates (V123 and V133) exhibited increased hemolytic activity under iron-limiting conditions compared to iron-rich conditions (Appendix D).

4.5 Protease production in *Aeromonas hydrophila*

The relationship between the iron concentrations and protease production of the *A. hydrophila* isolates were studied to determine the role of proteases in the iron acquiring process of these species. Appendix E summarizes the results of the proteolytic activity in *A. hydrophila*.

4.5.1 Protease assay with the azocasein method

All the clinical isolates of *A. hydrophila* were found to be protease producers under the conditions tested. All but two environmental isolates were non-proteolytic; the two isolates did not produce proteases at both 28°C and 37°C (Appendix E). More high protease producers were found among the clinical isolates than the environmental ones. Among the clinical isolates, there are more high protease producers at 28°C than 37°C (Table 4.21).

Table 4.21

The clinical and environmental isolates of *Aeromonas hydrophila* grouped into different categories of protease producers under iron-limiting and iron-rich conditions.

	Clinical isolates						Environmental isolates					
	28°C			37°C			28°C			37°C		
	TSB + EDDA	TSB	TSB + $FeSO_4$	TSB + EDDA	TSB	TSB + $FeSO_4$	TSB + EDDA	TSB	TSB + $FeSO_4$	TSB + EDDA	TSB	TSB + $FeSO_4$
Non-protease producer (%)	–	–	–	–	–	–	2 (14.3 %)	2 (14.3 %)	2 (14.3 %)	2 (14.3 %)	2 (14.3 %)	2 (14.3 %)
Low protease producer (%) ($A_{450} \leq 0.50$)	2 (11.1 %)	–	1 (5.6 %)	7 (38.9 %)	5 (27.8 %)	6 (33.3 %)	4 (28.5 %)	3 (21.4 %)	7 (50.0 %)	7 (50.0 %)	6 (42.9 %)	9 (64.3 %)
Medium protease producer (%) ($0.50 < A_{450} < 1.00$)	11 (61.1 %)	9 (50.0 %)	9 (50.0 %)	10 (55.6 %)	13 (72.2 %)	12 (66.7 %)	8 (57.1 %)	9 (64.3 %)	4 (28.6 %)	5 (35.7 %)	6 (42.9 %)	3 (21.4 %)
High protease producer (%) ($A_{450} \geq 1.00$)	5 (27.8 %)	9 (50.0 %)	8 (44.4 %)	1 (5.6 %)	–	–	–	–	1 (7.1 %)	–	–	–
Total (%)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	14 (100 %)	14 (100 %)	14 (100 %)	14 (100 %)	14 (100 %)	14 (100 %)

Table 4.22

Comparison between the protease production of the *Aeromonas hydrophila* isolates under iron-limiting and iron-rich conditions.

	Clinical isolates						**Environmental isolates**					
	28°C			**37°C**			**28°C**			**37°C**		
	TSB + EDDA	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**
No. of positive isolates (%)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)
Mean ± SD*	0.84 ± 0.25	0.92 ± 0.23	0.90 ± 0.28	0.63 ± 0.22	0.60 ± 0.23	0.60 ± 0.25	0.54 ± 0.25	0.60 ± 0.24	0.53 ± 0.26	0.48 ± 0.22	0.47 ± 0.21	0.39 ± 0.18
Student's T-test	Non-significant			Non-significant			Non-significant			Non-significant		
Student's T-test		Non-significant			Non-significant			Non-significant			Non-significant	

* Mean ± standard deviation absorbance value at 450nm of reactants.

4.5.2 Protease production under iron-limiting conditions

No significant difference was observed in the protease production of the *A. hydrophila* isolates under iron-limiting conditions compared to iron-rich conditions. The addition of 100μM $FeSO_4.7H_2O$ to the growth media did not alter the protease production of the isolates (Table 4.22).

4.5.3 Effect of temperature on protease production

Under iron-limiting conditions (achieved by the addition of 100μM EDDA to the growth media), the clinical isolates produced 34% more proteases at 28°C than 37°C. When cultivated in TSB without the addition of iron chelators, the increase was 50% at 28°C compared to 37°C. With 100μM $FeSO_4.7H_2O$ added to the growth media, the clinical isolates produced 52% more proteases at 28°C than at 37°C. Although the environmental strains also consistently produced more proteases at 28°C under all conditions tested (Table 4.23), this difference was found to be statistically non-significant ($p > 0.05$).

Table 4.23

Comparison between the protease production of the *Aeromonas hydrophila* isolates at 28°C and 37°C.

	Clinical isolates						Environmental isolates					
	TSB + EDDA		TSB		TSB + $FeSO_4$		TSB + EDDA		TSB		TSB + $FeSO_4$	
	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C	37°C
No. of positive isolates (%)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	18 (100 %)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)	12 (85.7%)
Mean ± SD*	0.84 ± 0.25	0.63 ± 0.22	0.92 ± 0.23	0.60 ± 0.22	0.90 ± 0.28	0.60 ± 0.25	0.54 ± 0.25	0.48 ± 0.22	0.60 ± 0.24	0.47 ± 0.21	0.53 ± 0.26	0.39 ± 0.18
Student's T-test	t = 2.60 p < 0.01		t = 4.15 p < 0.01		t = 3 30 p < 0.01		Non-significant		Non-significant		Non-significant	

* Mean ± standard deviation absorbance value at 450nm of reactants.

Table 4.24

Comparison between the protease production of the clinical and environmental isolates of *Aeromonas hydrophila*.

