Iranian Journal of Basic Medical Sciences
Vol. 13, No. 3, Summer 2010, 69-75
Received: Jan 21, 2010; Accepted: May 26, 2010

IJBMS

Original article

Detection of Neuraminidase Activity in *Pseudomonas aeruginosa* PAO1

*[1]Ciamak Ghazaei, [2]Malahat Ahmadi, [3]Nima Hosseini Jazani

Abstract

Objective(s)
Some properties of neuraminidase produced by *Pseudomonas aeruginosa* PAO1 growth in a defined medium (BHI) were examined and evaluated for its features.
Materials and Methods
The obtained supernatant enzyme of *P. aeruginosa* PAO1 cultures was used in a sensitive fluorometric assay by using 2'-(4-methylumbelliferyl) α-D-N acetylneuraminic acid as substrate. As hydrolyzing MUN with neuraminidase; free N-acetylneuraminic acid and 4-methylumbelliferone were formed with a shift in the fluorescence spectra from 315/374 nm (substrate) to 365/450 nm (product). Enzyme activity was then measured by the fluorescence of 4-methylumbelliferone at 450 nm.
Results
Among the culture media to determine the enzyme production, the highest production of *P. aeruginosa* PAO1 neuraminidase was found in BHI culture media. Neuraminidase production in *P. aeruginosa* PAO1 paralleled bacterial growth in defined medium (BHI) and was maximal in the late logarithmic phase of growth but decreased during the stationary phase, probably due to protease production or thermal instability. The neuraminidase of *P. aeruginosa* PAO1 possessed an optimum temperature of 56 °C and the activity was pH-dependent with maximal activity at pH 5. Heating the enzyme at 56 °C for 45 min in the presence of bovine serum albumin destroyed 33.1% of the activity while the addition of Ca^{+2}, EDTA and N-acetyl neuraminic acid (NANA) decreased activity markedly.
Conclusion
Overall, the results indicated that neuraminidase of *P. aeruginosa* PAO1 is more an extracellular enzyme than *K. pneumonia* neuraminidase is.

Keywords: Fluorescence spectrometry, *Klebsiella pneumoniae*, Neuraminidase, *Pseudomonas aeruginosa* PAO1, Specific activity

1- Department of Microbiology, Faculty of Veterinary, University of Urmia, Urmia, Iran
*Corresponding author: Tel: +98-441-2770508; Fax: 0441-2771926; email: ciamakghazaei@yahoo.com
2-Department of Microbiology, Faculty of Veterinary Medicine, University of Urmia, Urmia, Iran
3- Department of Microbiology, Immunology and Genetics, Faculty of Medicine, Urmia University of Medical Sciences, Uremia, Iran

Introduction

Pseudomonas aeruginosa produces neuraminidase which releases terminal sialic acids residues from glycoproteins, glycolipids and gangliosides expressed on host cells (1, 2). Neuraminidase has been implicated as a virulence factor and may serve as a marker for determining virulent *P. aeruginosa* strains (1). This enzyme plays an important role in bacterial attachment and subsequent invasion into host cells, and particularly into epithelial cells (1, 2).

It has been shown that treating mucus with neuraminidase decreases its viscosity, thereby enhancing the colonization of the underlying tissues by *Streptococcus pneumoniae*. Furthermore, removal of NANA by the neuraminidase could expose surface receptors for *S. pneumoniae* (1, 3). The inhibition of bacterial neuraminidases provides a mechanism to prevent bacterial pneumonia.

The aim of the present study was to prepare enzymes from two different sources in different conditions and compare their activities. The intent was also to assay and determine the best culture conditions for neuraminidase production and activity, using different media including brain heart infusion broth, Brucella broth, M9, pepton water, and tryptose soy broth. In addition, the neuraminidase activity in *P. aeruginosa* PAO1 was compared with the neuraminidase activity of *Klebsiella pneumonia ATCC 1003.* Meanwhile, the effects of different environmental conditions such as pH, temperature, added cation (Ca^{+2}), and components (NANA, EDTA) on the expression and activity of the neuraminidase were investigated.

