Vet.Med.J.,Giza. Vol.55, No. 2. (2007):623-637.

THE USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN SEPARATION AND PURIFICATION OF LP 64 KDA BAND OF MYCOPLASMA GALLISEPTICUM IN LARGE QUANTITIES

EL-SHATER S. A. A., S. I. EISSA and A. M. HASSAN
Animal Health Research Institute, Agricultural Research Center, Dokki, Egypt.

Received: 18. 11. 2006.
Accepted: 11. 12. 2006.

SUMMARY

High performance liquid chromatography (HPLC) size exclusion technique (SEC) was used for separation and purification of the LP 64 kDa band of *Mycoplasma gallisepticum* (MG) in large quantities. We found that the use of 210 nm was the best wave length for isolation and gave highest peak and the best resolution. The use of 200 mM of NaH_2PO_4 as a mobile phase improved chromatographic profile. Injection volume of 200 µl resulted in the highest peak and the best resolution. The flow rate of 5ml /min increased the peak height and profile resolution. The use of pH 5.7 for the *Mycoplasma gallisepticum* culture also improved the height of the chromatographic peak and the profile of resolution and was the optimum pH for production and purification. The use of HPLC-SEC could be adapted to extract and purify the intended LP64 kDa band of MG.

INTRODUCTION

Mycoplasma gallisepticum is one of the most important disease agents affecting poultry causing high losses to poultry farms. The LP64 kDa is a lipoprotein playing major role in cytadherence of *Mycoplasma gallisepticum* (Forsyth et al., 1992). Many methods for isolation and purification of this lipoprotein were used such as pH of the medium, buffer and salts, detergents and chelators or metal ions but the most accurate is high performance liquid chromatography (HPLC) (Jansen and Rydén, 1998). Size-exclusion chromatography (SEC) has widely been used for separation and purification of proteins. SEC is an entropically controlled separation technique that depends on the relative size of macromolecules with respect to the size and shape of the pores of the packing. High performance size-exclusion chromatography (HP-SEC) columns are favored because of their speed, high resolution and high sensitivity (Laing

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et al., 2001). Optimization of HP-SEC conditions such as wave length of the detection, ionic strength of the mobile phase, sample injection volume, flow rate and pH of the MG culture (Engelhardt and Ahr, 1983). Our work was to optimize the HPLC-size exclusion technique for production and purification of the LP 64 kDa band of *Mycoplasma gallisepticum*.

MATERIALS AND METHODS

Materials:

A Model P 580 pump and Model UVD 170 S detector were used (Dionex, USA). The column employed for separation by size exclusion (SEC) was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax). The fraction collector was Foxy Jr (Isco,Inc., USA).

Methods:

1- Preparation of media:

The basal broth dehydrated culture media was added to 100 ml distilled water, then sterilized by autoclaving at 121°C for 15 min. After cooling to 50°C other enrichment ingredients; horse serum, yeast extract, DNA and inhibitors (Thallium and penicillin G sodium), were added aseptically. Sterility was checked by incubation of the media at 37°C over night then stored at 4°C for use within 2-3 weeks (Frey et al., 1968)

2- Culture passage

M. gallisepticum, strains F (vaccinal strain), R, PG31 (ATCC type strain) and A5969 (high - passage chicken isolate), were grown in Frey's medium with 12% swine serum. All cultures were grown at 37°C and harvested by centrifugation at 12000x g for 20 min.

3-Integral membrane proteins extraction

Extraction of integral membrane protein was accomplished with triton X-114 by the method of (Bricker et al, 1988), withsome modifications briefly pellets of M. gallisepticum (1.25 mg protein/pellet) were resuspended in 1 ml of ice cold. 1.0% (v/v) Triton X-114 was added in (10mM Tris, 150 mM NaCl buffer, pH 7.49 (TS buffer) with 2mM phenylmethylsulphonyl floride (PMSF, Sigma chemical co.) these were incubated in at 4°C for 30 min. Insoluble materials was pelleted by two cycles of centrifugation at 4°C for 15 min. at 13000 g. Supernatants were incubated at 37°C for 8 min. and centrifuged at 10000 g for 5 min at room temperature. The aqueous phase was readjusted with 10% Triton X-114 to 1.0% (v/v). The detergent phase was brought to the original volume with TS buffer. The previous method was repeated five times and the detergent phase was used for HPLC after dialyzing against three changes of 150 mM phosphate buffer saline (PBS) and stored at - 20°C until used.

