CHAPTER 4

RESULTS

4.1 SCREENING, ISOLATION AND SELECTION OF ALKALINE LIPASE PRODUCING BACTERIA

The alkaline lipase production of three hundred and fifty bacterial strains isolated from forty soil and five wastewater samples was primarily investigated on Trioleoylglycerol Rhodamine B Agar plates. The highest group of alkaline lipase producers were composed of five strains (1.42%), the medium and the lowest group were 31.43 and 67.14%, respectively, as given in **Table 4.1.** Five of those isolates were named, ALP-20.6, ALP-35.8, ALP-40.3, ALP-41.9 and ALP-42.11. The results of spectrophotometrical measuring of lipase activities were shown in **Table 4.2.** Two bacterial strains from the highest group were chosenas the selected bacterial strains and named ALP-40.3 and ALP-42.11 for further studies. From Table 4.3, both of the selected bacterial strains were short rod shape and gram-negative. ALP-40.3 was isolated from hydrocarbon contaminated soil obtained from gas station and ALP-42.11 isolated from wastewater in leather bleaching industry and they were identified as ***Pseudomonas sp.*** Morphology of the colonies and cells of two selected bacterial strains are illustrated in **Figure 4.1-4.4.** Furthermore, some biochemical characteristics of both bacterial isolates were also performed as given in **Table 4.4** and **Figure 4.5-4.10.**

Table 4.1 Alkaline lipase production in cell-free supernatant of bactrial isolates[a] examined on TRA plates by fluorescent halo (Kouker and Jaeger, 1987).

Diameter of Orange Fluorescent Halo (cm)	No. of Strains	Percent
0.50 - 1.00	110	31.43
1.01 - 1.50	235	67.14
> 1.51	5	1.43
Total	350	100

[a]All bacterial isolates were cultivated in LOB with pH 9.0 by using average inocubum size; (5.57±2.04) $*10^9$ cell/ml, and incubate at 37°C.

Table 4.2 Alkaline lipase activity of five highest alkaline lipase producing strains measured confirm by spectrophotometric method.

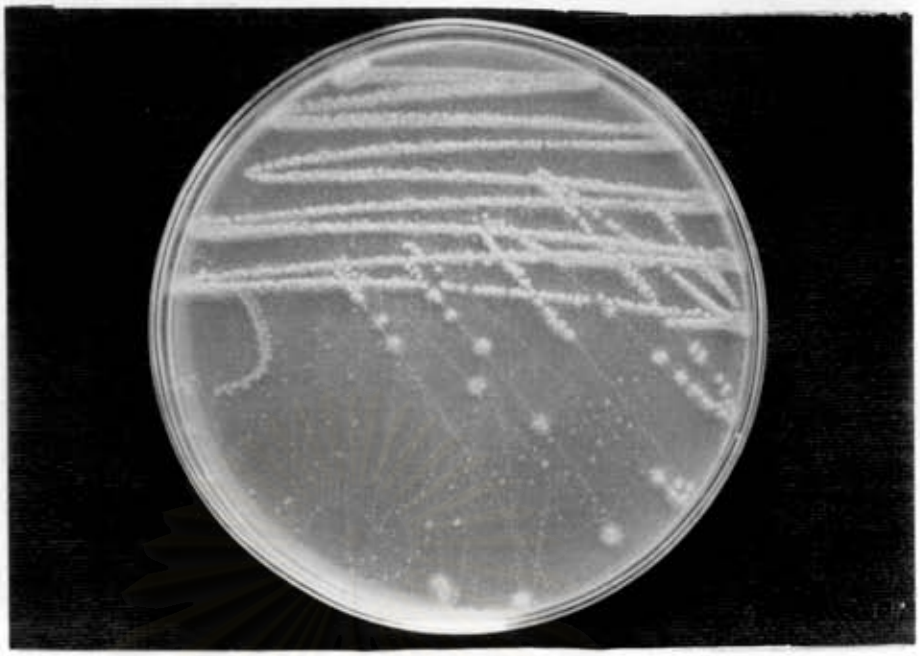
Baeterial[a] Strains	Lipase activity (Unit[b] /ml)
ALP 20.6	252.67±15.95
ALP 35.8	261.00±2.69
ALP 40.3	13639.33±3401.47
ALP 41.9	241.33±39.55
ALP 42.11	700.67±161.01

[a]Bacterial strains were cultivated in LOB with pH 9.0 as well as average inoculum size; $(8.61\pm0.79)*10^8$ cell/ml , and incubated at 37 °C.

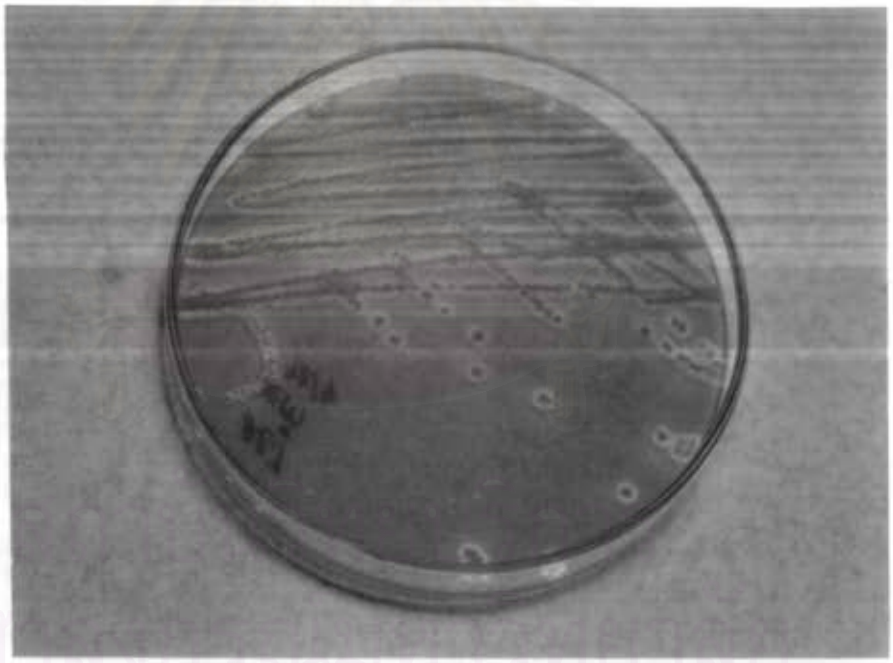
[b]Unit : nmole of pNP liberated per minute.

Table 4.3 Some characteristics and identification of two alkaline lipase producing bacterial isolates.

Bacterial isolates	Sources (Sampling Site)	Characteristics of		Identified as
		Colony	Morphology	
ALP 40.3	Soil (S1)	~3 mm in diameter, yellow brown, irregular lobate and unbonate	Short rod-shape gram-negative, 1.7 μm	***Pseudomonas sp.***
ALP 42.11	Wastewater (WW1)	~4 mm in diameter, light yellow, circular, undudate and round	Short rod-shape gram-negative, 1.1 μm	***Pseudomonas sp.***

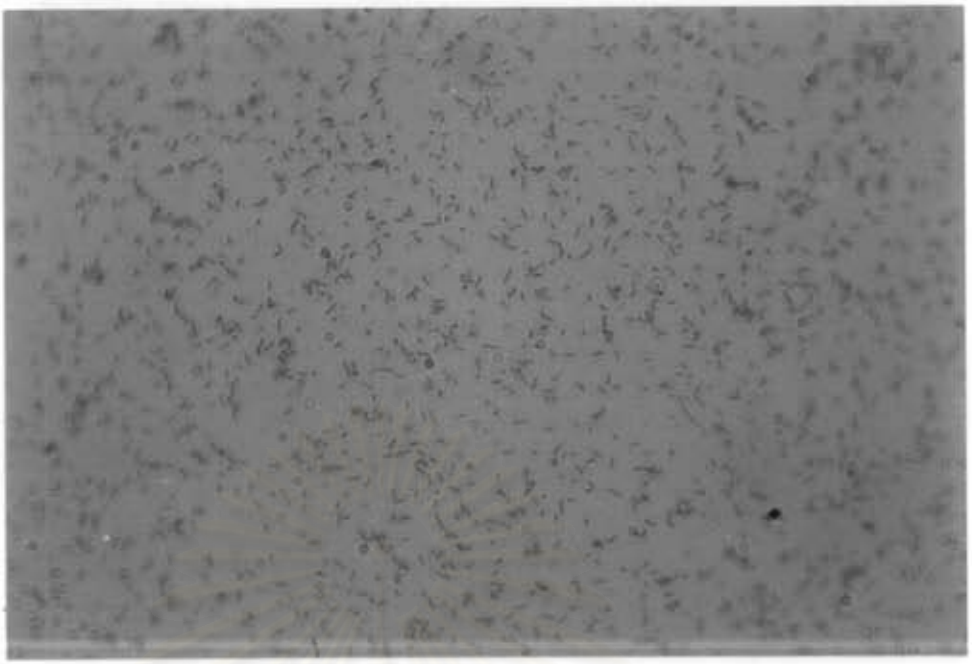


(a)



(b)

Figure 4.1 Colonial characteristics of alkaline lipase producing bacterial strain ALP-40.3 (Pseudomonas sp.) grown on LRA (a) and LRA upon UV irradiating at 350 nm, showing orange fluorescent halo around colonies (b) incubated at 37°c for 48 hr.



