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Research Article

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In vitro anticancer activity of L-arginase produced from *Idiomarina sediminum; H1695*

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ABSTRACT :

The aims of the present study were to purify, characterize and evaluate anticancer activity of l-arginase against cancer cell lines. The cells of the marine bacterial species were entrapped in hybrid beads made up of PVA and sodium alginate. And the production of enzyme was carried out using immobilized cells of idiomarina sp. (Gene Bank Accession Number JF346667) in a sea water based mineral arginine medium. The enzyme thus obtained was purified to near homogeneity (29.87 fold) with a molecular weight of 37 k Da. It showed an optimal pH and temperature of about 8 and 35 ^{0} C. When compared to other metal ions, manganese was the most efficient metal ion for enzyme activity. Whereas other ions were found to repress the activity. Out of various substrates used, l-arginase showed highest substrate specificity for l-arginine. The enzyme was strongly inhibited by thiol compounds such as dithiothrietol, reducing agents like 2-mercaptoethanol, and chelating agents like EDTA. The yield of the enzyme thus produced from the immobilized cells were almost near to the yield obtained from that of the free cells. Hence cell immobilization technology can be adopted for the enzyme production. Of the various other methods adopted, use of PVA- sodium alginate beads were considered as a suitable one .The purified enzyme showed good range of activity against Hela cells with IC50 value 0.5U/ml. Hence, l-arginase can be a potential candidate as an anticancer agent.

Keywords: L-Arginase, *Idiomarina sediminum; H1695*, polyacrylamide gel electrophoresis, PVA- sodium alginate hybrid matrix, MTT method, cervical cancer.

INTRODUCTION

The marine biosphere is considered as the one of the richest sources of diverse type of microorganisms especially bacteria. Large number of therapeutic proteins of economic and therapeutic importance were isolated from potential marine sources. L-Arginase is one among them. It was found to arrest the growth of wide range of arginine dependent cancer cells [1-5]. Human cervix cancer cell line were used for the present study because it is the second most common cancer effecting women worldwide [6]. Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease [7]. It is most common cancer in women aged 15–44 years. India also has the highest age standardized incidence of cervical cancer in South Asia at 22, compared to 19.2 in Bangladesh, 13 in Sri Lanka, and 2.8 in Iran.

The presence of the l-arginase was reported from various sources but as per our knowledge, there is no work on isolation and production of l-arginase from marine sources. The present study aims at the large scale production of l-arginase from noval marine bacterial species i.e. *Idiomarina sediminum; H1695* and evaluation of its anticancer activity against Hela cell lines. The use of free cells for the enzyme production is often associated with a laborious process of purification steps making the downstream process very costlier. These problems can be avoided by entrapping the cells in a semi-permeable matrix which restricts the cell movement and allows the free movement of the substrates and the products. It further aids in the easy recovery of the product. Various methods were adopted for immobilizing the cells like polyacrylamide gel, agar-agar and gelatin etc but none of them were suitable. For the present study, cells were immobilized in PVA-sodium alginate beads [8]. Production was carried out in an optimal medium using immobilized cells [9], purified and evaluated for its anticancer activity against cancer cell lines. MTT assay was followed for the activity [10] .

EXPERIMENTAL SECTION

Microorganisms and cultures maintenance: The halophilic bacterial species *Idiomarina sediminum; H1695*, a potential source of L-arginase was employed in the present study. The slants were maintained fresh and stored at 4^0C.

Preparation of inoculum: Loop full of microorganism was inoculated in a sterile nutrient broth and incubated for 48 hours at 37^0C.The growth thus formed was centrifuged and the bacterial pellet was weighed, washed and suspended in a sterile sodium chloride solution. The suspension was used as an inoculum.