4- Optimization of HPLC-SEC conditions:

4-1-Standard protein

64 kDa protein was isolated by electroelution after SDS-PAGE electrophoresis and then was used as slandered protein for the separation by HPLC-

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SEC procedure. The retention time of the protein was determined (from 6-11 min.) and 3 fractions could be separated having the same retention time.

4-2-Optimization of the wavelength.

Different wavelength 210 nm, 220 nm, 240 nm and 280 nm were used to optimize the wavelength, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax). The mobile phase is 0.2 M di-sodium phosphate, pH 7.0.

4-3-Optimization of the strength of mobile phase.

Two mobile phase 100 mM and 200 mM NaH_2PO_4 were used to improve the chromatographic profile of separated protein, size exclusion GF-250Xl column 21.2 mm ID X 250 mm (Zorbax) was used. The injected sample was 200µl, the flow rate 5 ml / min. and detection was by UV absorption at 210 nm.

4-4-Optimization of the sample injection volume.

Different volumes of the protein samples (20 , 40, 200 and 300 µl) were injected ,the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M disodium phosphate, pH 7. 0 ,. The flow rate 5 ml / min. and detection was by UV absorbtion at 210 nm. The fraction collector was Foxy Jr (Isco,Inc., USA).

4-5-Optimization of the flow rate.

Different flow rates (3 , 5 and 15ml/min) were used to improve the chromatographic profile of separated protein, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M di-sodium phosphate, pH 7. 0. injected sample 200 µl. and detection was by UV absorption at 210 nm. The fraction collector was Foxy Jr (Isco, Inc., USA)

4-6-Optimization of the pH of M. gallisepticum culture

Different pH of M. gallisepticum culture (7.4, 6.4, 5.7 , 5.45 , 5.25 and 5 pH) were used to obtain the highest peaks of separated protein, the column employed for separation by size exclusion was GF-250Xl column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M sodium phosphate, pH 7.0 . injected sample 200 µl. and The flow rate 5 ml / min detection was by UV absorbtion at 210 nm. The fraction collector was Foxy Jr (Isco, Inc., USA)

5-Analysis of Eluted Fractions:

A- SDS-PAGE. for the eluted fractions.

Electrophoresis was performed as described by Laemmli (1970). The gels were stained with Coo-

massie brilliant blue R-250 (Sigma), and de-stained with mixture of 45% methanol, 10% acetic acid and 45% distilled water .

B-Immunological activity of the eluted fractions.

Electrophoretic transfer to nitrocellulose (NTC) paper was accomplished as described by Towbin et al., (1979).After transfer to NTC, the membranes were stained with Ponceau's (Sigma), and the molecular weight standards were marked. The NTC blots were blocked with 5 % bovine serum albumin (BSA), 20 % fetal bovine serum, in Tris-buffer saline (10 mM Tris, pH 7.3, 0.9% NaCl), for 1 hr at 37°C with gentle rocking, The Mg antisera was diluted (1:500) in TBST (10 mM Tris pH 7.2, 150 mM NaCl, 0.05% Tween 20). Secondary antibody (horse radish peroxidase) conjugate was diluted 1:1000. The blots were developed for 5-10 minutes using H_2O_2 and 4-chloro-1-naphthol substrate.

6-Calculations: resolution for two adjacent peaks (m&n) can be calculatedas follows:

$$\text{Resolution} = \frac{2(t_n - t_m)}{w_n + w_m}$$

(t_n,t_m retention times wn,wm-band width)

RESULTS AND DISCUSSION

The LP 64 kDa is a lipoprotein playing major role in cytadherence of *Mycoplasma gallisepticum* (Forsyth et. al., 1992), so it is the part of cell responsible for its virulence. For obtaining this lipoprotein by HPLC the most suitable method was the size exclusion technique in order to obtain this lipoprotein according to its molecular weight (64 kDa).

