



ISSN: 0975-766X
CODEN: IJPTFI
Research Article

Available Online through
www.ijptonline.com

PRODUCTION OF RIBONUCLEASE BY VARIOUS ISOLATES

J.Venkateswararao*¹, T.Prabhakar², G.Girijasankar² and S.Vidyadhara¹

¹Chebrolu Hanumaiah Institute of Pharmaceutical sciences, Chowdavaram, Guntur -19.

²University college of Pharmaceutical Sciences, Andhra University, Visakhapatnam.

Email: venkateswararao.j@gmail.com

Received on 04-12-2011

Accepted on 18-12-2011

Abstract

Several bacterial and fungal cultures were isolated from soil, stagnant water samples collected in Chowdavaram village. A plate assay was used to detect Ribonuclease producing strains. The substrate RNA was included in the medium; the plates were incubated at 37⁰C for 48 hr and at 28⁰C for 3 days for bacteria, fungal strains respectively. After incubation period, the plates were flooded with a precipitant, perchloric acid. Colonies with clear zones were considered to produce the enzyme. It was found that 7 bacterial, 5 fungal strains have shown the enzymatic activity. Then these strains were isolated, preserved and used for the production of Ribonuclease by using suitable production media. Activity was assayed and found that the fungal strains F1, F4, F5 and F8 have shown the maximum enzyme activity obtained in 5 days of production at 30⁰C and the bacterial strains PW3, PW4 and CS3 were shown the maximum activity on the 2nd day of production. Beef extract, peptone and ammonium sulfate are the superior nitrogen source. Glucose found to be the excellent carbon source for RNase production. In the present study we also investigated the effect of various factors on the production and activity of the enzyme.

Introduction

Ribonucleases (RNase) catalyze the digestion of RNA and widely exist in organisms. RNases are nucleases produced by a diversity of organisms including fungi, plants, sub mammalian vertebrates, and mammalian liver, kidney, brain, placenta, pancreas, milk and semen [1]. Ribonucleases have gained importance in recent times due to their therapeutic effects (Newton *et al.*, 1992). It has been used in molecular biology for the isolation of DNA,

RNase protection assays and for mapping single base mutations in RNA. Ribonucleases may exhibit activities other than ribonucleolytic activity, such as anti mitogenic [2], antibacterial, antifungal [3], antiproliferative, antiviral activity [1,4], HIV-I reverse transcriptase inhibitory[5], translation inhibitory[6] and angiogenic[7] activities. As the demand for DNA vaccines and biological drugs increases, usage of ribonuclease attached to a solid support (immobilized) is better way which has many advantages over free enzymes [8]. RNase has extensive applications as an analytical enzyme, for the determination of structure, nearest neighbour sequence and sequence of RNA (Sambrook and Russell, 2001). In single cell protein production they are used to remove RNA from cell [9]. These enzymes are produced by numerous microorganisms among which the fungi are the most potent producers. RNase has also been used for the synthesis of oligonucleotides and in the manufacture of nucleotides 2', 5'-cyclic phosphates (Rowe and Smith, 1972; Chacko and Shankar, 1998). Many RNases are highly cytotoxic. Recently, a series of scientific studies showed that RNases had important biological functions, in controlling the tumor formation [10].

In recent years, the RNase functions related to the control of gene expression, cell growth and differentiation, cell protection from pathogens, and apoptosis induction have received special attention [11]. In view of the numerous applications of RNase, the work presented, addresses the exploitation of microorganisms for extracellular RNase producing microorganisms.

Materials and Methods

Commercial yeast RNA was purchased from Sisco Research Laboratories, Mumbai, India. Ribonuclease-A Type (I-A) procured from Sigma Chemical Co, St. Louis, MO, USA. Uranyl acetate was a product of Loba Chemie, Mumbai, India. Pre-sterile disposable Petri plates, potato dextrose agar [PDA] and nutrient agar [NA] were purchased from Hi Media Laboratories Pvt. Ltd, Mumbai, India, and all other chemicals used were of analytical grade. Several bacterial and fungal cultures were isolated from soil, stagnant water samples collected in Chowdavaram village. The fungal strains were routinely maintained on potato dextrose agar slants and bacterial strains on nutrient agar slants.

Preparation of media for screening

Media was modified for use, as shown in the following schematic representation.