Materials and Methods

Bacterial strains and culture media

Pseudomonas aeruginosa PAO1 was kindly provided by Dr. E. A. Worobec, (Department of Biology, Faculty of Sciences, University of Manitoba, CA), and *K. pneumonia* ATCC 10031 was obtained from the American Type Culture Collection. Bacteria were cultured in brain heart infusion broth (Merck Laboratories, Detroit, MI) at 37 °C with continuous shaking up to the stationary phase of growth, which was monitored by measuring cell turbidity at 600 nm (1, 2, 4). The culture was harvested by centrifugation at 12,000×g for 15 min at 4 °C. The supernatant was filtered through a membrane filter (0.45 um; Millipore Corp.) and dialyzed against 0.1 M acetate buffer (pH 5.5) for a period of 48 hr at 4 °C (1, 4, 5). The other culture media for some experiments was Brucella broth, M9, pepton water, and tryptose soy broth to assay as described above.

Assays for neuraminidase activity

Neuraminidase activity was measured by fluorescence spectroscopy with a F-2500 Hitachi fluorescence spectrophotometer (fluorimeter) using 4-methylumbelliferyl-α-D-N- acetylneuraminic acid (MUN) as a substrate (Sigma) (2, 3, 6, 7). Upon hydrolysis of MUN by neuraminidase, free N-acetylneuraminic acid and 4-methylumbelliferone were formed with a shift in the fluorescence spectra (excitation maximum/fluorescence maximum) from 315/374 nm (substrate) to 365/450 nm (product). Enzyme activity was then measured by the fluorescence of 4-methylumbelliferone at 450 nm.

A 10 mM stock solution of 4-methylumbelliferone (Sigma) was diluted to a 0.5 µM standard solution. Serial dilutions were made with 0.1 M sodium acetate buffer (pH 5.5) as a diluent, and these data were used to make the standard curve (6). A stock solution of 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid was prepared in distilled water at a concentration of 110 µmol/ml and stored frozen at -20 °C in 100 aliquots (6, 8). To quantitatively assay the samples for sialidase activity, 2'-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (110 µmol/ml) was mixed 1:1 with sodium acetate buffer (0.1 M). A test sample (50 µl) was added to the reaction mixture and incubated at 37° C for 20 min; then, 200 µl of a 1.33 M glycine buffer (pH 10.7) was added to the mixture to stop the reaction. A duplicate sample lacking substrate served as a negative control (3, 5, 8). Protein concentration was determined using the "Lowry" method with bovine serum albumin (Sigma Total Protein Kit) (9) as a standard (5, 6, 9, 10). Specific activity was defined as

micromoles of 4-methylumbelliferone formed per milligram of protein per minute at 37 °C. All assays were performed in duplicate (5, 8). The effect of pH, temperature, added cation (Ca^{+2}), and compound such as NANA and EDTA on neuraminidase activity as well as the effects of pH, Ca^{+2}, and EDTA on extracellular and cell-bound neuraminidase activity were tested in the system described above. In addition, the relationship between *P. aeruginosa PAO1's* growth curve and neuraminidase production, the effect of pH and temperature on the stability of the enzyme, and the influence of the growth medium on the production of neuraminidase by *P. aeruginosa* PAO1 were examined.

Determination of the relationship between growth curve and neuraminidase production

For this, at given intervals (1 hr), a portion of culture supernatant was removed, and bacterial growth was measured at 600 nm using a biophotometer (Eppendorf BioPhotometer plus). A portion (50 µl) of the culture's supernatant was filtered through a membrane filter (0.45 um; Millipore Corp.) and dialyzed against acetate buffer (0.1 M); then, the enzyme activity was determined (11, 12).