Cell immobilization:
10% of PVA solution was prepared and mixed up with 2% of sodium alginate solution drop by drop at 12000 rpm to form a homogenous solution. To this certain quantity of inoculum was added and mixed up at room temperature. The resultant mixture was collected in a syringe and drop by drop of it was added in to a 1000ml of solution containing a mixture of 4% boric acid and 2% calcium chloride. The beads were left as such in the mixture for 10-30 min and later collected , washed with buffer and kept for curing for one hour in the refrigerator.

Cultivation medium and cultural conditions: A volume of 10% of gel beads were inoculated into sterile production medium containing l-arginine- 2.0g**,** maltose- 1.5g, casein – 2g, K2HPO4 -0.1g, KH2PO4-0.1g, MgSO4 -0.1g, NaCl - 0.5g, aged sea water- 100ml. pH - 8 and incubated at 37^0C on an orbital shaker incubator at 120 rpm. After 120 hours, the medium was centrifuged and the supernatant liquid was used for estimation of enzyme activity.

Enzyme Assay: Arginase activity was measured in terms of the rate of hydrolysis of L- arginine to l-ornithine and urea by measuring the amount of urea released in the reaction. Urea was quantitated colorimetrically by the method of Archibald [11] .One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol of urea at 37^0C.

Determination of enzyme protein: Enzyme protein was measured according to the method of Lowry et al., [12] using folin ciocalteu's reagent.

Purification: L-Arginase was purified by ammonium sulphate fractionation followed by ion exchange and gel filtration chromatography. Ammonium sulphate fractionation was carried out at 4^0 C in an ice bath. Different concentration of solid ammonium sulphate was added to get 10% and then successively raised to 60% saturation. The precipitated protein was collected by centrifugation at 6000 rpm at 4^0 C.

The sample thus obtained was loaded in a Q-Sepharose column previously equilibrated with tris HCl buffer pH 7.2 containing 10mM Nacl and flow rate of 1 ml/minute was adjusted. Fractions of 2 ml were collected. The enzyme and protein content of each fraction was quantified.

Gel permeation chromatography was carried out loading 2ml enzyme solution from above step in Sephadex G-100 column previously equilibrated with Tris HCl Buffer pH 7.2 (0.01M). Flow rate of 1 ml/minute was maintained. Fractions of 2 ml each were collected and enzyme and protein content was quantified. Fractions containing maximal amount of the protein and enzyme were pooled, dialysed, concentrated by lyophilization and stored at -25^0C.

SDS-PAGE (Polyacrylamide gel electrophoresis): SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed as described by Laemmli, (1970) to check the purity of crude protein sample [13]. Protein was stained by silver staining method [14].

Enzyme characteristics: The purified arginase was characterized for its various properties. The characters analysed included effect of pH (6-10), temperature (25-45^0 C), Nacl (0-20%), various substrates, metal ions etc.

Evaluation of anticancer activity invitro: The purified enzyme thus obtained was evaluated for invitro cytotoxicity against HeLa cell lines. MTT assay was used for the evaluation. *HeLa* cell lines were obtained from Tata Memorial institute of cancer research, Mumbai, India. Cancer cells were inoculated into 96 well plate containing 5ml of medium and incubated for 48hours to get a sufficient growth. Different concentrations of the sample was added into the wells and incubated for 24 to 48 h at 37°C.After incubation, the wells were washed with buffer and 20µl MTT stock solution was added to each well, incubated for 4 h at 37 °C then the solution was decanted. To stop succinate-tetrazolium reductase activity and to solubilise formazan crystals, 100 µl of propanol was then added to each well. Absorbance was read on a plate reader at 540 nm. The percentage growth can be calculated in terms of % cell viability.

% cell viability = AT / AC of control cells × 100 %.

AT- absorbance of test
AC- absorbance of control.

RESULTS AND DISCUSSION

Enzyme production: The enzyme yield obtained using immobilized bacterial cells was almost equivalent to that of free cells. The crude extract showed the presence of 160.48 U of activity whereas activity of about 215.36U/ml was achieved with our previous study with free cells. Hence production of the enzyme can also be carried out using immobilization cells. PVA- sodium alginate hybrid matrix was considered as a suitable, biocompatible polymer for immobilizing the cells.