Optimization of the HPLC in separation and purification of LP 64 kDa band was studied including: wavelength , the mobile phase, sample injection, flow rate and pH of *M. gallisepticum culture.*

Optimization of the HPLC of wavelength on peak height using different wavelengths 210 nm , 220nm , 240 nm and 280 nm., results revealed that 210 nm gave the best peak height 58.91 mAU (Table 1) and the best resolution (Fig. 3). These results were in agreement with Calam and Davidson (1984), Laing et al., (2001) and Hayakawa et al., (2001).

To overcome the electrostatic effects between mobile phase and size-exclusion column matrix to improve the chromatographic profiles two aqueous buffers containing 100 mM NaH_2PO_4 and 200 mM NaH_2PO_4 were used; the aqueous buffer containing 200 mM NaH_2PO_4 improved the chromatographic profiles (Table, 2). The use of buffer ionic strength in range of (0.05-0.5M) was found to be suitable to avoid interaction between solute and matrex, this was in agreement with Jansen and Ryden (1998), also Laing et al, (2001) discovered that albumin or myoglobin had proper

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size exclusion behavior at cocentrations from 25 to 300 mM.

Different injection volumes were tried 20, 40, 200, 300 µl to optimize the injection volume of protein sample on peak height .The injection of 200 µl resulted in peak height of 657.21 mAU and increased the resolution of the band (Fig. 4), while in case of injection of 20 µl,40 µl,300 µl the peak heights were 28.47 ,58.91 and 510 mAU ,respectively (Table 3).

The effect of varying flow rates on peak height , protein profile resolution revealed that the decrease of flow rate from 15 ml/min to 5 ml/min, increase peak height from 55.41 mAU to 114.63 mAU and also increase in the resolution of the curve (Table 4) and (Fig. 5).

The effect of pH of *Mycoplasma gallisepticum* culture used for separation revealed that the optimum pH was 5.7 which gave best result of peak height 173.55 mAU and best curve resolution (Table 5) and (Fig. 6).

Electrophoretic pattern and immunoblot of *M. gallisepticum* protein fractions separated by HPLC revealed the presence of LP 64 kDa in fraction 3 with high concentration and pure form (Figures 1 and 2).

In conclusion LP 64 kDa lipoprotein of Mycoplasma gallisepticum have been separated by size-exclusion (SEC) HPLC (GF-250Xl column 21.2 mm ID X 250 mm (Zorbax)). Mobile phase was 0.2 M sodium phosphate, pH was 5.7. The flow rate was 5 ml / min. and detection was by UV absorption at 210 nm. and recovered with its immunological activity as seen by immunoblot. The results obtained by SDS-PAGE suggests that this may be a valuable method for isolation and purification as we could separate the target lipoprotein in a pure form, so we can use HPLC (SEC) as a simple, convenient, rapid, reproducable, and reliable method for seperation of *M. gallisepticum* LP 64 kDa in highly pure form.

Standard protein chromatogram	Chromatogram	Integration
		See table below

No.	Ret. time min	Peak Name	Height mAU	Rel. Area %
1	6.800	P1	12.957	19.61
2	9.721	P2	10.914	24.69
3	10.981	P3	9.379	55.70
Total			033.25	100.00

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Fig. (1): Electrophoretic pattern of M. gallisepticum protein fractions separated by HPLC.

Lane 1: low molecular weight standard (Pharmacia)

Lane 2: *M. gallisepticum* whole cell protein

Lane 3: *M. gallisepticum* (fraction 1, batch 1)

Lane 4: *M. gallisepticum* (fraction 2, batch 1)

Lane 5: *M. gallisepticum* (fraction 3, batch 1)

Lane 6: *M. gallisepticum* (fraction 1, batch 2)

Lane 7: *M. gallisepticum* (fraction 2, batch 2)

Lane 8: *M. gallisepticum* (fraction 3, batch 2)

Lane 9: *M. gallisepticum* whole cell protein.