Determination of secreted and cell-bound neuraminidase activity

To determine the amount of secreted and cell-bound neuraminidase of *P. aeruginosa* PAO1 *and K. pneumoniae* ATCC 10031, the cultures of these organisms were harvested by centrifugation at 12,000×g. The cell pellet was suspended in phosphate-buffered saline (PBS; 0.01 M [pH 7.4]) to 1:25 of the original volume. The suspension was divided into three parts and treated as follows: (i) Tween 80 (0.1%) was added to the cell suspension. The mixture was shaken at 30 °C for 30 min. (ii) The cells in PBS suspension were lysed by twice freezing and thawing the suspensions in liquid nitrogen; they were allowed to thaw completely before re-freezing (13). (iii) The cell suspensions in PBS were shaken at 30 °C for 30 min. These suspensions were further centrifuged at 27,000×g for 8 min at 4 °C. The supernatant from each portion was passed through a millipore filter (pore diameter, 0.45, um), dialyzed against acetate buffer (0.1 M), and assayed for neuraminidase activity (4, 12, 13).

Results

Effect of various concentrations of N-acetylneuraminic acid on neuraminidase production by P. aeruginosa* PAO1 *in M9 media

Data showed that a 4 mg concentration of N-acetyl neuraminic acid has the greatest effect on production of *Pseudomonas aeruginosa* PAO1 neuraminidase. Further incremental increases had no effect on enzyme production.

***Influence of growth medium on the production of neuraminidase by P. aeruginosa* PAO1**

The greatest enzyme yield was in BHI (4.06 unit/total mass of protein) with more complicated components whereas less enzyme was produced in M9 (1.37 unit/total mass of protein) media.

Relationship between P. aeruginosa* PAO1 *growth and neuraminidase production

The pattern observed for neuraminidase production closely paralleled the growth curve of the organism. Enzyme production increased dramatically as the organism entered the logarithmic phase of growth and was maximal at peak of this phase. A decline in enzyme activity was seen when the organism entered the late stationary phase of growth.

***Effect of growth temperature on the production of neuraminidase in P. aeruginosa* PAO1**

The results indicate that the enzyme production is maximal at 37 °C in contrast to the lower yield at 42 °C and 22 °C.

Relationship between amount of the enzyme and neuraminidase activity of P. aeruginosa PAO1

The pattern observed for neuraminidase activity closely paralleled the total amount of enzyme. The enzyme activity increased up to a concentration of 0.25 mg/ml protein and followed an almost constant linear rate.

***Relationship between incubation time and neuraminidase activity of P. aeruginosa* PAO1**

As the incubation time increased, the activity of the enzyme increased too and a linear pattern was maintained until 20 min. Under the conditions of the assay, 4-methylumbelliferone release decreased within 40 to 60 min. The assay is linear with respect to enzyme concentration.

The optimum temperature and pH of P. aeruginosa* PAO1 *neuraminidase activity

A peak of maximal activity occurred at 56 ˚C, but more than 95% of the enzyme activity was destroyed at 65 ˚C. An optimal activity was achieved at pH 5.

Thermal stability of P. aeruginosa* PAO1 *neuraminidase

Heating the enzyme in the presence and absence of albumin led to approximately %30 and %42.5 reduction of the *P. aeruginosa* PAO1 neuraminidase activity respectively within 45 min at 56 ˚C (Figure 1).

Stability of P. aeruginosa* PAO1 *neuraminidase at different pH after 1 hr

Incubation of the enzyme at various pHs showed that the lowest and highest stability of the enzyme occurred at pH 7 and 2 respectively.

Effects of Ca^{2+}, EDTA and N-acetyl neuraminic acid on neuraminidase activity of P. aeruginosa* PAO1 *with 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (MUN) as substrate

Added cation and other reagents were examined for their effects on the neuraminidase activity. Ca^{2+}, EDTA and N-acetyl neuraminic acid (NANA) led to a decrease in activity. The results indicated that Ca^{2+}, EDTA and NANA have inhibitory effects on the enzyme activity (Table 1).

***Presence of intracellular, extracellular and cell-bound neuraminidase of P. aeruginosa* PAO1**

The supernatant fraction (extracellular enzyme) possessed a specific activity of 4.06 µmol of 4-methylumbelliferone released per min per mg of protein. On the contrary, the whole-cell fraction had cell-bound (treated with shaking and Tween 80) and intracellular neuraminidase which possessed a lower specific activity (Table 2).