(a)

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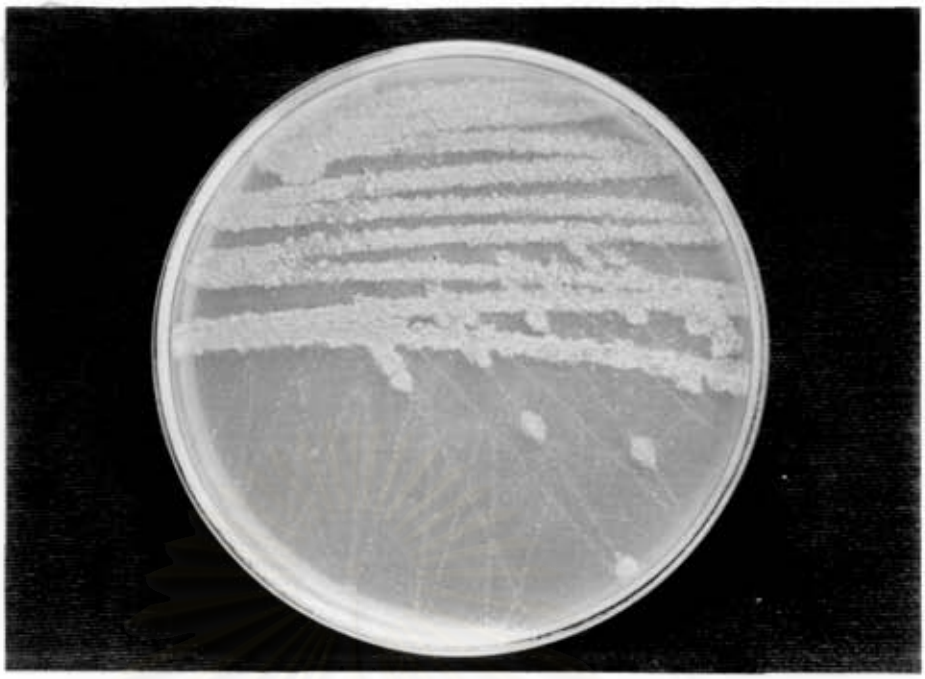
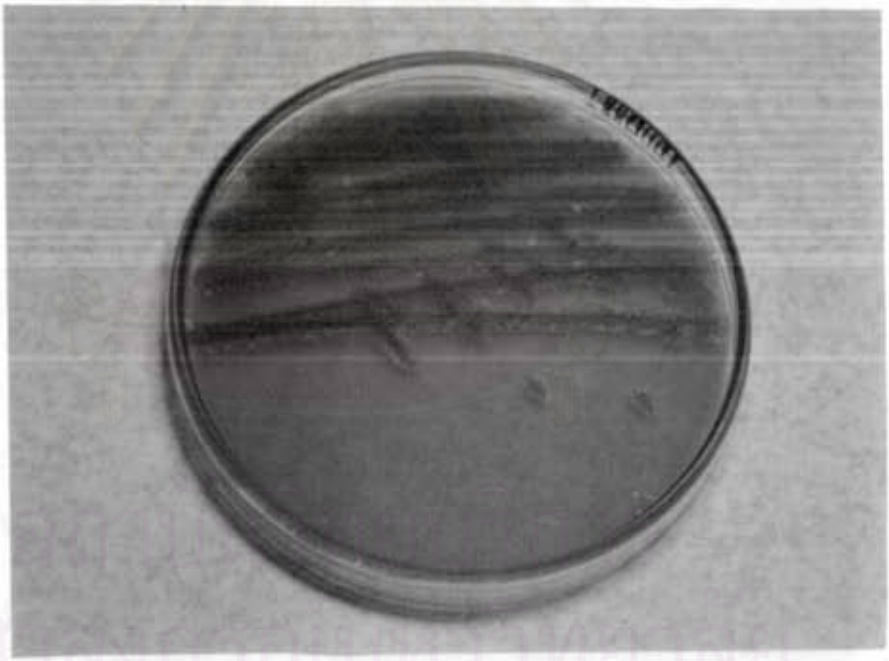
Figure4.2 Gram staining (a) and high revolution scanning electron micrograph (b) of alkaline lipase producing bacterial stain ALP-40.3 (x 10000).

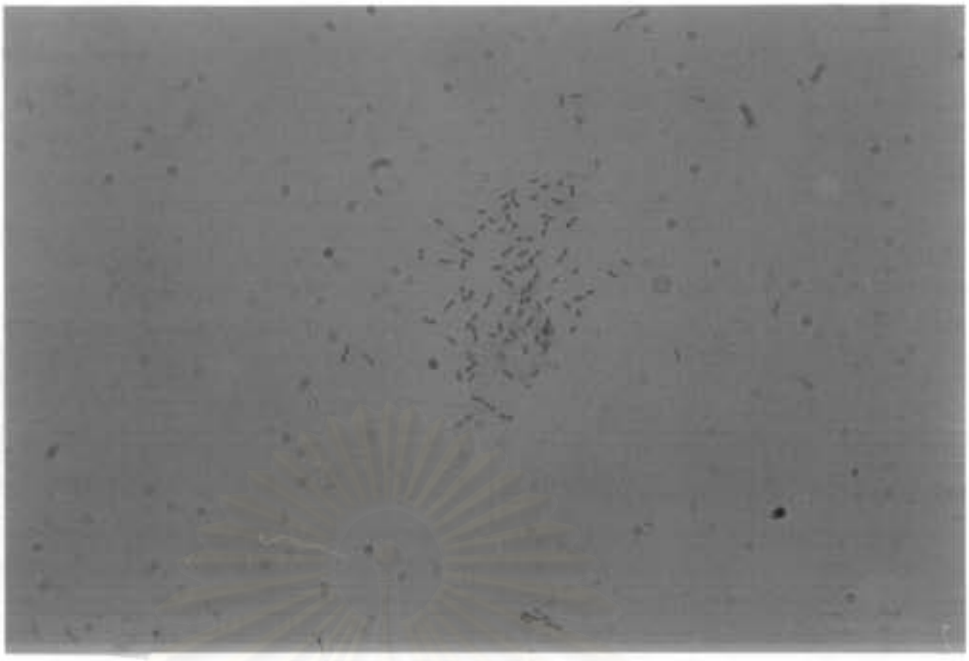


(a)

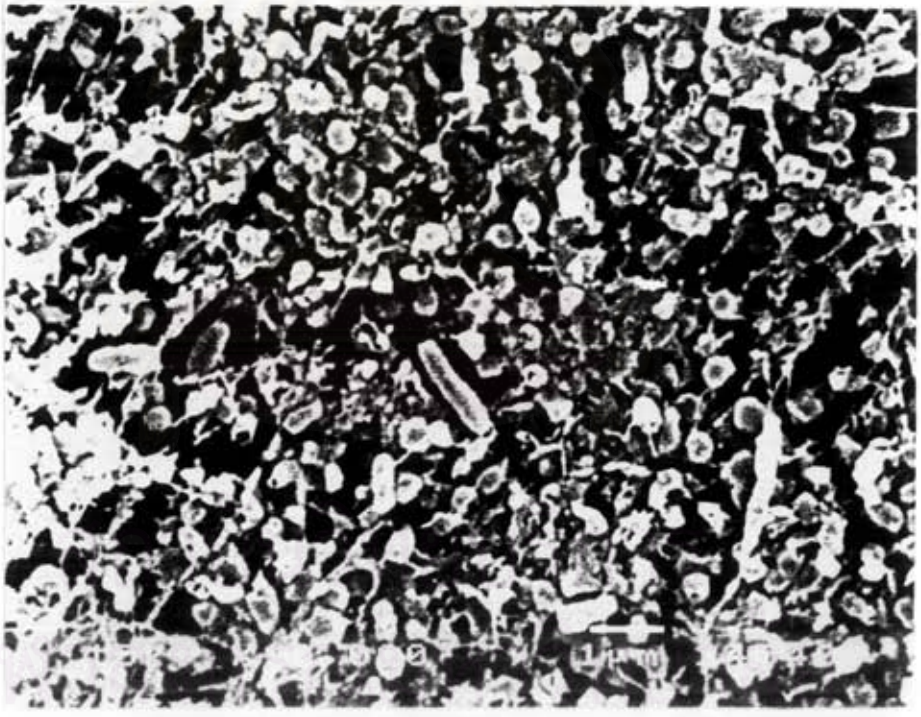


(b)

Figure 4.3 Colonial characteristics of alkaline lipase producing bacterial strain ALP-42.11 (Pseudomonas sp.) grown on LRA (a) and LRA upon UV irradiating at 350 nm, showing orange fluorescent halo around colonies (b) incubated at 37°c for 48 hr.



(a)



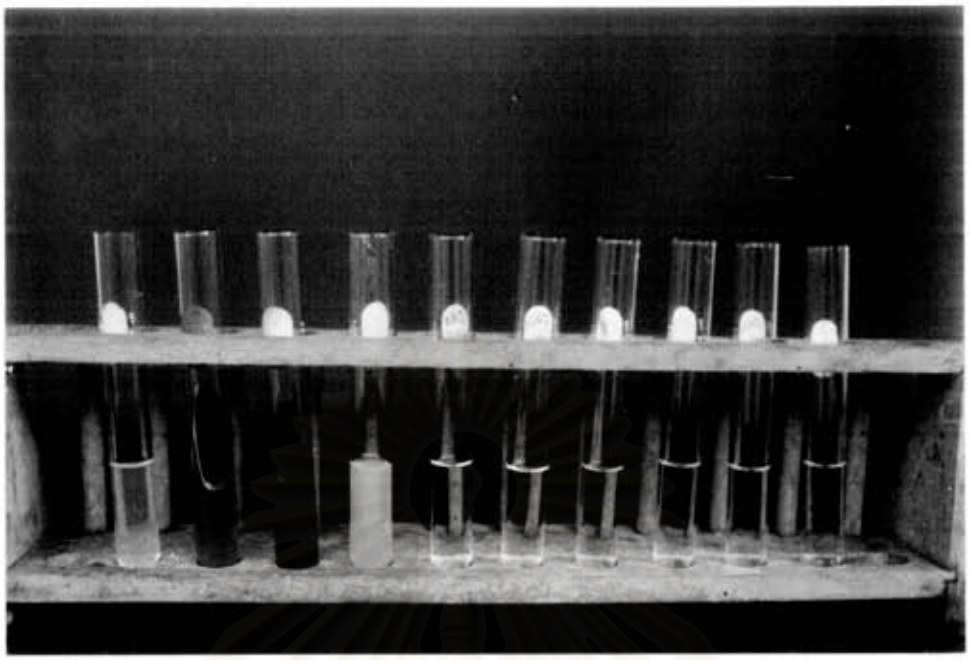
(b)

Figure4.4 Gram staining (a) and high revolution scanning electron micrograph (b) of alkaline lipase producing bacterial stain ALP-42.11 (x 10000).

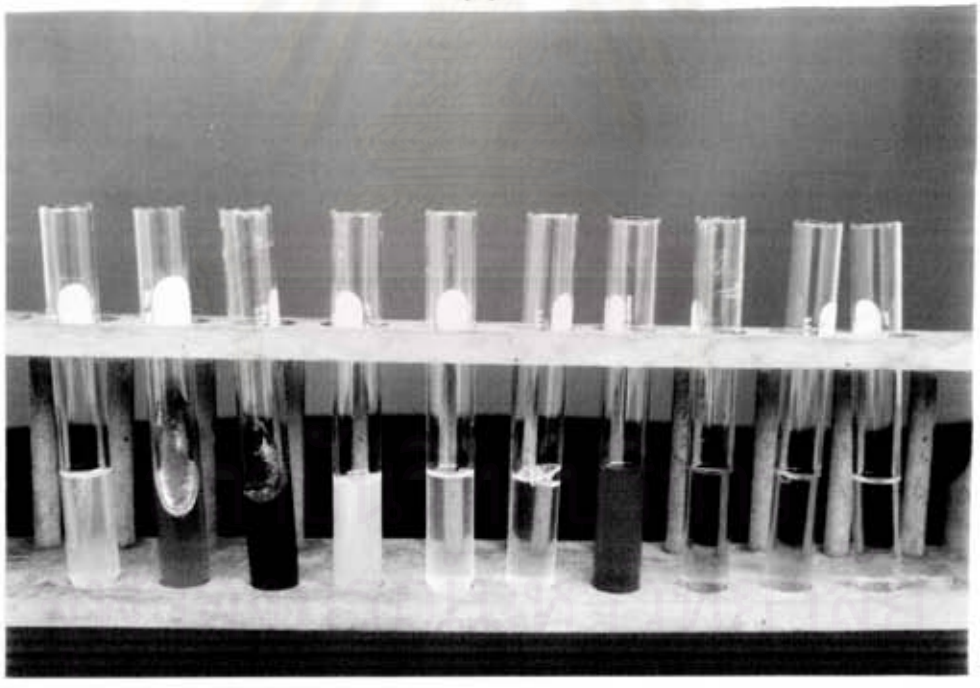
Table 4.4 Some biochemical tests and selective media for Identification of characteristics of the selected bacterial strains.