	28°C			37°C		
	TSB + EDDA	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**
Clinical isolates (Mean ± SD)*	0.84 ± 0.25	0.92 ± 0.23	0.90 ± 0.28	0.63 ± 0.22	0.60 ± 0.23	0.60 ± 0.25
Environmental isolates (Mean ± SD)*	0.54 ± 0.25	0.60 ± 0.24	0.53 ± 0.26	0.48 ± 0.22	0.47 ± 0.21	0.39 ± 0.18
Student's T-test	t = 3.11 p < 0.01	t = 3.54 p < 0.01	t = 3.52 p < 0.01	Non-significant	Non-significant	Non-significant

* Mean ± standard deviation absorbance value at 450nm of reactants.

4.5.4 Comparison between the protease production of clinical and environmental isolates

At 28°C, clinical isolates were found to produce more proteases than environmental isolates ($p < 0.01$). Under iron-limiting conditions, the clinical isolates produced 56% more proteases than the environmental isolates (Table 4.24). When grown in TSB without the addition of iron chelators, the clinical isolates possessed 52% more protease activity than environmental isolates. With the addition of 100μM $FeSO_4.7H_2O$ to the growth media, the clinical isolates produced 70% more proteases than the environmental isolates. Although the clinical isolates still produced higher levels of proteases at 37°C compared with the environmental isolates under all the conditions tested, this difference was found to be statistically non-significant ($p > 0.05$).

4.6 Protease production in *V. cholerae*

The effect of iron on the production of proteases in *V. cholerae* was investigated to determine the role of the *V. cholerae* hemagglutinin/protease in iron scavenging. The proteolytic activity of the *V. cholerae* isolates are given in full in Appendix F.

Table 4.25

The *Vibrio cholerae* isolates grouped into different categories of protease producers under iron-limiting and iron-rich conditions.

	28°C			37°C		
	TSB + EDDA	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**
Non-protease producer (%)	2 (6.9%)	2 (6.9%)	2 (6.9%)	3 (10.3 %)	3 (10.3 %)	3 (10.3 %)
Low protease producer (%) ($A_{450} \leq 0.50$)	4 (13.8 %)	2 (6.9 %)	_	4 (13.8 %)	1 (3.4 %)	
Medium protease producer (%) ($0.50 < A_{450} < 1.00$)	23 (79.3 %)	25 (86.2 %)	26 (89.7 %)	22 (75.9 %)	25 (86.2 %)	24 (82.8 %)
High protease producer (%) ($A_{450} \geq 1.00$)	_	_	1 (3.4 %)	_	_	3 (6.9 %)
Total (%)	29 (100 %)	29 (100 %)	29 (100 %)	29 (100 %)	29 (100 %)	29 (100 %)

Table 4.26

Comparison between the protease production of *Vibrio cholerae* isolates under iron-limiting and iron-rich conditions.

	28°C			37°C		
	TSB + EDDA	**TSB**	**TSB + $FeSO_4$**	**TSB + EDDA**	**TSB**	**TSB + $FeSO_4$**
No. of positive isolates (%)	27 (93.1 %)	27 (93.1 %)	27 (93.1 %)	26 (89.7 %)	26 (89.7 %)	26 (89.7 %)
Mean ± SD*	0.65 ± 0.13	0 76 ± 0.13	0.86 ± 0.10	0.69 ± 0.14	0.77 ± 0.12	0.81 ± 0.12
Student's T-test	t = 3.11 p < 0.01			t = 2.21 p < 0.025		
Student's T-test		t = 2.69 p < 0.01			t = 1.15 Non-significant	

* Mean ± standard deviation absorbance value at 450nm of reactants.

4.6.1 Protease assay with the azocasein method

All but two of the 29 *V. cholerae* isolates were positive for protease production at both 28°C and 37°C. The two isolates (V114 and V1400) failed to produce any proteases at both temperatures. One isolate (V1398) which produced proteases at 28°C failed to show any protease activity at 37°C (Appendix F). Most of the *V. cholerae* isolates were medium protease producers under all the conditions tested (Table 4.25).

4.6.2 Protease production under iron-limiting and iron-rich conditions

Protease production of the *V. cholerae* isolates increased under iron-rich conditions compared with iron-limiting conditions (Table 4.26). At 28°C, the isolates produced 18% more proteases when grown in TSB without the addition of iron chelators compared to the protease production in TSB with the addition of 100μM EDDA. At 37°C, the isolates possessed 12% more siderophore activity when grown in TSB without the addition of iron chelators compared to the protease production in TSB with the addition of 100μM EDDA. When cultured in the presence 100μM $FeSO_4.7H_2O$, the protease production increased 12% compared to when cultured in TSB alone. The protease production of the isolates was similar at both 28°C and 37°C under all the different conditions tested (Table 4.27).

Table 4.27

Comparison between the protease production of the *Vibrio cholerae* isolates at 28°C and 37°C.

	TSB + EDDA		TSB		TSB + $FeSO_4$	
	28°C	**37°C**	**28°C**	**37°C**	**28°C**	**37°C**
No. of positive isolates (%)	27 (93.1 %)	26 (89.7 %)	27 (93.1 %)	26 (89.7 %)	27 (93.1 %)	26 (89.7 %)
Mean ± SD*	0.65 ± 0.13	0.69 ± 0.14	0.76 ± 0.13	0.77 ± 0.12	0.86 ± 0.10	0.81 ± 0.12
Student's T-test	Non-significant		Non-significant		Non-significant	

* Mean ± standard deviation absorbance value at 450nm of reactants.