Other systems of immobilization like entrapment techniques of polyacrylamide gel, agar-agar and gelatin do not prove to be efficient in terms of kinetic characteristics of the immobilized enzyme. A similar trend has been observed by Adinarayana et al., (2005) who immobilized *Bacillus subtilis* into these matrices to produce alkaline protease [16]. Sol-gel hybrid systems do not interfere with enzyme activity and can also be an efficient matrix for efficient immobilization [17]. Now-a-days preparation of new hybrid matrices were found to be one of the noval and promising techniques for the immobilization of cells.

Enzyme purification: The crude extract consisted of large number of impurities which were removed by step by step procedure of purification process. The crude extract showed the presence of 102.68 mg of total protein with specific activity of 1.56U/mg .Upon purification, specific activity was increased to 46.61 U/mg with 44.44 % of recovery and 29.87 times purity (**Table 1**).

Table 1: Results of purification of l-arginase

S. No.	Method	Total enzyme activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Purification fold	Yield
1	Crude extract	160.48	102.68	1.56	0	100
2	Ammonium sulphate	135.64	10.94	12.39	7.94	84.37
3	Q-Sepharose	88.07	4.57	19.27	12.35	54.87
4	Sephadex G-	71.32	1.53	46.61	29.87	44.44

This indicates that, the methods adopted for the purification process were effective in removing all forms of impurities .The impurities were removed with subsequent loss of 55.56 % of total activity.

SDS-PAGE: The molecular weight of the purified enzyme was found to be around 37kD by SDS –PAGE analysis which is close to the molecular weight of the l-arginase obtained from other sources [5].

Enzyme characteristics:

Arginase isolated from various sources were found to have optimal pH in the alkaline region. [18, 19, 20, 21]. However, some exceptions have been found as well. Like E. coli expressed *Helicobacter pylori* arginase was found to have optimal acidic pH of 6.1 as reported by McGee et al. [22].The enzyme obtained in our study was active over a wide range of pH 6-10 with optimal pH at 8.0 (**Figure 1**) .The activity considerably decreased at both low pH (5.0) and high pH (10) .