Biochemical Tests	Selected bacterial strains	
	ALP-40.3	ALP-42.11
Motility		
Catalase	+	+
Oxidase	+	+
TSI	+	+
Citrate	K/N	K/N
Urease	+	+
Nitrate	-	+
KCN,noKCN	+	-
Indole	+,+	+,+
EMB	-	-
MA	no growth	growth,pink
PSIA	growth	growth
SSA	growth	growth
O-F-Test	growth	growth
Glucose		
Dextrose	F	F
Lactose	F	F
Maltose	-	-
Sucrose	F	-
MR	-	-
VP	-	-
	-	-

F = Ferment, + = positive, - = negative



(a)



(b)

Figure 4.5 Biochemical test (left to right; motility , TSI, citrate utilization, urease, KCN, no KCN, nitrate reduction, indole, MR and VP test) of alkaline lipase producing bacterial strain ALP-40.3 with control (a) and results (b).

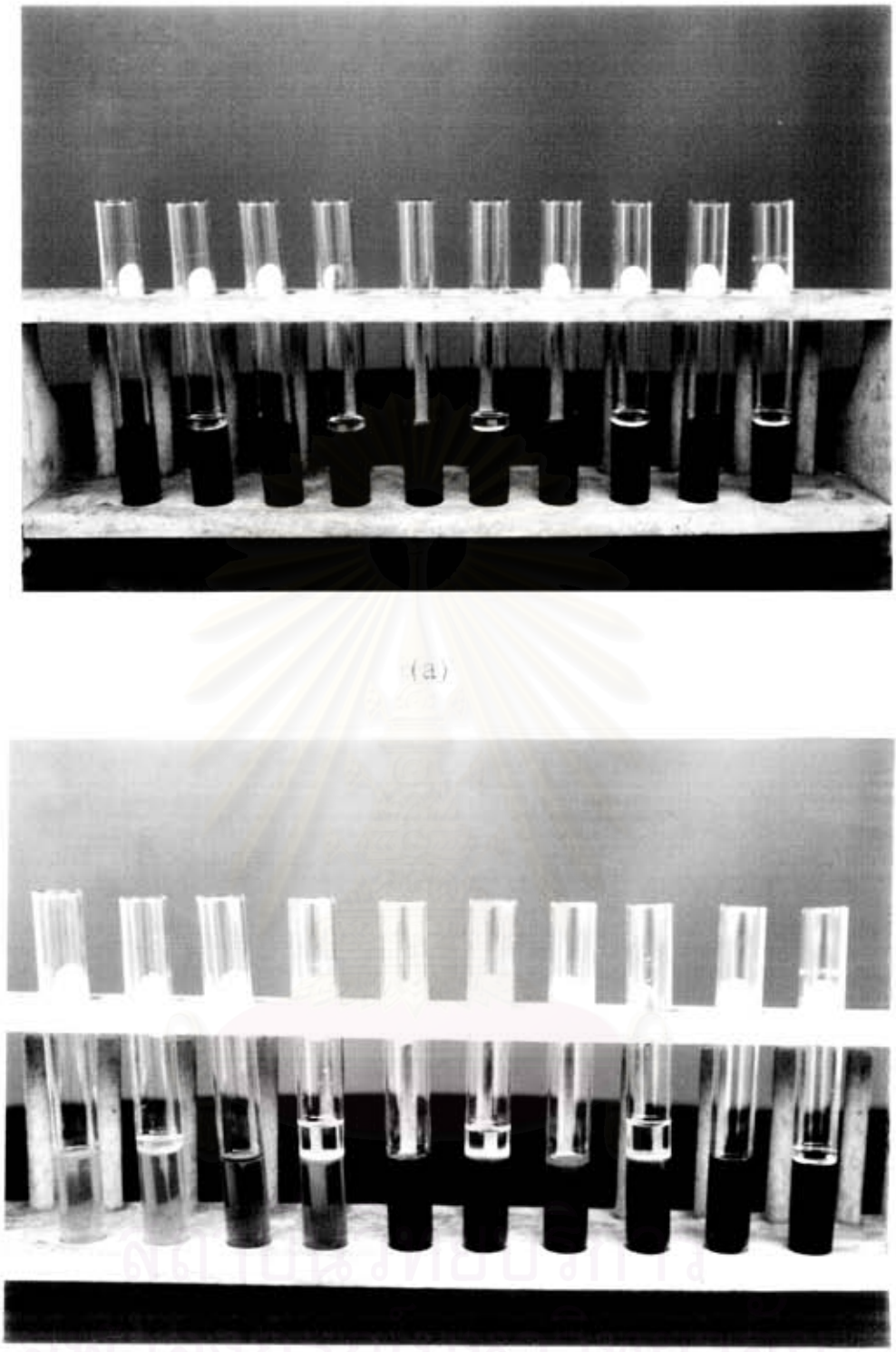
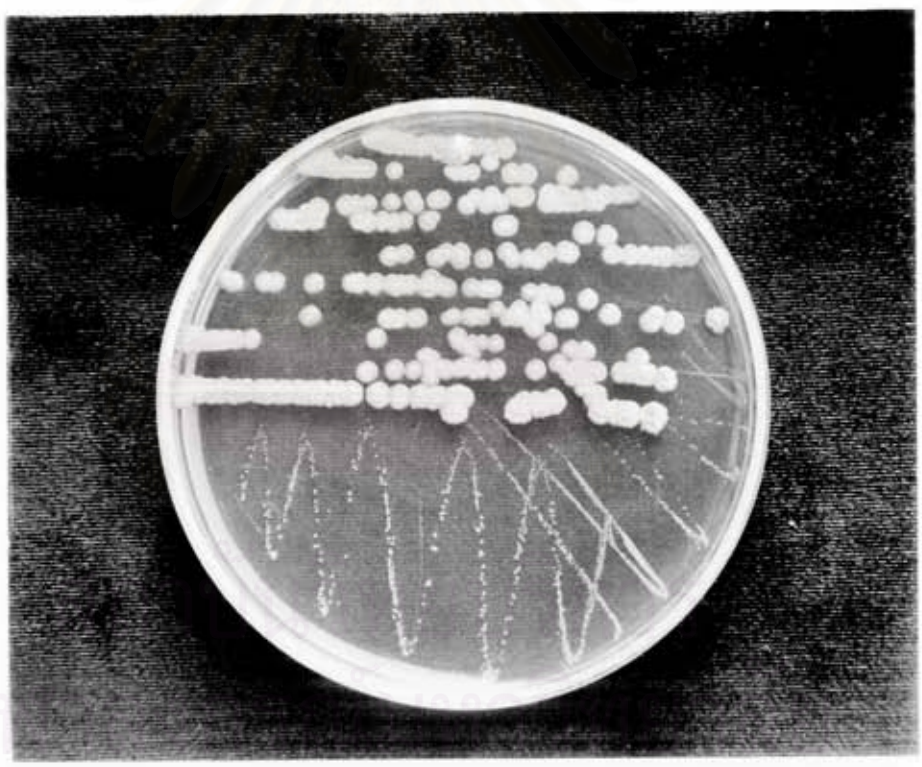


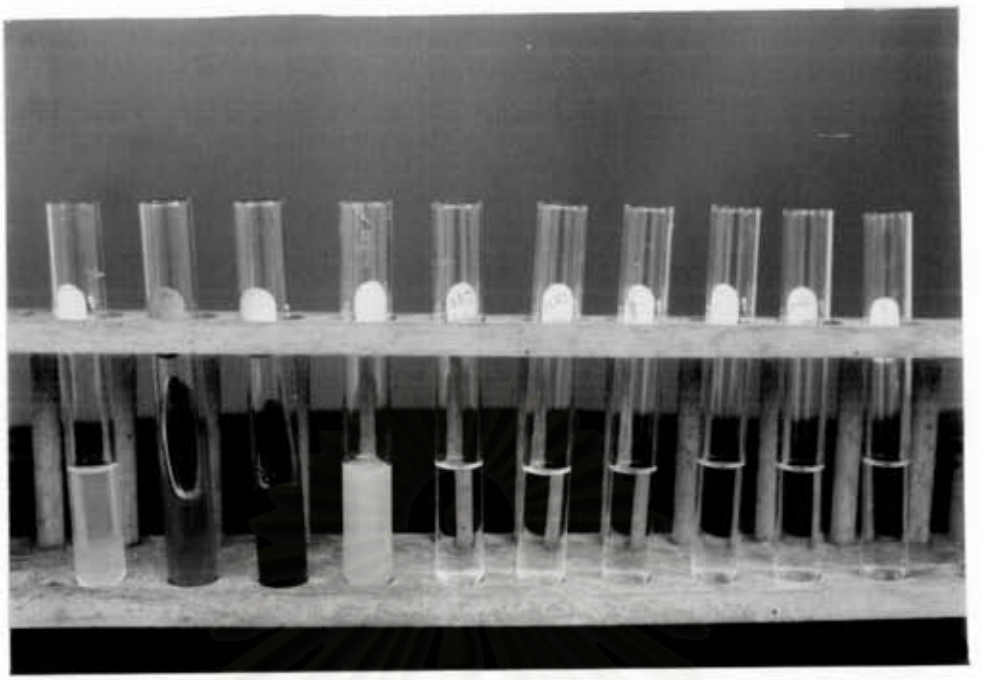
Figure 4.6 Oxidation-Fermentation test (left to right; glucose, dextrose, lactose, maltose and sucrose) of alkaline lipase producing bacterial strain ALP-40.3 with control (a) and results (b).