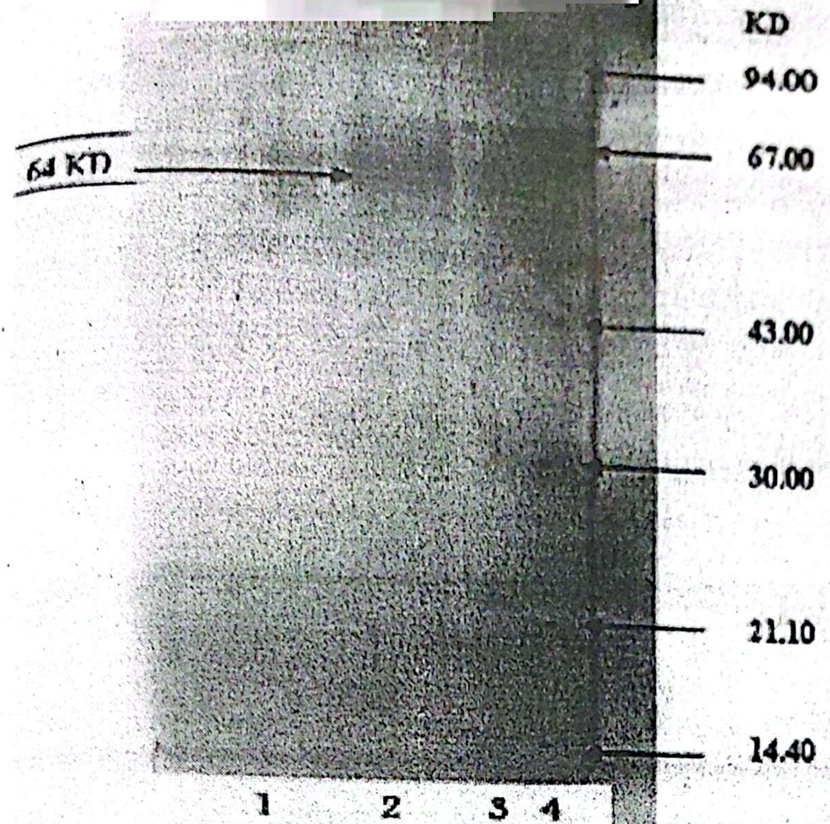

Fig. (2): Immunoblot of *M. gallisepticum* protein fractions separated by HPLC against *M. gallisepticum* positive serum.

fractions

Lane 1: M. gallisepticum (Fraction 3).

Lane 2: M. gallisepticum (Fraction 2).

Lane 3: M. gallisepticum (Fraction 1).

Lane 4: Low Molecular weight standard (Pharmacia).

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Table (1): Optimization of the wavelength.

Wave length	Chromatogram	Integration
210 nm		see below
220 nm		see below
240 nm		see below
280 nm		see below

210 nm

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	5.99	P1	15.93	10.74
2	7.31	P2	13.25	23.91
3	9.78	P3	13.69	20.05
4	11.11	P4	16.05	45.30
Total			58.91	100.00

220 nm

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	5.99	P1	9.38	11.79
2	7.30	P2	7.38	24.81
3	9.77	P3	7.53	20.89
4	11.09	P4	8.17	42.51
Total			32.46	100.00

240 nm

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	5.99	P1	6.28	51.35
2	7.03	P2	1.25	6.77
3	9.77	P3	3.68	40.38
4	11.09	P4	0.61	1.50
Total			12.26	100.00

280 nm

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	5.99	P1	13.31	53.82
2	7.39	P2	1.9	5.44
3	9.75	P3	6.08	19.36
4	11.12	P4	4.10	21.38
Total			25.39	100.00

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Table (2): Optimization of mobile phase concentration.

Mobile phase Conc.	Chromatogram	Integration
0.1 mNa H_2PO_4		see integration table below
0.2 mNa H_2PO_4		see integration table below

0.1 mNa H_2PO_4

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	6.87	P1	25.96	85.14
2	11.04	P2	4.56	14.44
3	19.72	P3	0.00	0.42
Total			30.53	100.00

0.2 mNa H_2PO_4

No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
1	6.522	P1	30.062	24.12
2	10.490	P2	17.573	22.25
3	11.838	P3	44.219	37.64
4	13.402	P4	4.128	1.12
5	15.761	P5	4.386	5.33
6	17.389	P6	3.019	9.54
Total			103.388	100.00

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Table (1): Optimization of the wavelength.