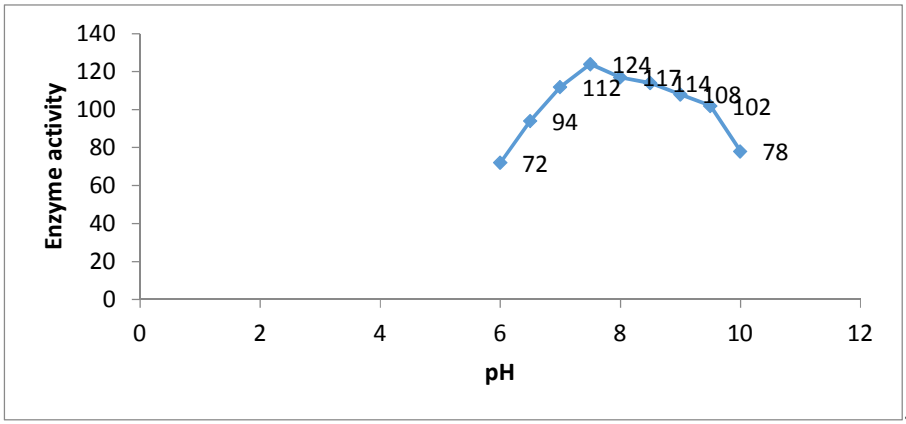

Figure 1: Effect of pH on enzyme activity

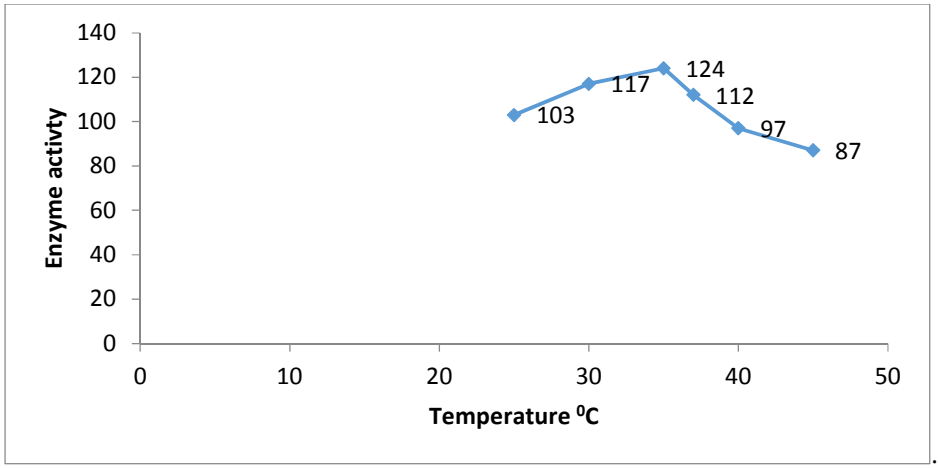

Figure 2: Effect temperature on enzyme activity

Arginase isolated from other sources was found to be active at higher temperatures with an optimal temperatures of 25, 30, 35 and 37 ^{0}C [23, 24, 25]. In our study, the enzyme showed good activity between temperature range of 25 -45 ^{0} C with optimum activity at 35^{0}C (**as shown in Fig 2**).

Metal ions acts as co-factor for the enzymes. Manganese was the most efficient metal ion for enzyme activity (**Figure 3**). Other ions were found to repress enzyme activity. Similar results were reported by others in their studies , when the manganese ions were replaced by other ions [25, 26].

Out of various substrates used, l-arginase showed highest substrate specificity for l-arginine, very low specificity towards d-arginine and very less activity towards others (**Figure 4**). Similar results were reported by Nakamura et al and others [27].

The enzyme was strongly inhibited by thiol compounds such as Dithiothrietol, reducing agents like 2-Mercaptoethanol, and chelating agents like EDTA [28, 29]. Our results confirm the previous findings (**Figure 5**).