(a)

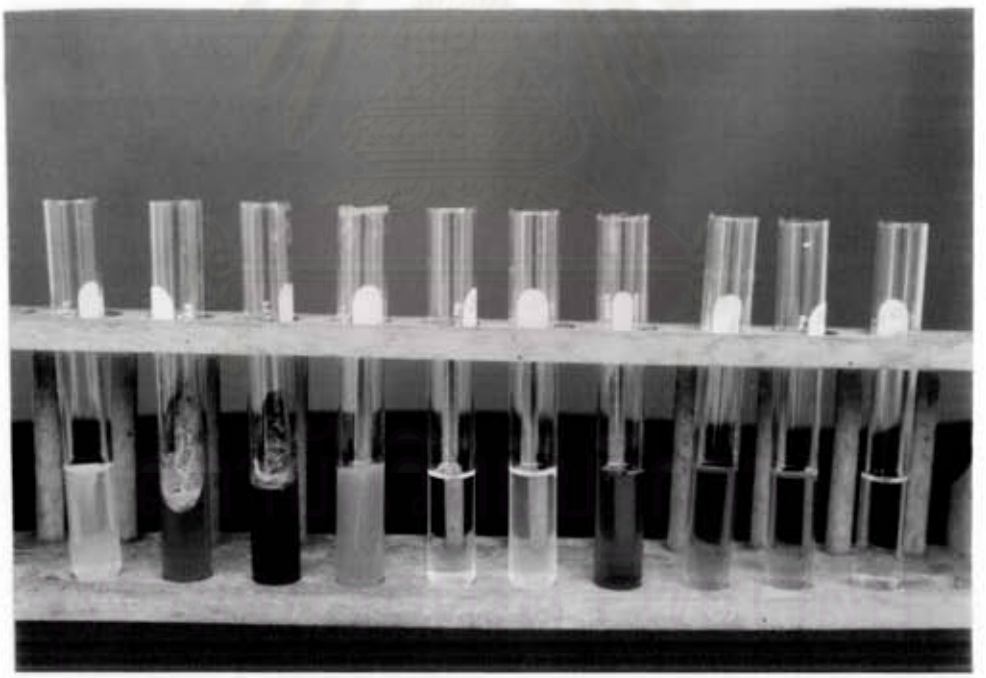


(b)

Figure 4.7 Clear zone resulted from hydrolysis of starch(a) and colonial characteristics of alkaline lipase producing bacterial strain ALP-40.3 grown on SSA (b), incubated at 37 ^{0}C for 24 hr.

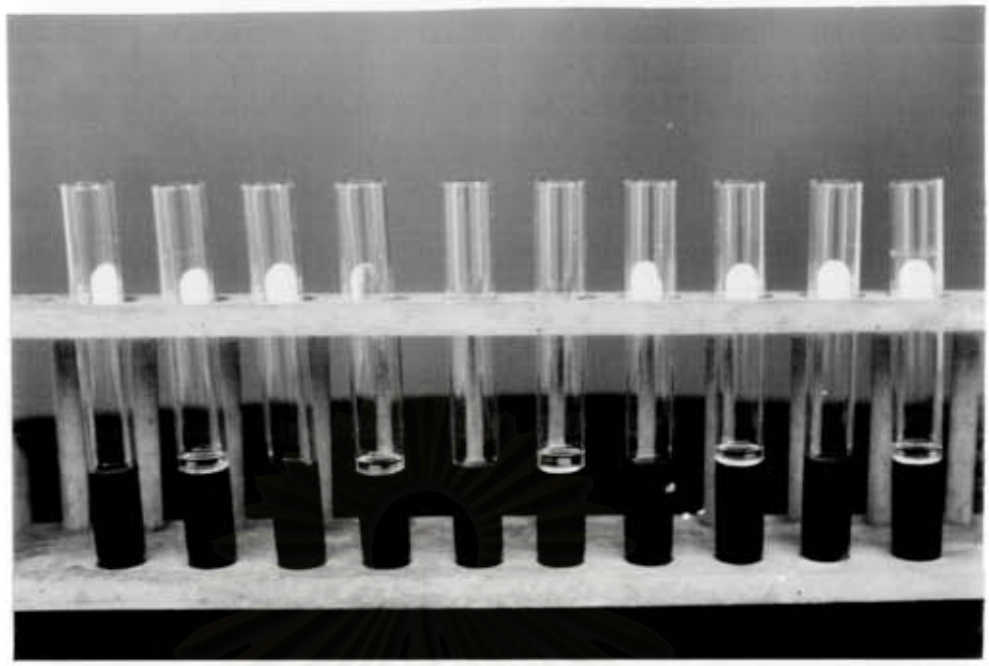


(a)

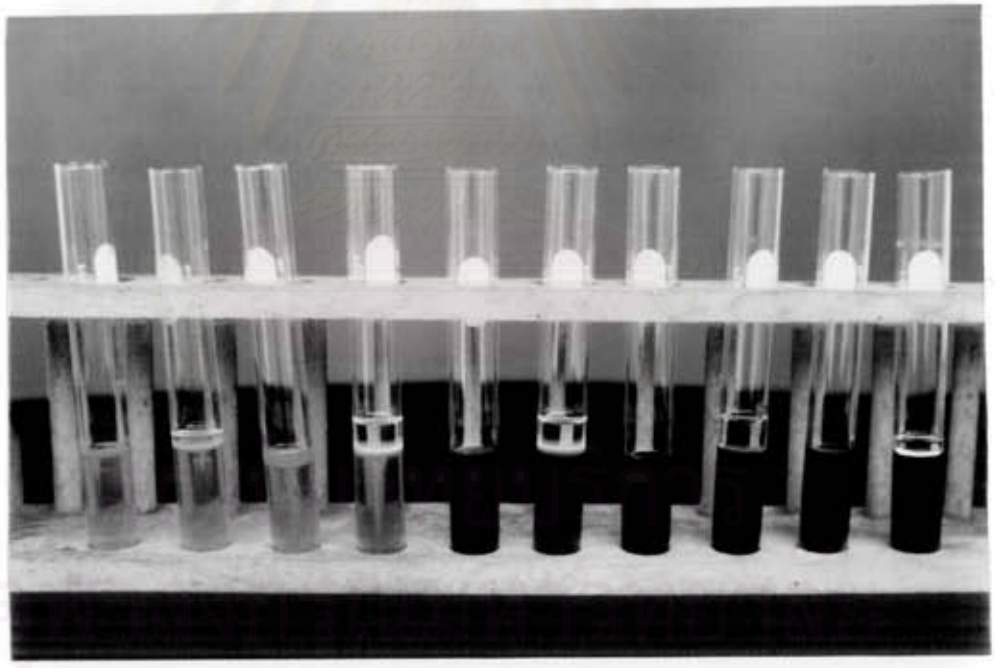


(b)

Figure 4.8 Biochemical test (left to right; motility , TSI, citrate utiliztion, urease, KCN, no KCN, nitrate reduction, indole, MR and VP test) of alkaline lipase producing bacterial strain ALP-42.11 with control (a) and results (b).

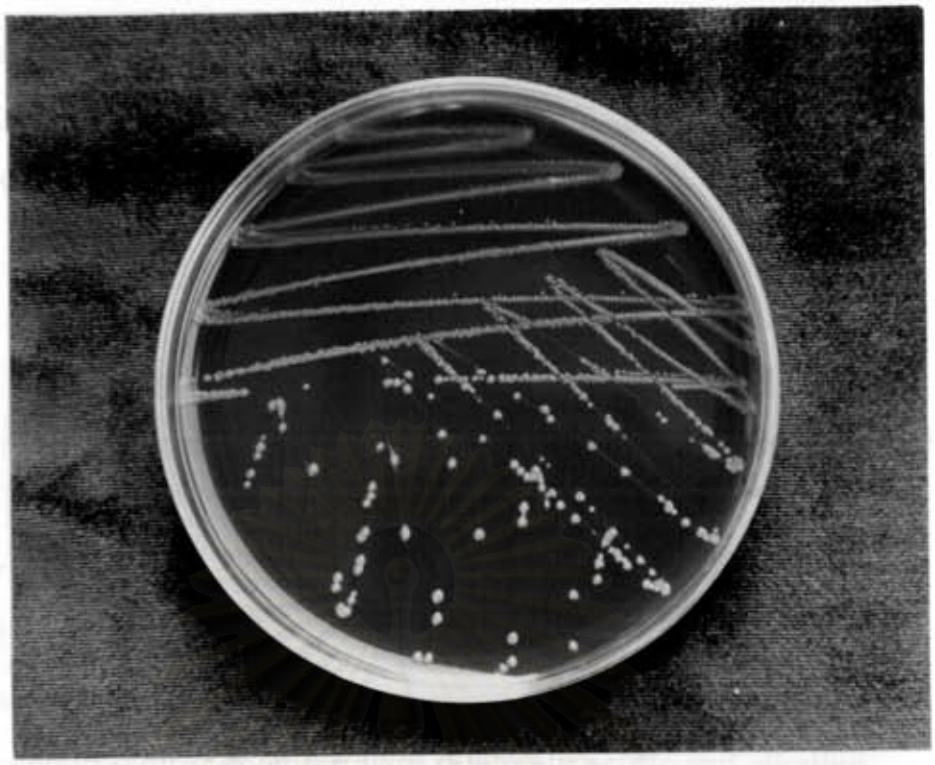


(a)

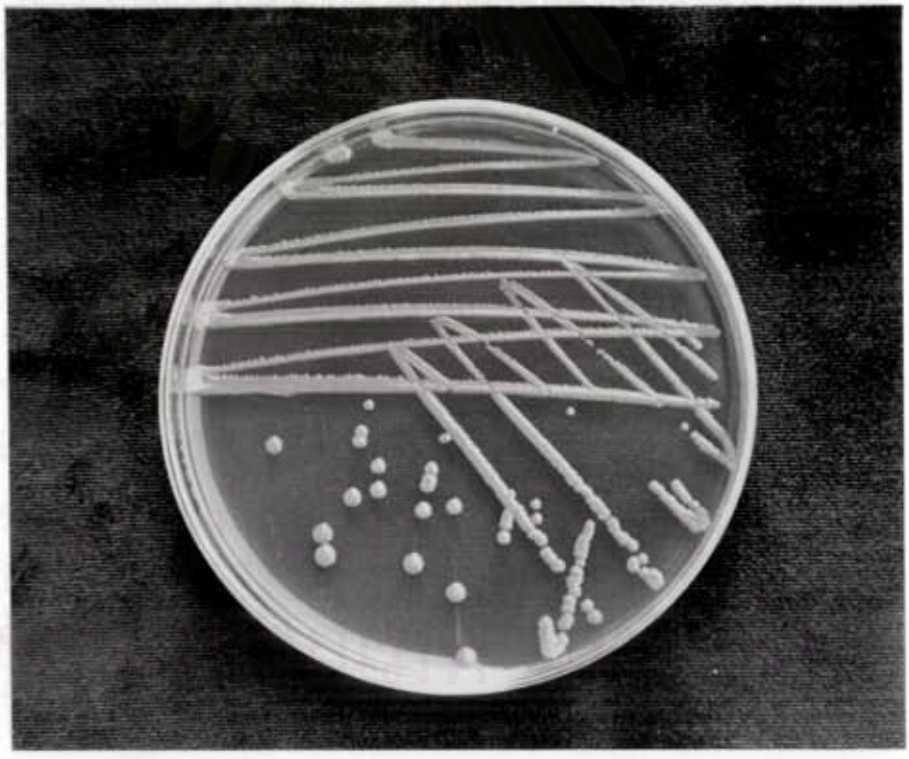


(b)

Figure 4.9 Oxidation-Fermentation Test (left to right; glucose, dextrose, lactose, maltose and sucrose) of alkaline lipase producing bacterial strain ALP-42.11 w ith control (a) and results (b).