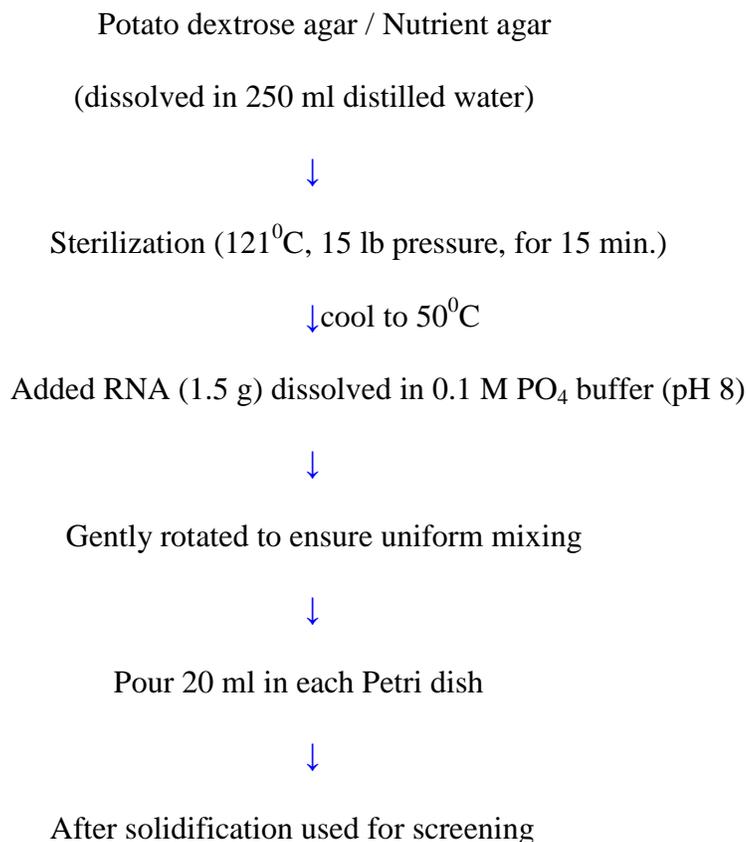


Fig.1 preparation of screening medium for RNase.

Screening for Ribonuclease:

Different cultures (fungi, bacteria) were prepared as suspensions and screened for extracellular RNase, by inoculating into the cups made in respective plates containing modified agar medium as indicated above. The plates were then incubated at required temperatures until growth was clearly visible. After incubation the plates were flooded with 3 ml of the precipitant (perchloric acid) and left to stand for 5 min. The plates were then visualized for transparent halos formed around the grown colonies, against an opalescent RNA background. These cultures, which produced clear zones were preserved and used for the fermentation.

Production by fermentation

The fermentation process was carried out for the isolated bacterial, fungal cultures separately in 500ml Erlenmeyer flasks with 100ml working volume. For each bacterial culture inoculum was developed the medium containing glucose(1.5%), yeast extract (0.5%), beef extract (0.5%), peptone (1.0%), MgSO₄ .2H₂O (0.05%), CaCl₂.2H₂O (0.01%) and traces of RNA, p^H was adjusted to 7.0 and incubated at 37⁰ C on a rotary shaking incubator at 150 rpm for 48hr. Fungal isolates were grown in the medium contains 100ml sterile medium glucose (3.0%), beef extract (0.5%), peptone (1.0%), MgSO₄ .2H₂O (0.05%), CaCl₂.2H₂O (0.01%). Seed cultures were prepared by incubating the flasks with spores suspension (1ml, about 10⁷ spores) was inoculated into each flask for 24hr at their respective temperatures. The fermentation medium, which is similar to the inoculum, inoculated with 10% (v/v) seed culture. The flasks were placed on a rotary shaker at 180 rpm 30°C for 120hr., to produce RNase by fungal strains. Similarly, bacterial strains at 150 rpm 37⁰C for 48hr. After fermentation, the biomass was separated from the culture fluids by filtration and then the filtrates were used to determine the enzyme activity. The growth of bacteria and fungus were measured and expressed as their cell dry weight.

Determination of Ribonuclease Activity

Ribonuclease activity was assayed by modified Kalnitsky *et al.* method. The rate of hydrolysis of yeast RNA is determined by measuring the amount of acid soluble oligonucleotide released under defined conditions.