Table 1. Effect of Ca^{2+}, EDTA and NANA on neuraminidase activity of *P. aeruginosa PAO1*.

Additive	Percent decrease
2 mM $CaCl_2$	36.2
20 mM $CaCl_2$	58.31
0.05 mM EDTA	49.78
0.5 mM EDTA	36.66
10 mM NANA	39.8
20 mM NANA	54.8
40 mM NANA	63

Table 2. Neuraminidase activity (secreted, intracellular and cell-bound) in *P. aeruginosa PAO1*.

Fraction	*Specific activity
Extracellular	4.06
Intracellular	2.32
Cell-bound (treated with shaker)	0.58
Cell-bound (treated with Tween)	0.79

*Specific activity is expressed as micromoles of 4-methylumbelliferone released per minute per milligram of protein.

Effect of pH on extracellular and cell-bound neuraminidase activity in P. aeruginosa* PAO1 *and K. Pneumonia ATCC 10031

In *P. aeruginosa* PAO1, increasing the pH led to an initial drop in activity at pH 6 followed by an increase, whereas in *K. pneumoniae* ATCC 10031, the enzyme activity increased with increasing pH and an appreciable drop was observed at pH 7 (Figure 2).

Effect of Ca^{2+} on extracellular and cell-bound neuraminidase activity in P. aeruginosa* PAO1 *and K. pneumonia ATCC 10031

In *P. aeruginosa PAO1,* addition of Ca^{+2} up to 25 mM led to an appreciable decrease in extracellular activity followed by an increase. Also in *K. pneumoniae* ATCC 10031, similar results were observed (Figure 3).

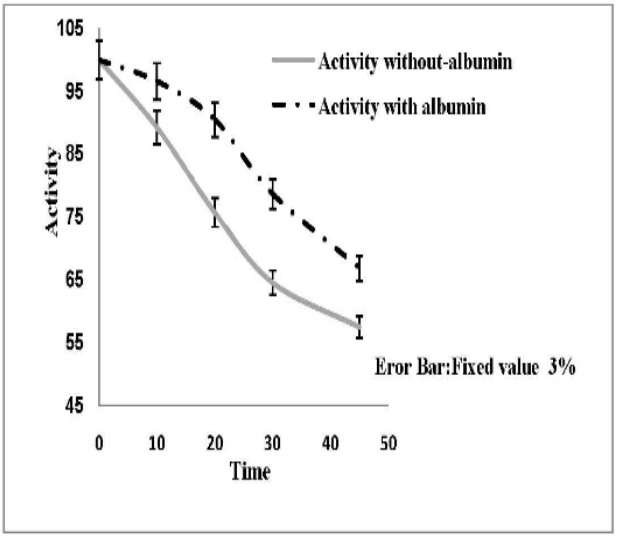

Figure 1. Thermal stability of *P. aeruginosa* PAO1 neuraminidase in the presence and absence of bovine serum albumin.

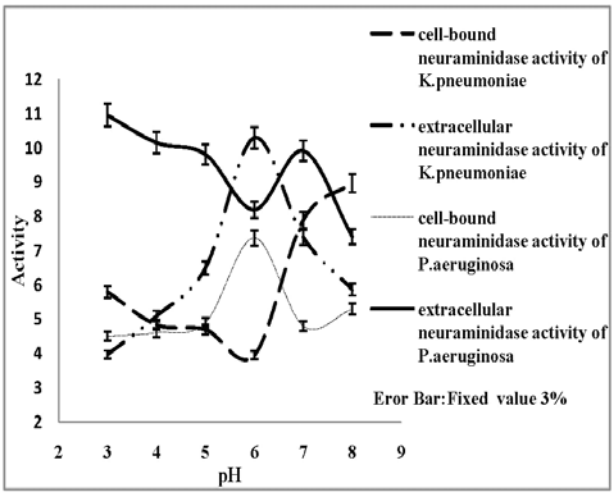

Figure 2. Effect of pH on extracellular and cell-bound neuraminidase activity of *P. aeruginosa* PAO1 and *K. pneumoniae* ATCC 10031.