(a)



(b)

Figure 4.10 Colonial characteristics of alkaline lipase producing bacterial strain ALP-42.11 grown on EMB (a) and SSA (b), incubated at 37 ^{0}C for 24 hr.

4.2 EFFECT OF SOME ENVIRONMENTAL FACTORS ON GROWTH OF ALKALINE LIPASE PRODUCING BACTERIAL ISOLATES.

4.2.1 Effect of pH

Both of the selected bacterial isolates showed good growth with optimum pH value 8. However, ALP-40.3 strain was able to grow in a wide pH range from 6.0 to 10.0 whereas the other, ALP-42.11, was able to grow in a wide pH range from 5.0 to 10.0 (Summarized in **Table 4.5**). No growth of ALP 40.3 strain was observed in culture medium with relatively acidic and alkaline condition, i. e., pH 5.0 and pH 10.0, after overnight incubation at 37°C. Effects of pH on growth of the selected alkaline lipase producing bacteria were also given in **Figure 4.11.**

4.2.2 Effect of Temperature

The optimum temperature on growth of the two selected bacterial isolates was summarized in **Table 4.6**. They were shown the largest number of cells at 35°C for ALP-40.3 strain and 25°C for ALP-42.11 strain. Growth of both bacterial isolates was inhibited remarkably at 45°C to 65°C. It is possible to say that the selected bacteria strains, ALP-40.3 and ALP-42.11, to be mesotrophs.

Table 4.5 Effects of pH on growth of the bacterial isolates

[a]Bacterial strains	Initial log no. of organism (cells/ml)	Log no. of bacterial cells (cells/ml) at various pH						
		5	6	7	8	9	10	11
ALP 40.3	4.78	[b]ND	7.56	9.11	9.18	8.91	8.41	ND
ALP 42.11	5.86	9.38	9.41	9.51	9.57	7.08	5.88	5.45

[a]Bacterial strains were cultivated in 250 ml-flasks containing LPM with various pH values, maintained with appropriate buffer solution described in 3.6.2.1, and incubated at 37°c for 24 hrs on rotary shaker (200rpm).

[b]ND : not detect

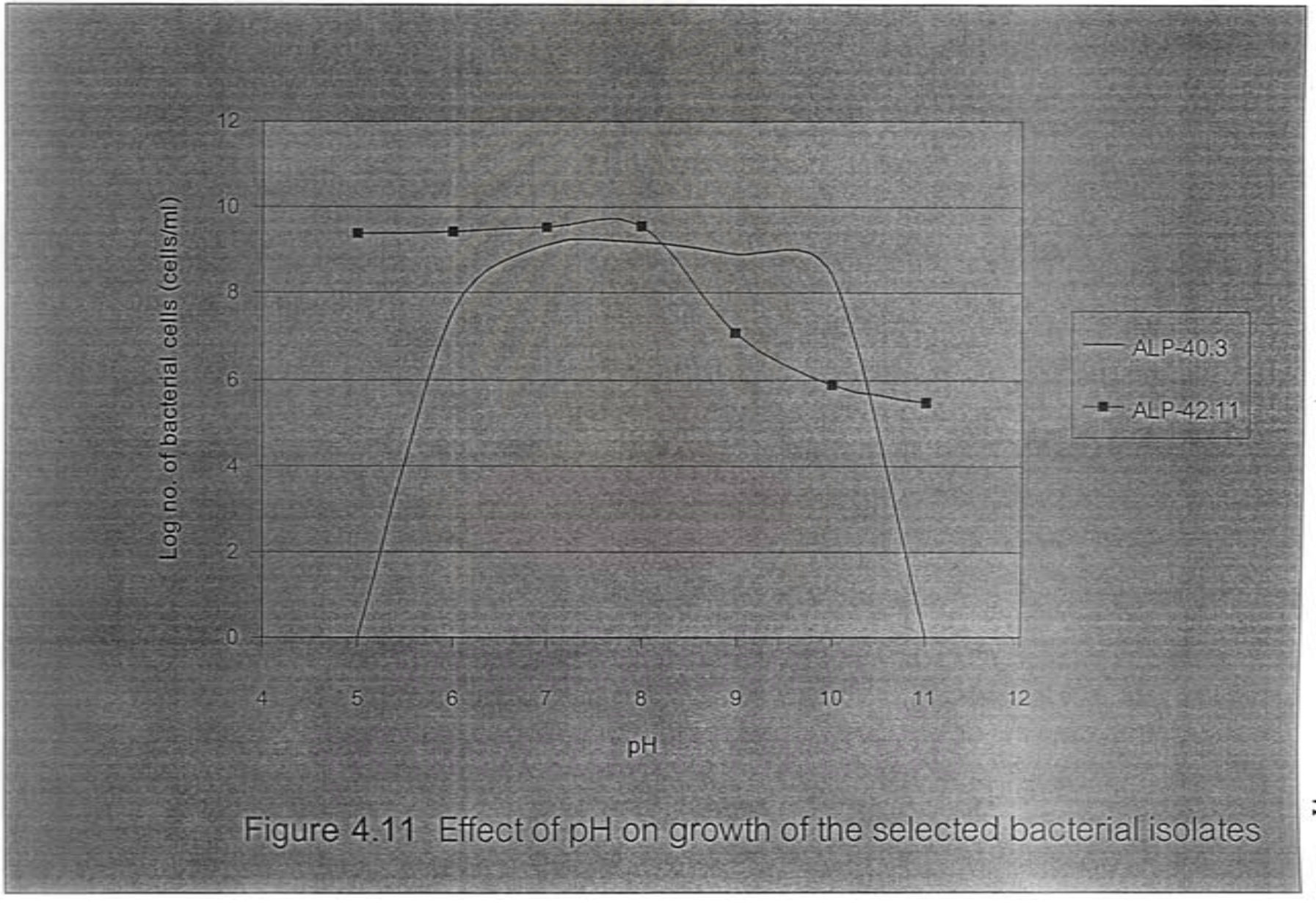


Figure 4.11 Effect of pH on growth of the selected bacterial isolates

Table 4.6 Effect of temperature on growth of the selected bacterial strains.

[a]Bacterial strains	Initial log no. of organisms (cells/ml)	Log no. of bacterial cells at various Temperature (cells/ml)				
		25°C	35°C	45°C	55°C	65°C
ALP 40.3	5.74	7.76	9.15	[b]ND	ND	ND
ALP 42.11	5.86	9.52	9.40	ND	ND	ND

[a]Bacterial strains were cultivated in 250-ml flask containing LPM with pH 8.0 and incubated for 24 hrs.

[b]ND : not detect

4.3 GROWTH CONDITION OF SELECTED BACTERIAL STRAINS

4.3.1 Growth Curve of ALP-40.3 Strain

The result of lipase production relating to growth of ALP-40.3 strain was summarized in **Table 4.7** and shown in **Figure 4.12.** The number of viable cells was highest at 12 hours in stationary phase, as well as the maximum production of lipase was also found in the same period.

4.3.2 Growth Curve of ALP-42.11 Strain

Unlike the bacterial strain ALP-40.3, the maximum growth of bacterial strain ALP-42.11 was shown at 10 hours whereas lipase production was decreased and again increased after 48 hours of incubation (given in **Table 4.8** and **Figure 4.13**).

Table 4.7 Relationships between growth and lipase production of [a]bacterial strain ALP-40.3

Time (hr)	Log no. of bacterial cells (cells/ml)	Lipase activity ([b]Unit/ml)
O	7.31	0
1	7.41	[c]NM
2	7.64	NM
4	8.88	40.00±10.58
8	9.42	456.67±25.11
10	9.50	NM
12	9.58	1808.67±120.96
18	9.53	301.33±50.60
24	9.51	673.33±25.17
30	9.46	135.33±17.01
48	9.41	476.67±91.09

[a]Bacterial strain 40.3 was cultivated in MGM with 1% olive oil at 35°C on incubation shaker (200 rpm) for 48 hrs.

[b]Unit : nmol of pNP released per min.

[c]NM : no measurement

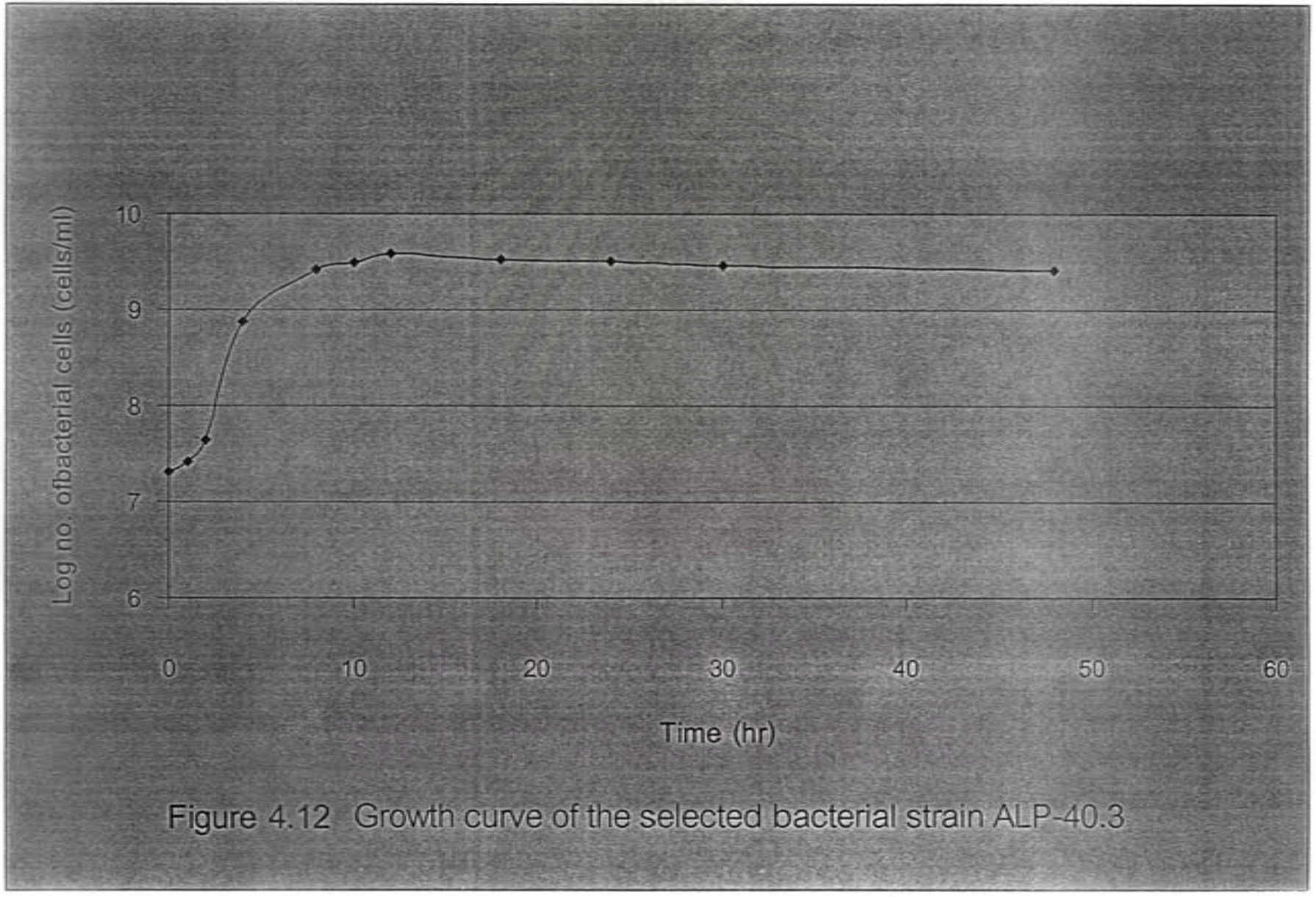


Figure 4.12 Growth curve of the selected bacterial strain ALP-40.3

Table 4.8 Relationships between growth and lipase production of bacterial strain ALP-42.11