Wave length	Chromatogram	No.	Ret. Time min	Peak Name	Height mAU	Ret. Area %
210 nm		1	5.99	P1	15.93	10.74
		2	7.31	P2	13.25	23.91
		3	9.78	P3	13.69	20.05
		4	11.11	P4	16.05	45.30
		Total			58.91	100.00
220 nm		1	5.99	P1	9.38	11.79
		2	7.30	P2	7.38	24.81
		3	9.77	P3	7.53	20.89
		4	11.09	P4	8.17	42.51
		Total			32.46	100.00
240 nm		1	5.99	P1	6.28	51.35
		2	7.03	P2	1.25	6.77
		3	9.77	P3	3.68	40.38
		4	11.09	P4	0.61	1.50
		Total			12.26	100.00
280 nm		1	5.99	P1	13.31	53.82
		2	7.39	P2	1.9	5.44
		3	9.75	P3	6.08	19.36
		4	11.12	P4	4.10	21.38
		Total			25.39	100.00

Table (2): Optimization of the mobile phase concentration.

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Table (4): Optimization of flow rate.

flow Rate	No.	Ret. time min	Peak Name	Height mAU	Rel. Area %
Flow 15 ml/ min	1	1.26	P1	4.11	5.37
	2	2.18	P2	14.05	10.05
	3	2.44	P3	13.58	27.96
	4	3.40	P4	11.75	22.82
	5	3.86	P5	11.24	33.51
	Total			55.41	100.00
Flow 5 ml/ min	1	6.310	P1	36.81	13.60
	2	6.342	P2	36.65	16.73
	3	6.370	P3	0.843	0.14
	4	16.061	P4	6.77	13.61
	5	19.449	P5	33.18	55.91
	Total			114.63	100.00
Flow 3 ml/ min	1	10.795	P1	21.476	46.07
	2	11.592	P2	1.320	1.52
	3	17.414	P3	7.830	12.70
	4	19.684	P4	29.135	37.76
	5	22.480	P5	2.539	1.95
	Total			62.300	100.00

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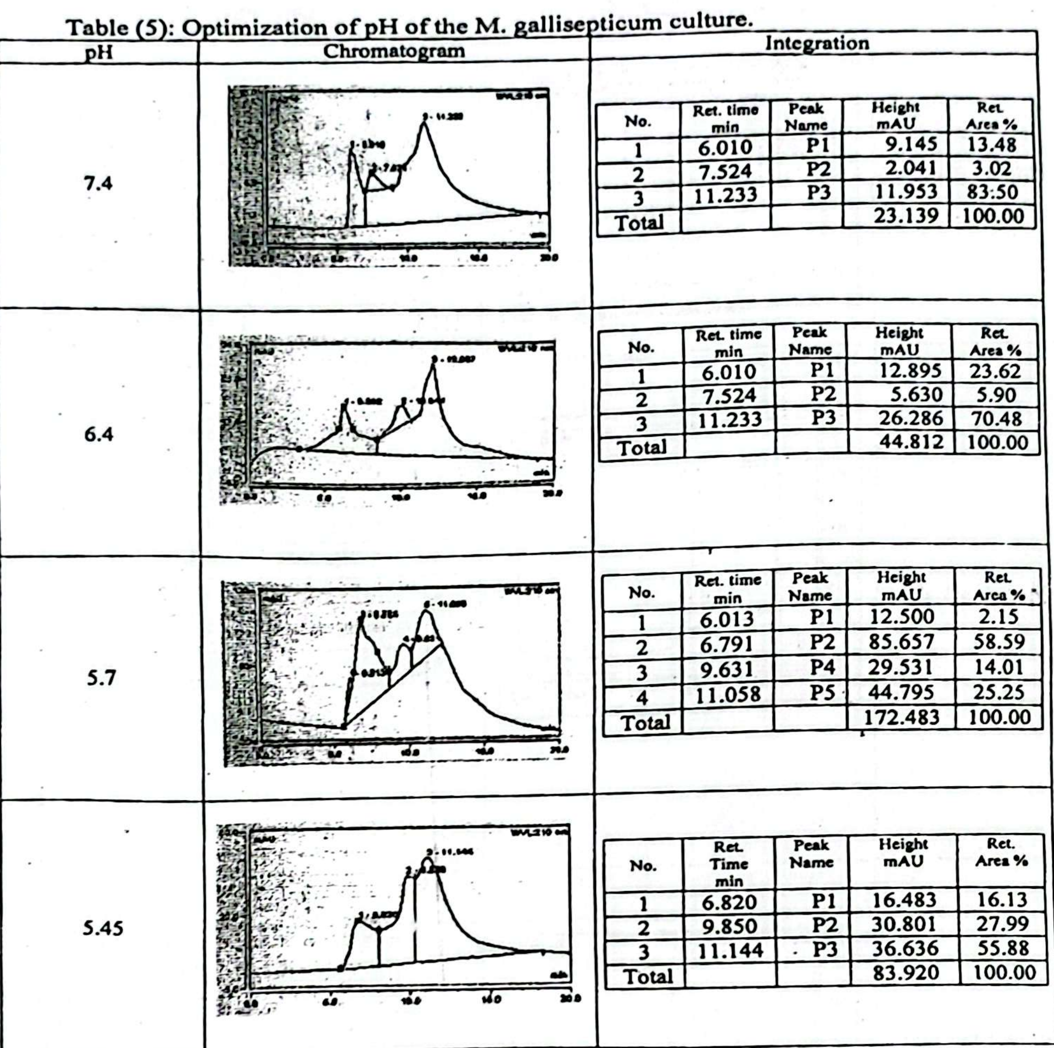
Table (5): Optimization of pH of the M. gallisepticum culture.