0.5 ml of suitably diluted enzyme solution was placed in 10 ml centrifuge tubes, Included a blank containing 1ml of 0.15 M tris buffer, pH 8.2 containing 0.25mM EDTA, added 1ml of 1% RNA , incubated all tubes at 37°C for 8-10 minutes and stopped the reaction by the addition of 1 ml of Uranyl acetate-Perchloric acid solution. Transferred to an ice-bath and cool for 5minutes. Clarified by centrifugation at 10000 rpm for 10 min., and diluted 0.5 ml of clear supernatant to 3.5 ml with reagent grade water. Read A₂₆₀ versus blank. All values are corrected for a zero time blank approx. 0.4 unit/ml. The amount of acid-soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 L.mol⁻¹cm⁻¹(4).

One unit causes an increase in absorbance of 1.0 at A₂₆₀ at 37°C under the specified conditions.

Results and Discussion

33 bacterial, 08 fungal strains were isolated from the soil, stagnant water samples collected in and around chowdavaram. Out of these 6 bacterial, 5 fungal strains have shown the ability to produce Ribonuclease, especially the bacterial strains PW3, PW4 and CS3 and the fungal strains F1, F4, F5 and F8 were shown good enzymatic activity.

PW3 and F5 were selected for further study, because of their high enzymatic activity when compared with others. Fermentation process was carried out for the above cultures separately in 500ml Erlenmeyer flasks with 100ml working volume. Both bacterial, fungal cultures were grown in their specified production media and studied their growth, production activities at different nutritional, physical parameters to optimize them.

Effect of different carbon and nitrogen sources for RNase production:

Various carbon sources such as glucose, maltose, sucrose, and lactose, were tested, respectively, to find the optimal medium for ribonuclease production. For the maximal production of ribonuclease from bacterial strain PW3 and the fungal strain F5, glucose was found to be the most effective carbon source of all the different carbon sources used glucose was found to be the most suitable to induce the ribonuclease production. The carbon content was maintained constant in all experiments. But PW3 had shown the maximum enzymatic activity at 1.5 % and the F5 at 3.0% respectively.

Fig. 1 and 2 shows the effect of different carbon sources in the production medium of ribonuclease enzyme on enzyme activity, cell dry weight of PW3 and F5 respectively.

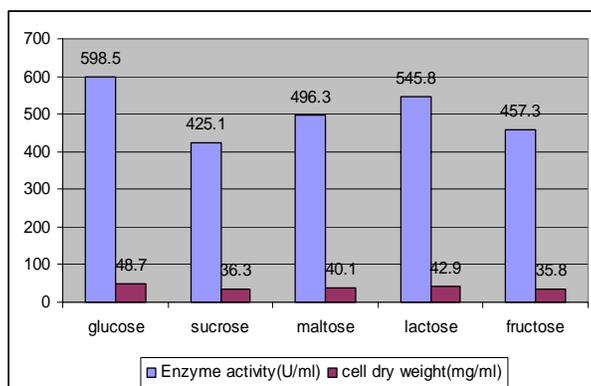


Fig.1 Effect of various carbohydrates on enzyme activity and cell dry weight of PW3.

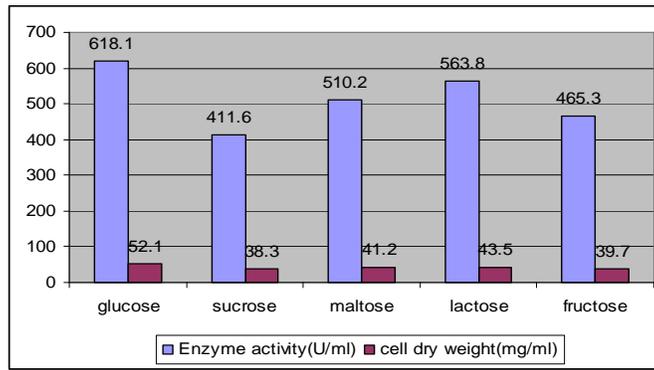


Fig.2 Effect of various carbohydrates on enzyme activity and cell dry weight of F5.

To optimize the nitrogen content various sources of nitrogen were used as shown in Fig. 3 and 4.