Time (hr)	Log no of bacterial cells (cell/ml)	Lipase activity ([b]Unit/ml)
0	7.58	0
1	7.81	[c]NM
2	8.04	NM
4	8.97	117.67±0.58
8	9.40	149.00±8.18
10	9.46	NM
12	9.36	107.33±11.02
18	9.07	62.67±4.16
24	8.89	103.67±9.50
30	8.81	66.67±10.41
48	8.58	198.67±0.76

[a]Bacterial strain 42.11 was cultivated in MGM with 1% olive oil, pH 8.0 and at 25°C on shaker (200 rpm) for 24 hrs.

[b]Unit : nmol of pNP release per min.

[c]NM : Not measurement.

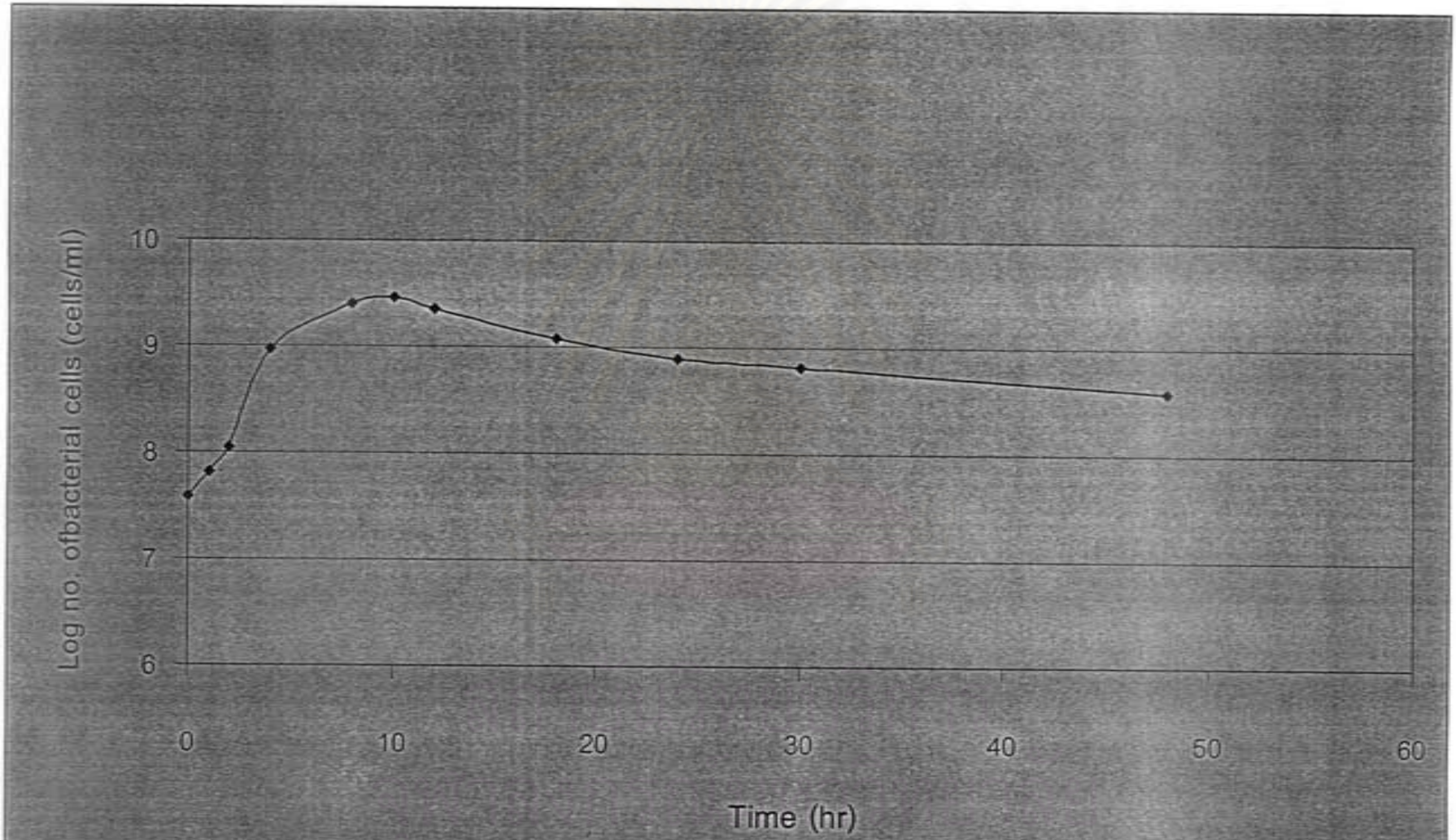


Figure 4.13 Growth curve of the selected bacterial strain ALP-42.11

4.4 EFFECT ON DIFFERENT MEDIA ON LIPASE PRODUCTION

Both selected bacterial strains were cultivated in media, namely, LPM and LOB with optimum pH and temperature at various times. It was found that the strain ALP-40.3 was able to produce a large amount of lipase in LPM at 18 hours of incubation. Moreover, the other also produced high quantities of lipase in LPM at the same incubation time. Therefore, the suitable medium and incubation time to be used for the production of alkaline lipases from the selected bacterial isolates were LPM and 18 hours of incubation time as shown in **Table 4.9.** However, those bacterial strains were cultivated at optimum temperature and pH for each strain.

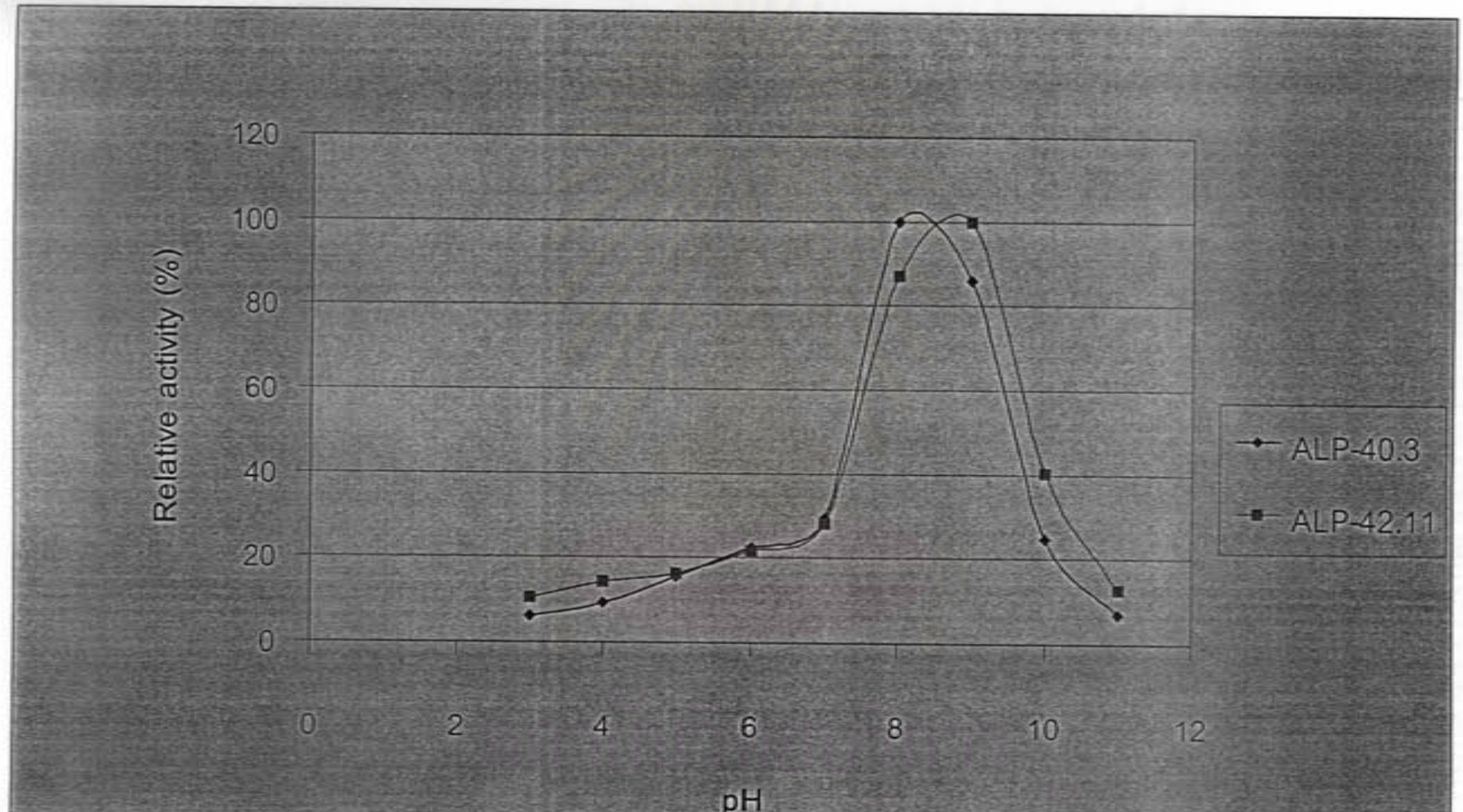


Figure 4.14 Effect of pH on activity of lipases of the selected bacterial isolates

4.5 EFFECT OF SOME ENVIRONMENTAL FACTORS ON ACTIVITY OF CRUDE LIPASES PRODUCED FROM THE SELECTED BACTERIAL ISOLATES.

It was found that the optimum pH for the highest activityof crude lipases produced by ALP-40.3 and ALP-42.11 were 8 and 9, respectively and both of them were able to grow in slightly alkaline condition (**Table 4.10** and **Figure 4.14**).