pH	Chromatogram	Integration
7.4		see below
6.4		see below
5.7		see below
5.45		see below

pH 7.4

No.	Ret. time min	Peak Name	Height mAU	Rel. Area %
1	6.010	P1	9.145	13.48
2	7.524	P2	2.041	3.02
3	11.233	P3	11.953	83:50
Total			23.139	100.00

pH 6.4

No.	Ret. time min	Peak Name	Height mAU	Rel. Area %
1	6.010	P1	12.895	23.62
2	7.524	P2	5.630	5.90
3	11.233	P3	26.286	70.48
Total			44.812	100.00

pH 5.7

No.	Ret. time min	Peak Name	Height mAU	Rel. Area %
1	6.013	P1	12.500	2.15
2	6.791	P2	85.657	58.59
3	9.631	P4	29.531	14.01
4	11.058	P5	44.795	25.25
Total			172.483	100.00

pH 5.45

No.	Ret. Time min	Peak Name	Height mAU	Rel. Area %
1	6.820	P1	16.483	16.13
2	9.850	P2	30.801	27.99
3	11.144	P3	36.636	55.88
Total			83.920	100.00

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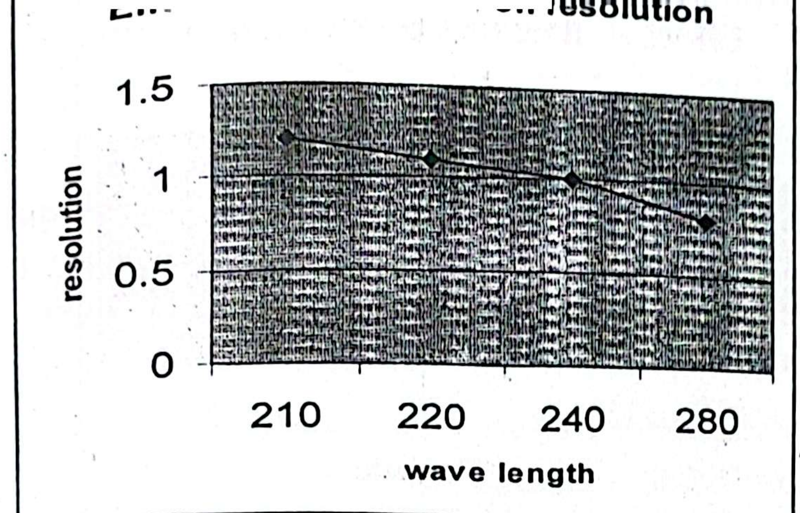


Fig. (3): Effect of wave length on the resolution.