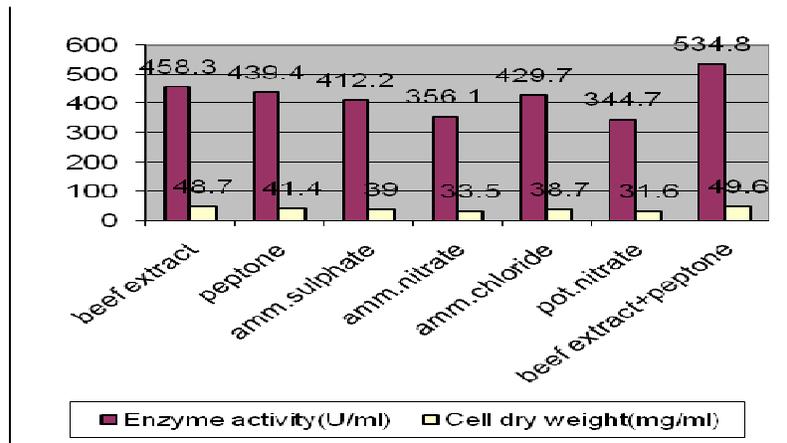


Fig.3 Effect of various nitrogen sources on enzyme activity and cell dry weight of PW3.

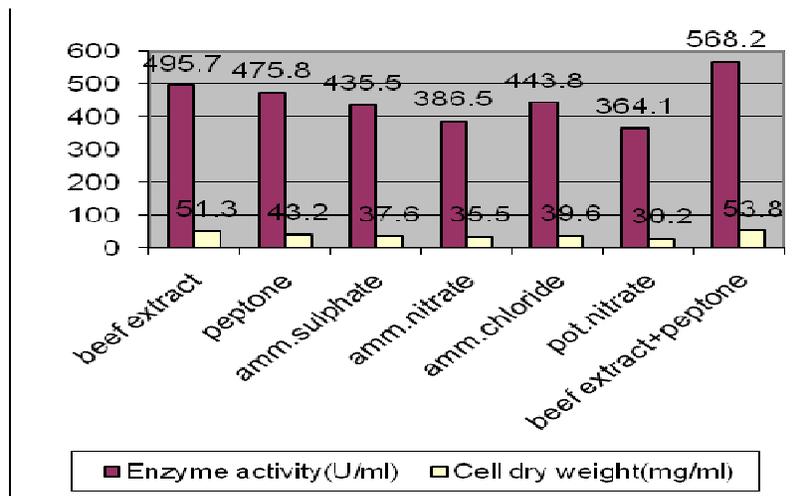


Fig.4 Effect of various carbohydrates on enzyme activity and cell dry weight of F5.

The rapidly metabolizable complex nitrogen sources are widely used for studying the growth and product formation characteristics of microorganisms in the laboratory as they provide a good source of various amino acids,

vitamins, minerals and other unknown growth factors to sustain good growth of microorganism. The nitrogen level was maintained the same in all cases as 177.5mg of nitrogen/100ml of media. Inorganic nitrogen sources like ammonium sulfate, ammonium nitrate, ammonium chloride, and potassium nitrate showed their positive effect on cell growth as well as on ribonuclease production. Both PW3 and F5 were shown good growth and considerable amount of ribonuclease production when grown on beef extract, peptone. PW3 produced higher amount of enzyme (534.8 U/ml) when we used the combination of beef extract with peptone as nitrogen source. It was found that the amount produced by peptone and beef extract were 458.3U/ml and 439.4U/ml respectively. Similarly, F5 produced higher amount of enzyme (568.2 U/ml) when we used the combination of beef extract with peptone as nitrogen source. It was found that the amount produced by peptone and beef extract were 495.7U/ml and 475.8U/ml respectively. Both of the above instances indicate that combined use beef extract and peptone gave the best results and was chosen as the suitable nitrogen source for ribonuclease production.

Effect of pH and temperature

The maximum enzyme production was studied at pH range of 3.5-6.5 for fungi and 4.5-7.5 for bacteria, found that the optimum pH is 6.0 for the fungal strain and that is 7.0 for the bacterial.

Similarly studied the effect of temperature for enzyme production and was found to be 30⁰C and 37⁰C respectively (Fig.7 and 8).