The stability of enzyme crudely extracted from both of the selected bacterial isolates was performed at various pH, ranging from 3 to 11, and both enzymes were stable in alkaline region from 8 to 11 for 24 hours, but in acid region, their residual activity was quite low (summarized in **Table 4.11** as well as **Figure 4.15**).

Optimum temperatures of alkaline lipases extracted from the isolated bacterial strains were given in **Table 4.12,** and the high activity of lipase produced by ALP-40.3 and ALP-42.11 were 702.33±186.39 units per ml and 561.33 unit per ml when they were incubated at 45 and 65°C, respectively. Effect of temperature of bacterial isolates was also shown in **Figure 4.16.**

After 20 minute-incubation, crude lipase extracted from ALP-40.3 strain ALP-40.3 was stable up to 65°C, and its activity was sharply decreased at 75°C. Thermostability of alkaline lipase from strain ALP-42.11 was also in the same range as lipase from strain 40.3 but lower in activity was shown at 75°C as illustrated in **Table 4.13** and **Figure 4.17**.

Table 4.10 Effect of pH on lipase activity from the selected bacterial strains.

[a]pH	Lipase activity ([b]Unit/ml)	
	Lipase from strain ALP- 40.3	Lipase from strain ALP-42.11
3.0	2.24±1.29	3.64±1.28
4.0	3.36±1.45	5.04±0.81
5.0	5.60±0.49	5.59±0.97
6.0	8.11±1.74	7.56±0.84
7.0	10.63±1.28	9.79±0.01
8.0	36.10±3.66	30.23±4.67
9.0	31.07±7.17	34.70±5.46
10.0	8.95±1.28	13.99±4.63
11.0	2.52±0.84	4.48±1.28

[a]pH of buffer systems (100mM) used ; glycine-HCl (pH3.0-4.0); phosphate buffer (pH5.0-6.0) ; Tris HCl (pH7.0-9.0) ; and glycine-NaOH (pH10.0-11.0).

[b]Unit : nmol of free fatty acid released per min.

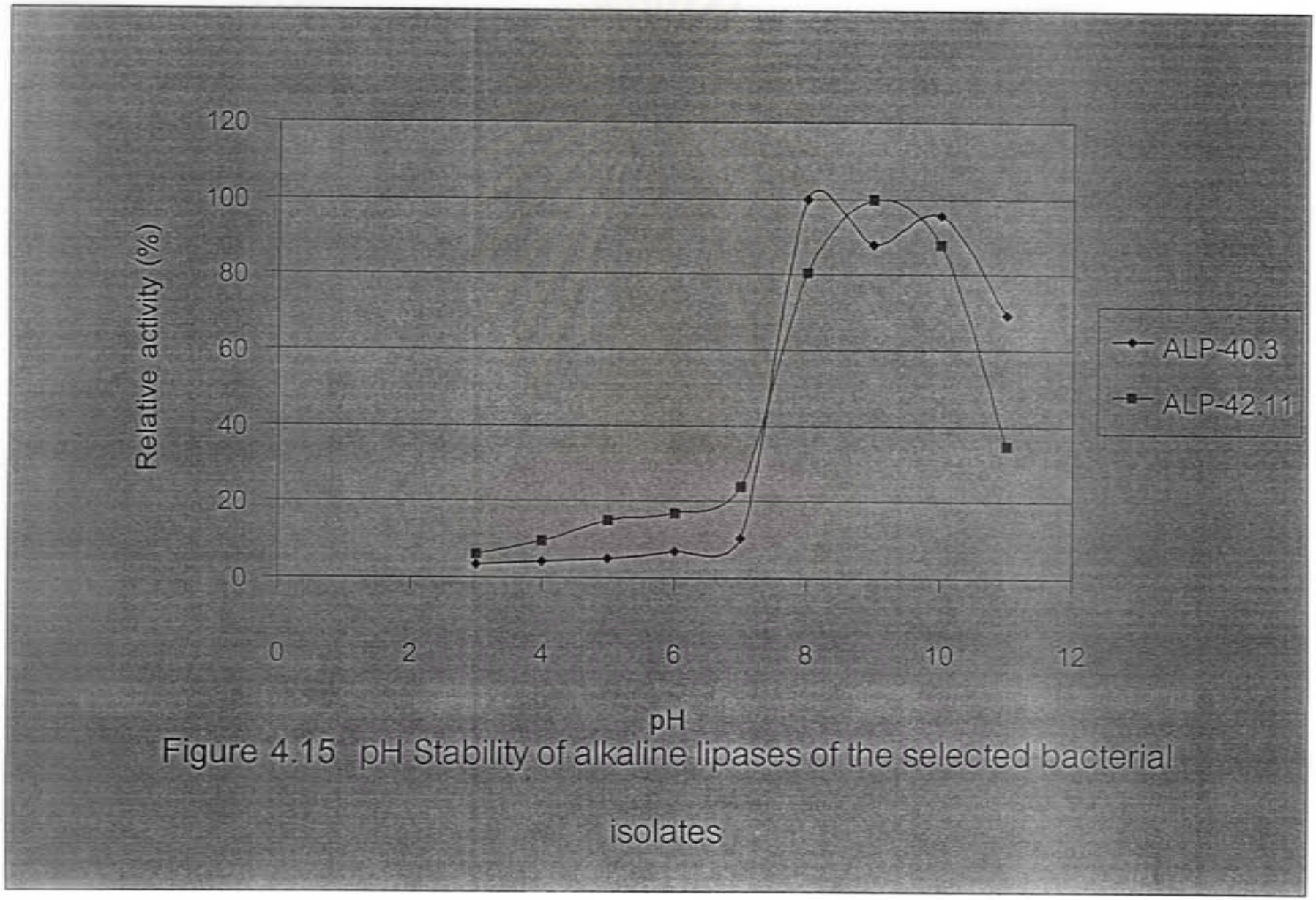


Figure 4.15 pH Stability of alkaline lipases of the selected bacterial isolates

Table 4.11 pH Stability of alkaline lipase from selected baeterial strains.

[a]pH	Lipase activity ([b]Unit/ml)	
	Lipase of strain ALP-40.3	Lipase of strain ALP-42.11
3	1.12±0.49	1.96±0.48
4	1.40±0.45	3.08±0.49
5	1.67±0.08	4.76±1.28
6	2.24±0.01	5.32±0.49
7	3.37±0.08	7.56±0.84
8	32.47±1.46	25.19±3.02
9	28.54±2.56	31.34±2.56
10	31.04±3.79	27.50±2.18
11	22.51±3.24	24.92±1.68

[a]pH of buffer systems (100mM) used; glycine-HCl (pH 3.0- 4.0); phosphate buffer (pH 5.0- 6.0); Tris-HCl (pH 7.0-9.0); and glycine-NaOH (pH 10.0-11.0). The lipase solution at various pH was kept at 5°C for 24 hrs.

[b]Unit : nmol of free fatty acid liberated per min.

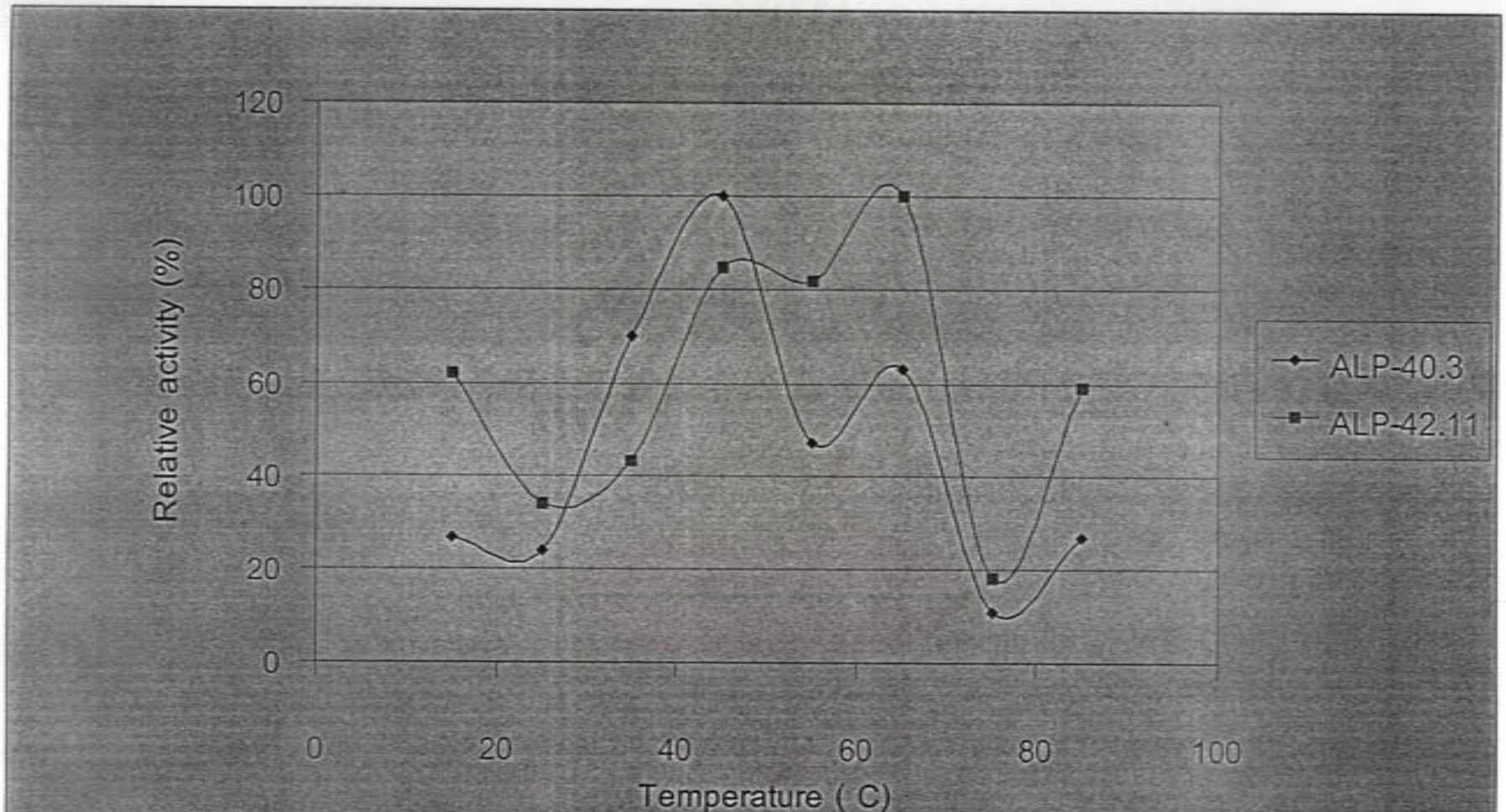


Figure 4.16 Effect of temperature on activity of alkaline lipases of the selected bacterial isolates

Table 4.12 Effect of temperature on alkaline lipases of the selected bacterial strains.