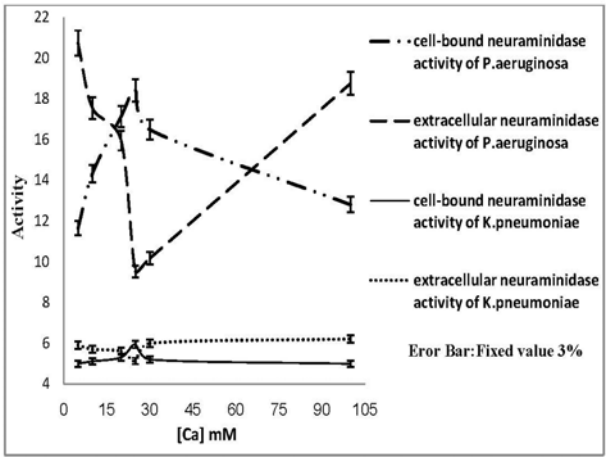

Figure 3. Ca^{2+} effect on extracellular and cell-bound neuraminidase activity of *P. aeruginosa PAO1*and *K . pneumoniae* ATCC 10031.

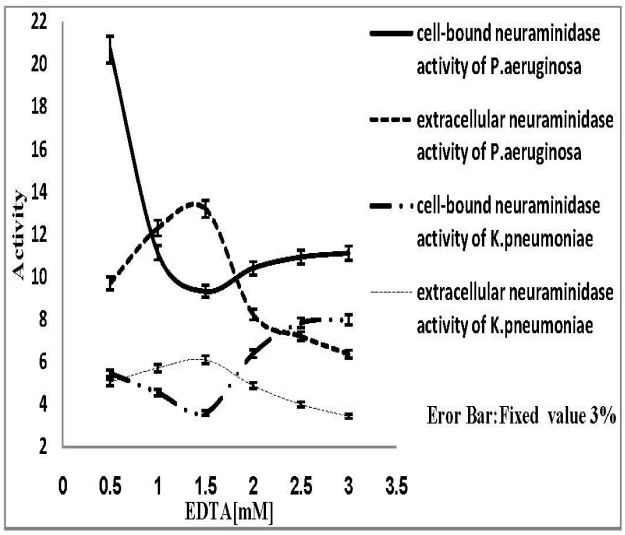

Figure 4. Effect of EDTA on extracellular and cell-bound neuraminidase activity of *P. aeruginosa* PAO1 and *K. pnumoniae* ATCC 10031.

Effect of EDTA on extracellular and cell-bound neuraminidase activity in P. aeruginosa PAO1 *and K. Pneumoniae ATCC 10031*

In *P. aeruginosa PAO1,* addition of EDTA up to 1.5 mM led to a markedly increase in extracellular activity followed by a decrease. Also in *K. pneumonia*, the similar pattern was observed (Figure 4).

Discussion

In this study, we examined the effects of multiple parameters on the production and activity of neuraminidase in *P. aeruginosa* and directly evaluated the activities of neuraminidases produced by *P. aeruginosa* PAO1 and *K. pneumoniae* ATCC 10031 under a variety of assay conditions. Both *P. aeruginosa* and *K. pneumoniae* are common sources of nosocomial and opportunistic infection in immunocompromised individuals and those with other chronic medical conditions (14, 15). *P. aeruginosa*, in particular, is a major public health concern, as it is widespread in hospital settings, is often resistant to antibiotics, and frequently is a cause of sepsis and death (14). As neuraminidase is an important determinant of the virulence of *P. aeruginosa* as well as that of *K. pneumoniae* (1, 16), it is a highly attractive target for the development of newer, more effective antibiotics. Defining optimal

production and assay conditions for the neuraminidases of these two organisms is critical for efficient development of drug screening assays.