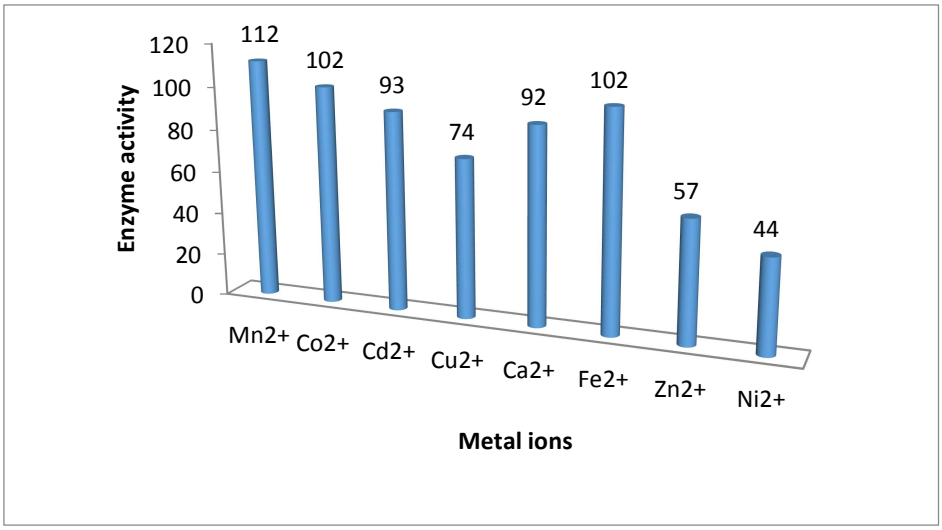

Figure 3: Effect of metal ions on enzyme activity

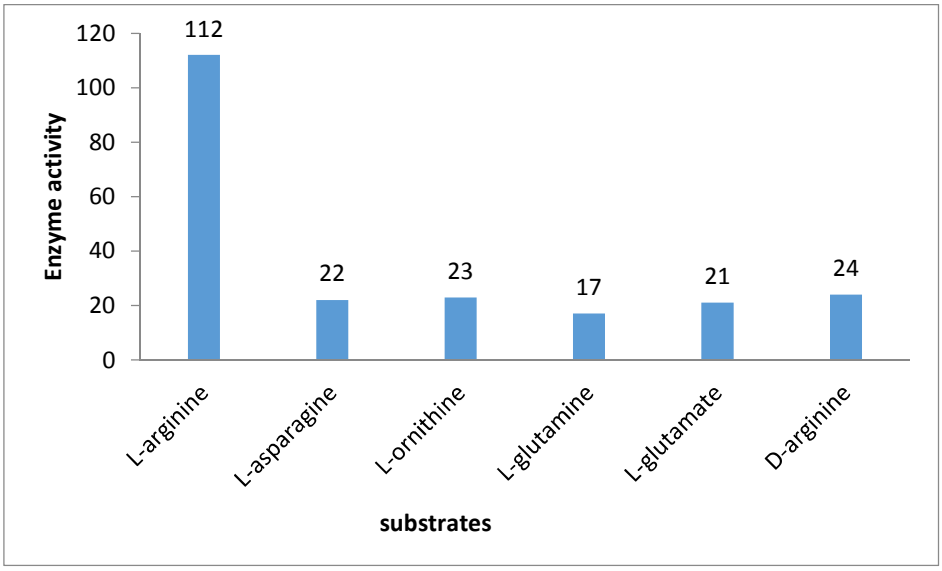

Figure 4: Effect of substrates on enzyme activity

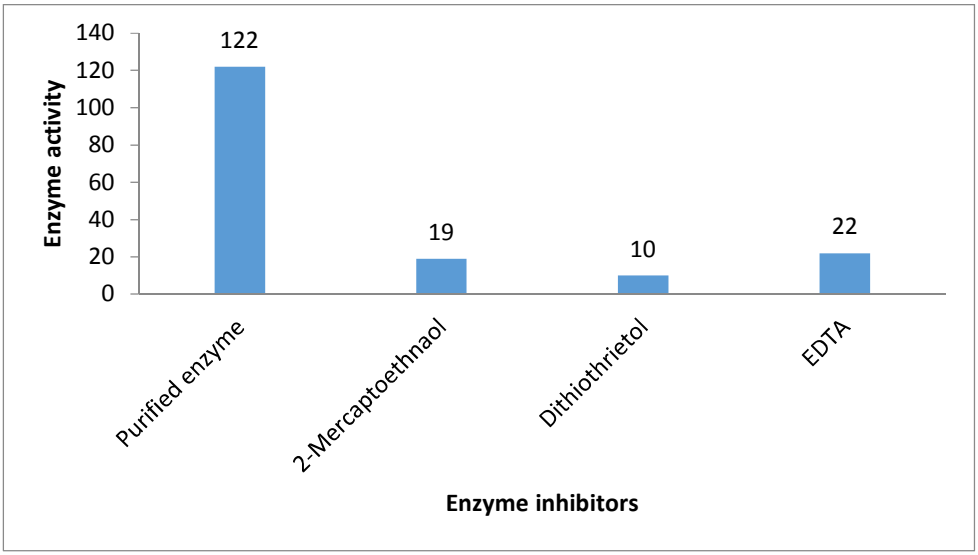

Figure 5: Effect of enzyme inhibitors on enzyme activity

Invitro cytotoxic activity :

Table 2: Determination of cytotoxicity of l-arginase on *HeLa* cell line by MTT Assay