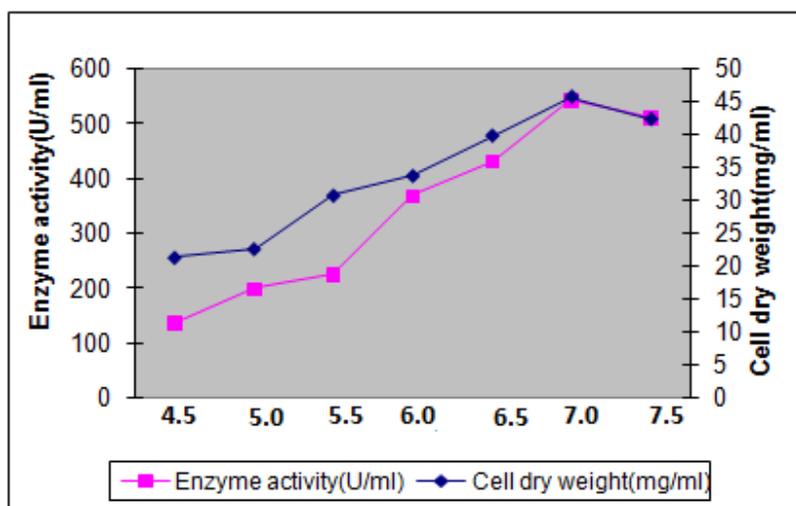


Fig.5 Effect of pH on enzyme activity and cell dry weight of PW3.

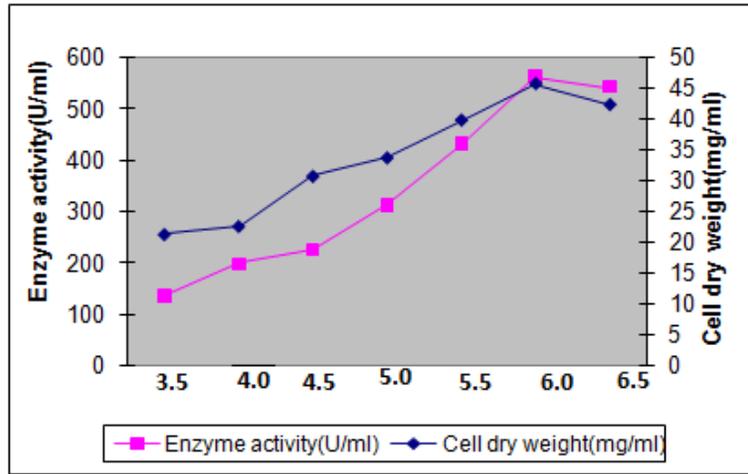


Fig.6 Effect of pH on enzyme activity and cell dry weight of F5.

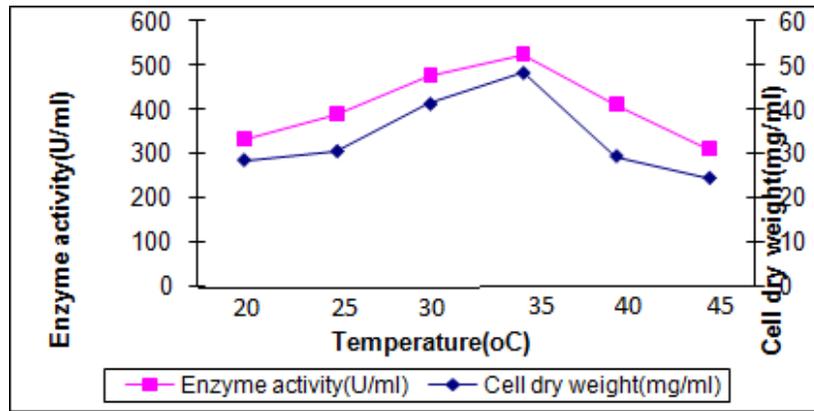


Fig.7 Effect of temperature on enzyme activity and cell dry weight of PW3.

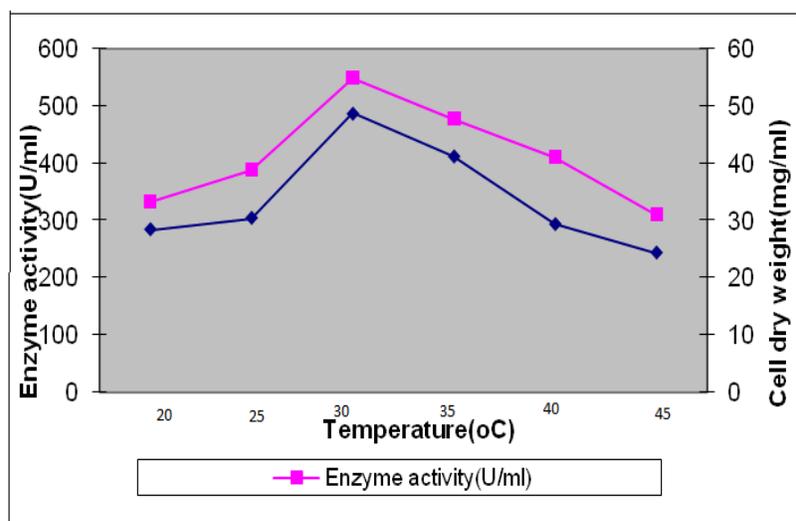


Fig.8 Effect of temperature on enzyme activity and cell dry weight of F5.