As a first step in defining optimal culture conditions for the production of neuraminidase from *P. aeruginosa PAO1*, we examined the effects of varying concentrations of N-acetyl neuraminic acid in the culture medium (17, 18), growth medium, and growth temperature on the production of neuraminidase, as assayed by its activity in culture supernatants. We found that neuraminidase activity increased with increasing concentrations of N-acetyl neuraminic acid up to 4 mg/ml, above which there was no further increase in activity. We also found greater neuraminidase production by cells cultured in the more complex BHI medium relative to M9 medium. Finally, enzyme production was higher at 37 °C than 42 °C or 22 °C. Optimum growth temperature of *P. aeruginosa* is 37 °C (19). Enzyme production closely paralleled growth, with the greatest rate of production occurring during the log phase of growth and with a sharp decline in production as the cultures reached stationary phase (Figure 1). In *Pasteurella multocida,* enzyme production increased gradually throughout the first 10 hr of growth but increased dramatically as the organisms entered the first stationary phase, this continued throughout the second stationary phase (12). Similarly, enzyme activity closely paralleled protein concentration in the supernatant, increasing linearly with protein concentration up to 0.25 mg/ml of protein.

We next examined the relationship between assay parameters, including temperature and pH, on both the activity and stability of neuraminidase. First, we verified that our assay was linear with respect to incubation time. Cumulative neuraminidase activity increased linearly for at least 20 min, and fell below the linear rate by 40 to 60 min. Maximal activity was achieved at 56 °C, with activity rapidly decaying at 65 °C, and the optimal pH was found to be 6.4. At 56 °C, 42.5% of activity was lost after 45 min. Addition of albumin slowed this decay to some extent, with only 30% activity loss after 45 min. Prolonged incubation of neuraminidase at various pHs revealed that it is maximally stable at pH 2 and maximally labile at pH 7. Furthermore, addition of Ca^{2+}, EDTA, and NANA all led to inhibition of activity (20).

Finally, we determined the relative amounts of secreted, membrane-bound, and intracellular neuraminidase (4) from *P. aeruginosa PAO1*, and the effects of both calcium and EDTA on the activities of both free and cell-associated neuraminidases from both *P. aeruginosa* PAO1 and *K. pneumoniae* ATCC 10031. As expected, the highest specific activity was found in the supernatants of *P. aeruginosa* PAO1 cultures, with far lower specific activities in the membrane-associated fractions derived from either detergent extraction or cell shaking, and an intermediate level in the intracellular fraction (Table 2). Interestingly, the neuraminidases from *P. aeruginosa* PAO1 and *K. pneumoniae* ATCC 10031 differed markedly in their responses to pH, Ca^{2+}, and EDTA, exhibiting nearly opposite sensitivities. Furthermore, there were dramatic differences between extracellular and membrane-bound forms (21, 22) for each organism. Extracellular neuraminidase from K. *pneumoniae* was found to be highly sensitive to pH, having a pH optimum around 6 and being significantly less active at the higher and lower pH, whereas neuraminidase from *P. aeruginosa* PAO1 exhibited an minimum activity around pH 6 and significantly higher activity at low pH. Similarly, extracellular enzyme from *K. pneumoniae* was stimulated by modest amounts of Ca^{2+} and inhibited by modest amounts of EDTA, whereas the *P. aeruginosa* PAO1 enzyme exhibited the opposite behavior. In both organisms, the cell-associated enzyme exhibited pH and divalent cation sensitivities (21, 23) that differed from those of the extracellular enzyme.

Conclusion

These results not only establish important baseline conditions for the optimal production and assay of neuraminidase from *P. aeruginosa*, but also reveal key differences in

the properties of enzymes from two different pathogens. The T- test comparing two organism specific activities (*P* value equals 0.0131 with 95% confidence interval of this difference) shows the difference to be statistically significant. Thus, the neuraminidase of *P. aeruginosa* PAO1 is more of an extracellular enzyme. The data are also important to understand the biology and pathogenesis of these organisms, and are also of importance for developing a production system for making large amounts of neuraminidase.

Acknowledgment

This work was supported by Department of Microbiology of Faculty of Veterinary, Urmia University. We are grateful to Department of Microbiology and Research Laboratory Center of Urmia University of Medical Sciences for invaluable technical assistance and also we thank Mrs. Zartoshti (Laboratory assistant) for helps throughout the course of this study.