S. No.	Concentration (IU/ml)	Absorbance (O.D)	Cell viability (%)
1	Cell control	0.52	100
2	0.01	0.48	92.3
3	0.1	0.45	86.5
4	0.25	0.41	78.8
5	0.5	0.26	50
6	1	0.19	36.53
7	2	0.13	25
8	2.5	0.9	17.3

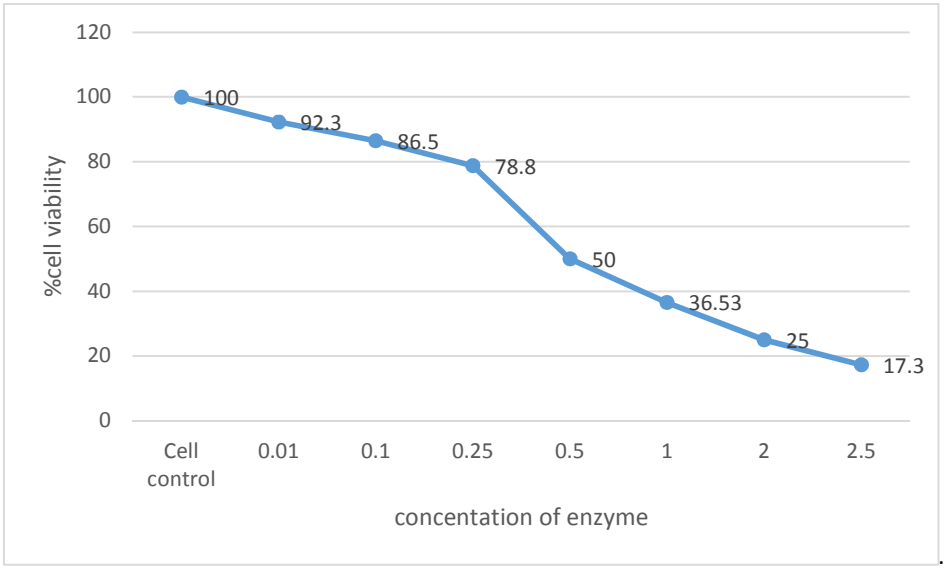

Fig 6: Cell viability in percentage and inhibition of growth in percentage. IC50 estimation in the *HeLa* cell line was 0.5U/ml for l-arginase

The results indicated dose dependent activity of l-arginase against cancer cells line. Increased in the concentration gradually decreased number of viable cells (**Figure 6**). The concentration of the enzyme required to show 50% was found to 0.5U/ml which is almost equal to 0.3U/ml as shown by R Philip etal on HeLa cell lines. [15].

CONCLUSION

Cell immobilization technique offers various advantages over free cell. The yield obtained was almost equivalent to free cells. L-Arginase was active and stable over a wide range of pH and temperature and is highly salt tolerant. The substrate specificity towards l-arginine is high which means that it could be used in low amounts to achieve the desired effect. And the enzyme shows optimal activity at physiological pH and temperature which makes it suitable for therapeutic use. Further, it showed profound activity against Hela cells. Based on the results obtained we conclude that the marine *idiomarina sediminum* has immense potential for large scale production of l-arginase which is having several beneficial properties for its use as an anticancer agent. Further studies can be carried out to develop l-arginase as an effective drug for cervical cancer.