Temperature (°C)	Lipase activity ([a]Unit/ml)	
	Lipase of Strain ALP- 40.3	Lipase of Strain ALP-42.11
15	189.67±4.93	349.33±40.80
25	169.78±112.25	188.33±4.51
35	490.00±37.27	239.67±14.57
45	702.33±186.39	475.33±31.18
55	327.67±18.23	457.67±37.69
65	440.67±90.97	561.33±16.01
75	75.67±4.72	98.67±15.28
85	188.33±3.78	329.33±23.67

[a]Unit : nmol of pNp released per min.

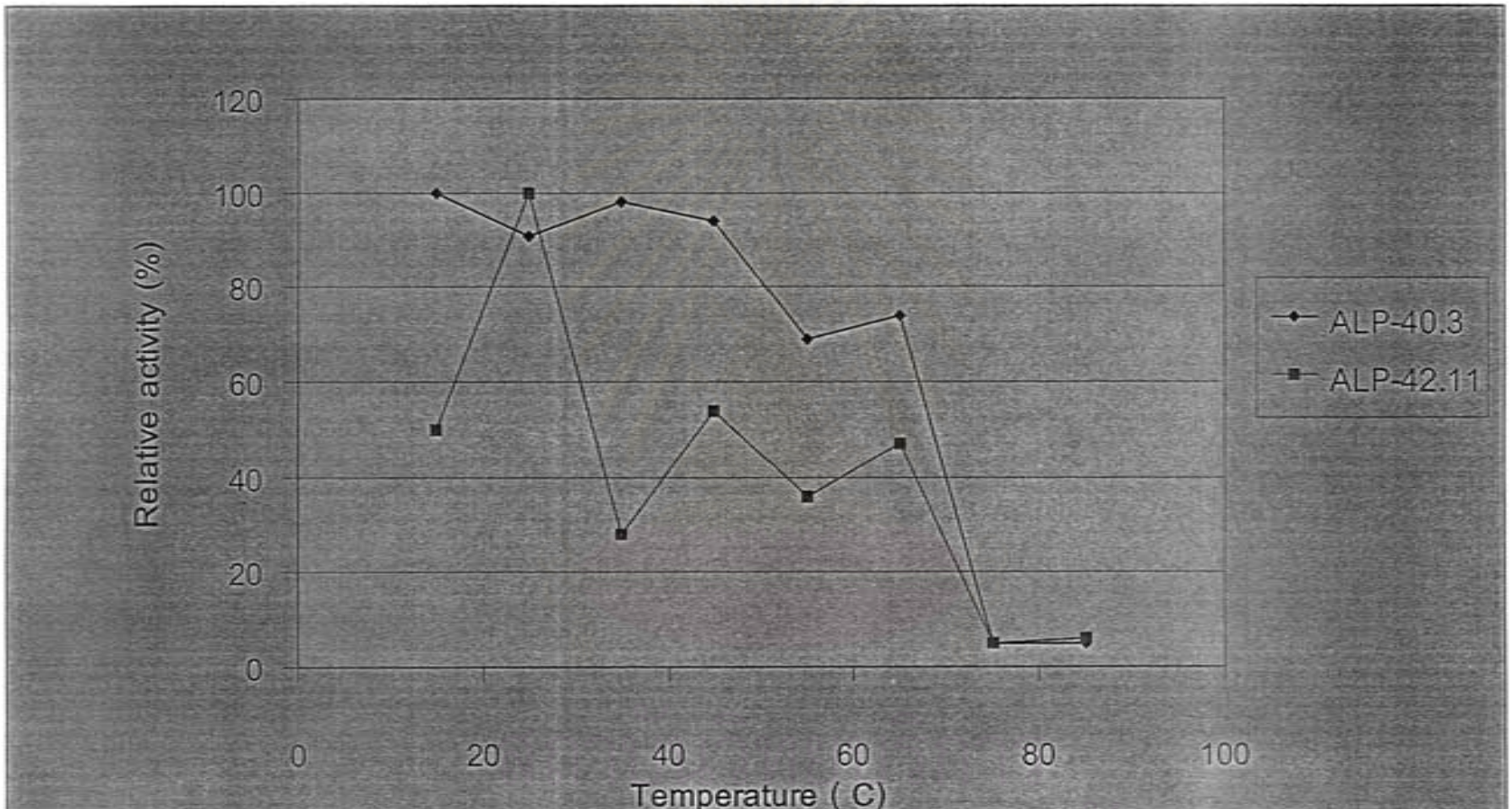


Figure 4.17 Thermostability of alkaline lipases of the selected bacterial isolates

Table 4.13 Thermostability of alkaline lipases of the selected bacterial strains.

Temperature (°C)	Lipase activity ([a]Unit/ml)	
	Lipase of strain ALP- 40.3	Lipase of Strain ALP- 42.11
15	498.67±37.43	452.67±12.10
25	452.00±19.05	902.23±68.13
35	489.00±40.78	250.33±57.98
45	470.33±50.00	485.33±30.66
55	344.33±17.90	325.00±56.71
65	371.00±9.54	427.67±69.56
75	10.67±5.77	43.00±13.53
85	25.33±3.06	52.33±12.66

[a]U: nmol of p-NP released per min.

[b]The enzyme solution of strain 40.3 in Tris-HCl (pH 8.0) was incubate at each temperature for 20 min, and the residual activity was measured by the method assay described in 3.8.1.

[c]The enzyme solution of strain 42.11 in Tris-HCl buffer (pH 9.0) was performed as the same as lipase of strain 40.3, except pH.

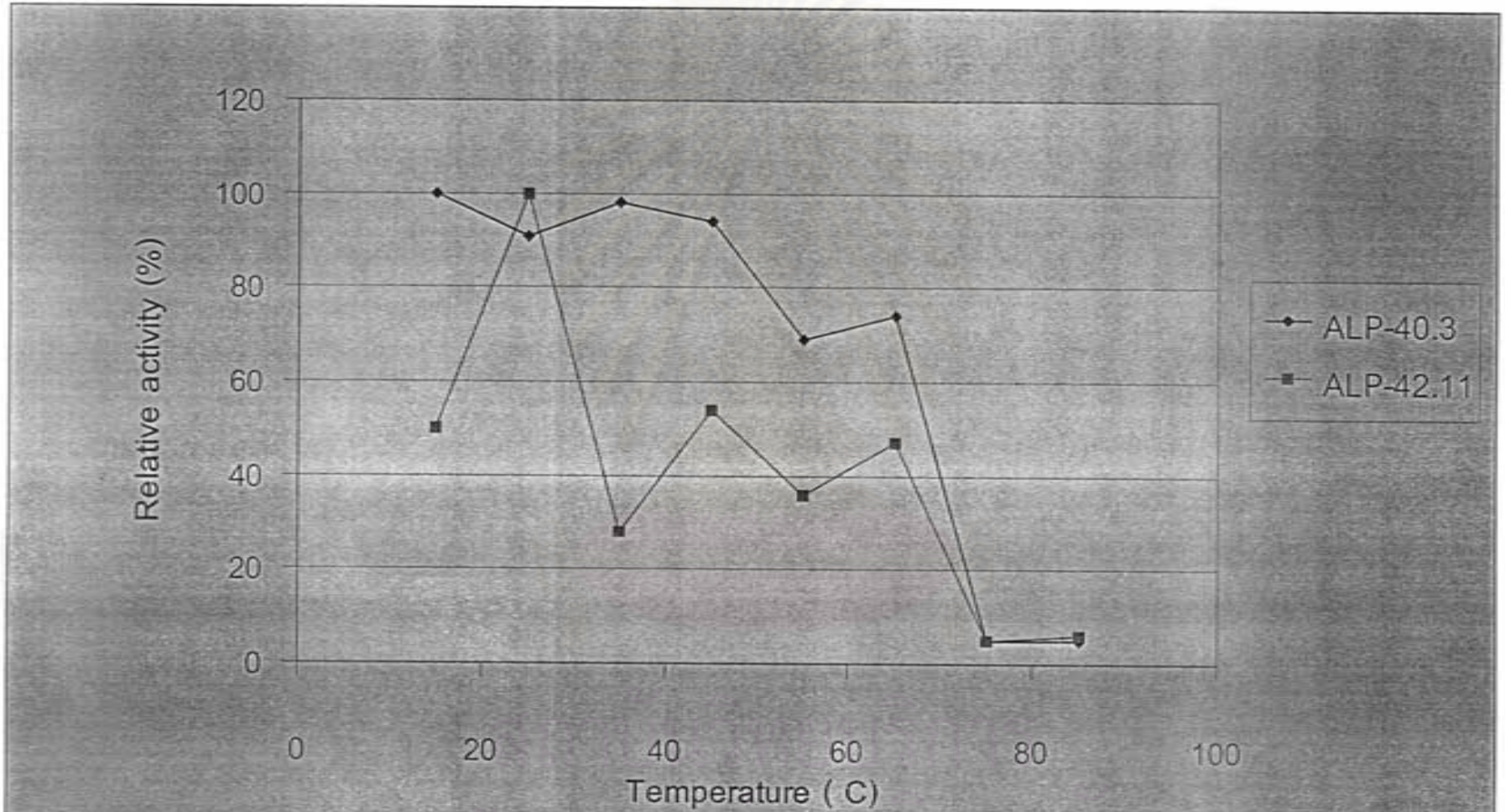


Figure 4.17 Thermostability of alkaline lipases of the selected bacterial isolates

4.6 DETERMINATION OF SUBSTRATE SPECIFICITY ON ALKALINE LIPASE ACTIVITY

Similarly to soybean oil, corn oil, olive oil, castor oil and palm oil substrates as shown in **Table 4.12** were used to examine the specificity of alkaline lipases extracted from the selected bacterial strains. It was shown that lipase extracted fromALP-40.3 and ALP42.11 prefered olive oil as substrate (161% hydrolysis) and palm oil (194% hydrolysis) comparing with corn oil.

Table 4.14 Relative rate of hydrolysis of oils by alkaline lipase from selected bacterial isolates.

Oils	Relative rate of hydrolysis (%)	
	Lipase of [a] strain ALP-40.3	Lipase of [b] Strain ALP-42.11
Soybean oil	6	77
Corn oil	100	100
Olive oil	161	129
Castor oil	44	8
Palm oil	6	194

[a] Reaction mixture of strain 40.2 lipase was incubated at 45°c for 1 hr, 100% activity corresponds to 677.33 ± 92.12 units at lipase activity assayed under condition described in 3.8.2.

[b] Reaction mixture of strain 42.11 lipase was incubated at 65°C for 1 hr, 100% activity corresponds to 268.67 ± 16.50 units of lipase activity assayed under condition described in 3.8.2.