References

1. Cacalano G, Kays M, Saiman L, Prince A. Production of *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J Clin Invest 1992; 89:1866-1874.
2. Soong G, Muir A., Gomez Marisa I, Waks J, Reddy B, Planet P, *et al*. Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. J Clin Invest 2006; 116:2297-2305.
3. Camera M, Boulnois GJ, Andrew PW, Mitchell TJ. A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. Infect Immun 1994; 62:3688-3695.
4. Shahjahan K, Nayeema A, Showkat A. Neuraminidase production by *Vibrio cholerae* o1 and other diarrheagenic bacteria. Infect Immun 1984; 49:747-749.
5. Straus DC, Portnoy-Duran C. Neuraminidase production by a *Streptococcus sanguis* strain associated with subacute bacterial endocarditis. Infect Immun 1983; 41:507-515.
6. Moncla BJ, Braham P. Detection of sialidase (neuraminidase) activity in actinomyces species by using 2'-(4-methylumbelliferyl) o1-d-n-acetylneuraminic acid in a filter paper spot test. J Clin Microbiol 1989; 27:182-184.
7. Winter AJ, Spiro DC, Osborne MP, Tarlow MJ, Stephen J, Andrew PE, *et al*. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. Infect Immun 1997; 65:4411–4418.
8. Briselden A, Moncla BJ, Stevens CE, Sharon LH. Sialidases (neuraminidases) in bacterial vaginosis and bacterial vaginosis-associated microflora. J Clin Microbiol 1992; 30:663-666.
9. Anon., Protein determination-lowry procedure. Available at: http://wwwbiodavidsonedu/people/jowilliamson/Techniques/Protocolweek5html 2000.
10. Berg JO, Lindqvist L, Andersson G, Nord CE. Neuraminidase in *Bacteroides fragilis*. Appl Environ Microbiol 1983; 46:75-80.
11. Popoff MR, Dodin A. Survey of neuraminidase production by *Clostridium butyricum*, *C. beijerinckii*, and *C. difficile* strains from clinical and nonclinical sources. J Clin Microbiol 1985; 22:873-576.
12. White DJ, Jolley WL, Purdy CW, Straus DC.Extracellular neuraminidase production by a *Pasteurella multocidaa*: 3 strain associated with bovine pneumonia. Infect Immun 1995; 63: 1703-1709.
13. Burns B, Mendz G, Hazell S. Methods for the measurement of a bacterial enzyme activity in cell lysates and extracts. Biol Proced Online 1998; 1:17-26.
14. Ryan K, Ray CG. Sherris medical microbiology.4th ed. McGraw Hill; 2004.
15. Murray P, Rosenthal K, Pfaller MA. Medical microbiology. New York, NY: Elsevier Press; 2005.
16. Moncla BJ, Braham, P, Hillier SL. Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria. J Clin Microbiol 1990; 28:422-425.
17. Flashner M, Kessler J, Tanenbaum SW. Structural requirements for neuraminidase induction in *Arthrobacter sialophilus*. J Bacteriol 1982; 151:1630–1632.
18. Vimr E, Kalivoda KA, Deszo EL, Steenbergen SM. Diversity of microbial sialic acid metabolism. Microbiol Mol Biol Rev 2004; 68:132-153.
19. Songer JG, Post k. W Veterinary microbiology. Elsevier Saunders; 2005.
20. Abrashev I, Dulguerova G. Neuraminidases (sialidases) from bacterial origin. Exp Pathol Parasitol 2001; 4:35-40.
21. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 2003; 67:1092-2172.
22. Prithiviraj B, Bais H, Weir T, Suresh B, Najarro E, Dayakar B , *et al*. Down-regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. Infect Immun 2005; 73:5319–5320.
23. Naomi IA, Masami O, Hiroshi O, Sadahiko I, Misuzu K. Interactions between cations in modifying the binding of hexokinases i and ii to mitochondria. Mol Cell Biochem 1988; 81:37-41.