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REFERENCES

[1] Yoon C ; Shim YJ ; Kim EH; et al, *Int J Cancer*. **2007**, 120(4): 897–905.
[2] Kim RH ; Coates JM ; Bowles TL, et al. *Cancer Res*, **2009**, 69(2): 700–708.
[3] Glazer ES; Stone EM ; Zhu C; Massey KL ; Hamir AN, Curley SA, *Transl Oncol*.; **2011**, 4(3): 138–146.
[4] Yau CC ; Chan P; Pang R, et al, *J Clin Oncol.;* **2010**, 28: e13503.
[5] Ashraf S. El-Sayed etal , *Archives of Pharmacal Research***,** DOI 10.1007/s12272-014-0498-y.
[6] Krauss, T., Huschmand, N.A., Viereck, V. and Emons, G . *Onkologie*. **(2001),** 24 (4): 340-345
[7] ICO Information Centre on HPV and cancer, *Human Papillomavirus* and Related Diseases in India (Summary Report 2014-08-22); **2014**.
[8] J. F. Zhan; S. T. Jiang and L. J. Pan, *Brazilian Journal of Chemical Engineering* ,Vol. 30, No. 04, pp. 721 - 728, October - December, **2013**.
[9] Rahamat unissa etal**,** *Int J Pharm Bio Sci* **2015** July; 6(2): (P) 506 – 517.
[10] Mosmann, T. *J Immunol Methods* 65, 55-63, **(1983).**
[11] Archibald. R.M. *J. Biol Chem*, **1945**, 157: 507- 518.
[12] Lowry, O.H, Rosebrough, N.N., Farr, A.L & Randall, R.Y. , *J. Biol. Chem.***1951**, 193, 265-275.
[13] Laemmli, U.K., *Nature*, **(1970)** , 227: 680-685.
[14] Wray; W. Boulikas; T., Wray; V. P. and Hancock, R. *Anal. Biochem.* , **(1981)**, 118: 197-203.
[15] R Philip; E Campbell and DN Wheatley,. *British Journal of Cancer* **(2003)** 88, 613 – 623.
[16] Adinarayana; K., Jyothi; B. and Ellaiah, P. *AAPS PharmaSc iTech* **, 2005** , 6(3): 391-397.
[17] Braun, S., Rappoport, S., Zusman, R., Avnir , D. and Ottolenghi, M. *Mater Lett.* , (1990), 10: 1-8.
[18] Okonji. R.E, Ehigie. L.O. and Soyelu .O.J., *African Journal of Biochemistry Research*. Vol. 8(4), pp .84-94, April, **2014**.
[19] Shimotohno ; K.W.; Miwa, I. and Endo, T. *Biosci Biotechnol Biochem.*, **(1997)**, 61(9): 1459-1464.
[20] Jenkinson; C.P. and Grigor, C.P. *Biochem Med Metab Biol.*, **(1994)** , 51**:** 156–165.
[21] Kuhn, N.J., Ward, S., Piponski, M. amd Young, T.W. *Arch Biochem Biophys*., **(1995)** , 320 (1): 24-34.
[22] McGee; D.J., Zabaleta; J., Viator; R.J., Testerman, T.L., Ochoa, A.C. and Mendz, G.L, *Eur J Biochem.,* **(2004)** , 271: 1952-1962.
[23] Grazi, E. and Magri, E. *Biochem J.*, **(1972)**, 126: 667-674.
[24] McGee, D.J., Zabaleta, J., Viator, R.J., Testerman, T.L., Ochoa, A.C. and Mendz, G.L., *Eur J Biochem.,* **(2004)** , 271**:** 1952- 1962.
[25] Dabir, S.; Dabir; P. and Somvanshi, B. *Int J. Biol Sci.* **(2005),** 1(3): 114-122.
[26] Muller, I.B., Walter, R.D. and Wrenger, C., *Biol Chem.*, **(2005)**, 386(2): 117-126.
[27] Nakamura; N., Fujita; M. and Kimura, K. *Agricultural and Biological Chemistry*, **(1973)** , 37(12): 2827-2833.
[28] McGee; D.J.; Zabaleta, J.; Viator, R.J., Testerman, T.L., Ochoa, A.C. and Mendz, G.L. *Eur J Biochem.,* **(2004)** , 271: 1952-1962.
[29] Arakawa; N.; Igarashi; M., Kazuoka; t., Oikawa, T. and Soda, K. *J Biochem*, 133: 33-42, **(2003).**