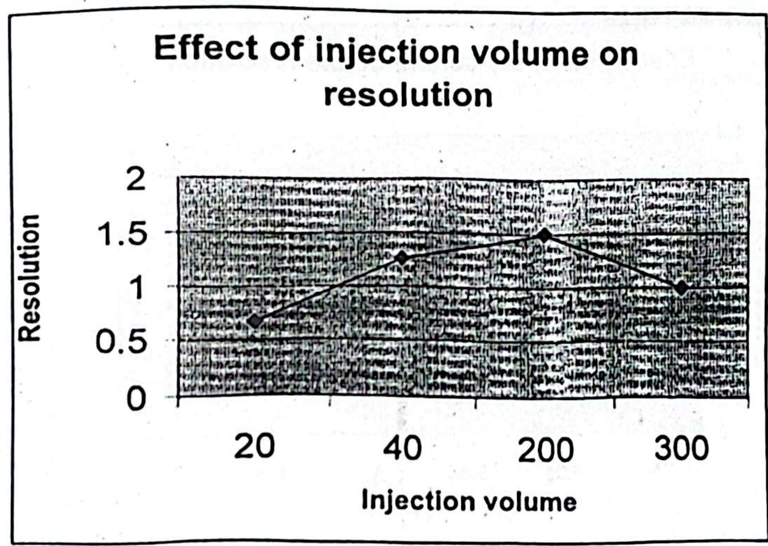


Fig. (4): Effect of injection volume on the resolution.

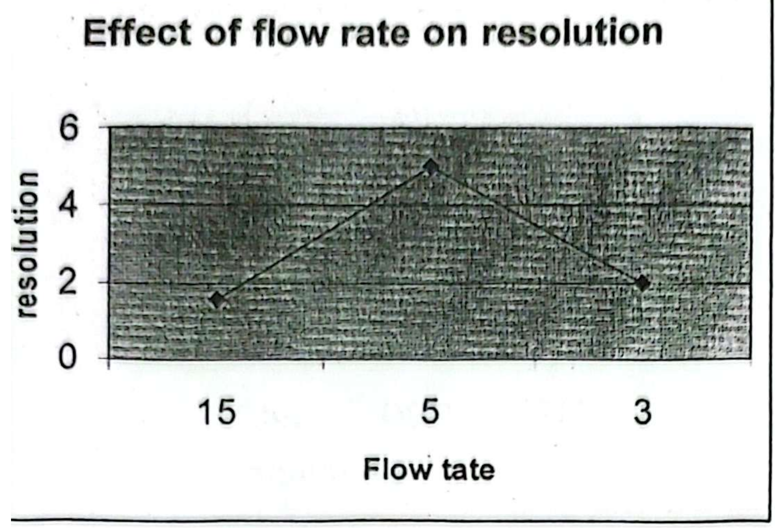
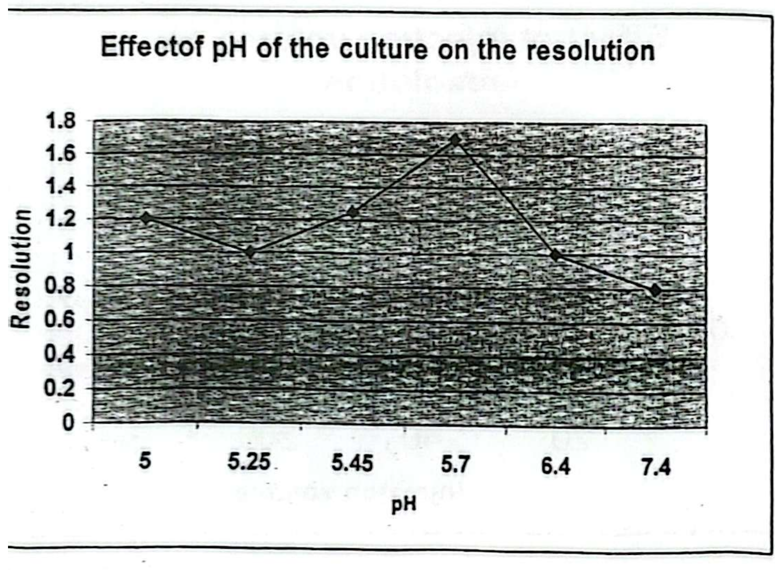


Fig. (5): Effect of flow rate on the resolution.



ig. (6): Effect of pH of the culture on the resolution.

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