Maximum production was observed on 5th day (120hr) of cultivation in the case of fungi and that is on the 2nd day (48hr.) for the bacteria.

These optimal conditions pH and temperature, carbon source and nitrogen source concentration will be used in our subsequent investigations for ribonuclease production by PW3 and F5.

Conclusion

In the present study, several bacterial and fungal cultures were isolated from soil and stagnant water samples. A plate assay was performed to detect Ribonuclease producing strains. This study indicating that soil, stagnant water samples provide a rich source of ribonuclease producing microorganisms. In the present study we also investigated beef extract, mixture of peptone and ammonium sulphate are the superior nitrogen source where as glucose is found to be excellent carbon source for RNase production. However, more detailed study is required to identify the species and to characterize the organisms as well as the enzyme. Even more efforts can be put on enzyme kinetics and immobilization of enzyme. Studies on the commercial production of the enzyme can be done.

Acknowledgment

The authors are sincerely thanking to the Management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur, for providing laboratory and technical support.

References:

1. G.P. Guan, H.X. Wang, and T.B. Ng, “ A novel ribonuclease with antiproliferative activity from fresh fruiting bodies of the edible mushroom *Hypsizygus marmoreus*”, *Biochimica et Biophysica Acta* , 1770, pp. 1593-1597, 2007.
2. P.H.K. Ngai, and T.B. Ng, “A ribonuclease with antimicrobial, antimitogenic and antiproliferative activities from the edible mushroom *Pleurotus sajor-caju*”, *Peptides*, pp. 11-17, 2004.
3. S.K. Lam, and T.B. Ng, “Isolation of a novel thermolabile heterodimeric ribonuclease with antiproliferative activities from roots of the sachi ginseng *Panaxnotoginseng*”, *Bioche. Biophys. Res. Commun*, 285, pp. 419-423, 2001.

4. Wen-Wen Zhou, and Tain –Gui Niu, “Purification and some properties of an extracellular ribonuclease with antiviral activity against tobacco mosaic virus from *Bacillus cereus*”, *Biotech-Lett* , 31, pp. 101-105, 2009.
5. H.X. Wang, and T.B. Ng, Quinqueginsin, “A novel protein with anti-human immunodeficiency virus, antifungal, ribonuclease and cell-free translation-inhibitory activities from American ginseng roots”, *Biochem. Biophys. Res. Commun*, 269, pp. 155-159, 2000.
6. T.B. Ng, and H.X. Wang, Panaxagin, “A new protein from Chinese ginseng possesses antifungal, antiviral, translation-inhibiting and ribonuclease activities”, *Life Sci*, 68: pp. 739-749, 2001.
7. R. Shapiro, and B.L. Valle, “Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin”, *Proc. Natl. Acad. Sci. U.S.A*, 84: pp. 2238-2241, 1987.
8. Mojca Bencina, Janja Babic, and Ales Podgornik, “Preparation and characterization of ribonuclease monolithic bioreactor”, *Journal of Chromatography A*, 1144: pp. 135-142, 2007.
9. G.D. Reddy, and V. Shankar, “Immobilized nucleases” *CRC Critical Review of Biotechnology* , 13: pp. 255-273, 1993.
10. R.J. Youle, Y.N. Wu, S.M. Mikulski, K. Shogen, R.S. Hamitton, D.Newton, G. Dalessio, and M. Gravell, “RNase inhibition of human immunodeficiency virus infection of H9 cells”, *Proceedings of the National Academy of Sciences of the United States of America*, 91: pp. 6012-6016, 1994.
11. O.N. Ilinskaya, and A.A. Makarov, “Whey Ribonucleases Induce Tumor Cell Death. *Molecular Biology*, 39: pp. 1-10, 2005.
12. S. Barindra, G. Debashish, S. Malay, and M. Joydeep, “Purification and characterization of an extracellular, uracil specific ribonuclease from a *Bizionia* species isolated from the marine environment of the sundarbans”, *Microbiological Research*, 163: pp. 31-38, 2006.

Corresponding Author:

J.Venkateswararao*,

Email: venkateswararao.j@gmail.com