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Miniaturized fiber-optic biosensor to monitor asparagine in clinical samples

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ABSTRACT

Asparagine biosensor was constructed by co-immobilized anti-leukemic enzyme (L-asparaginase) and absorptive pH sensitive indicator on to plastic chips of 5mm through hydro sol-gel approach. Asparagine is used as a biomarker for detection of acute lymphoblastic leukemia. In this research work asparagine biosensor was developed focusing on L-asparaginase activity as bioassay principle. L-asparagine gets hydrolyzed into L-aspartic acid and ammonia by enzyme L-asparaginases, thereby concentration of ammonia produced was monitored by miniaturized fiber-optic spectrophotometer. Anti-leukemic enzyme L-asparaginase used in the present research work was screened from Staphylococcus sp. isolated from sewage waste water. Fiber optic approach was used for substrate analysis using transparent chips; sample volume up to 5µl was used for sample study and absorption spectrum was observed. Developed biosensor was applied to clinical samples from the patients of different ages (Throat, blood cancer) to monitor asparagine concentration. Fiber optic based biosensor proved to be suitable approach for substrate breakdown with miniaturized level up to 5µl and detection limit of 0.1nM

Keywords: Asparagine; TMOS sol gel; Bacteria; Fiber optic approach; clinical samples

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INTRODUCTION

Enzyme L-asparaginase breaks down substrate into its products resulting in ammonia production forming the basis of asparagine biosensor development [1,2, 3]. It is an antineoplastic agent serving best enzyme for leukemia treatment. Neoplastic cells cannot synthesize L-asparagine due to the absence of L-asparagine synthetase [4,5]. It deprives leukemic cells from its source asparagine to divide in presence of L-asparaginase; Bacterial L-asparaginases are classified into two types based on their location with periplasmic subtype depicting higher substrate affinity than cytoplasmic subtype[3].Microbial sources are considered to be best for L-asparaginase production for its use in clinical trials[6].It is widely distributed, found in animals, plants and microbes [1,7]. It is the foremost enzyme with anti-tumor activity in human. Anti-tumor properties of guinea pig serum were first accounted for asparaginase activity [8,9, 10]. *E.coli* L-asparaginase II was the first antileukemic to be used clinically [11]. L-asparaginase from bacterial origin had been commonly reported; eukaryotic microorganisms also contribute potentially for its production [12,13,14] . L-asparaginase structural view had been derived from variety of organisms [15].Structure-function relationship of L-asparaginases from different sources is under investigation [16,17]. It is a tetrameric protein with four identical subunits, each subunit consisting of 326 amino acid residues; molecular weight of 141 kDa [18,19]. Primarly used in acute lymphoplastic leukemia treatment [20]and L-asparagine concentration determination [21]. L-asparaginase enzyme functions as a drug to suppress malignant tumor cell growth. Elspar, Oncaspar, Kidrolase and Erwinase are brand names of L-asparaginase used as adrug.FDA has approved this enzyme for anti-leukemic treatment [2]. Its analysis using biosensor with fiber-optic approach serves to be cheap and best technology to be relied upon with minimum sample treatment compared to other costly and tedious available

techniques as XPS, XRD [22]. L-asparaginase has been and continues to be focused as therapeutic enzyme for anti-leukemia treatment by researchers and scientists worldwide[2]. The present study deals with miniaturized fiber optic biosensor for asparagine concentration determination. Asparagine biosensor developed so far were on clinical basis with detection limit upto $\leq 10^{-4}$mol/l using on line gas dialyzer using potentiometric approach by [23] in1983 from *E.coli*sp. ; [24] in 1990 also developed potentiometric based biosensor with L-Asparaginase was determined in the range of 0.4-1.6 U in a 0.1 ml sample method to assay L-asparagine with an ammonia gas-sensing method and L-asparagine was determined with concentration range of $1x10^{-4}$-$1x10^{-3}$M. Asparagine biosensor for leukemia was developed with detection limit 10^{-9}-10^{-1} M using potentiometric approach; various immobilization strategies had been used for asparagine concentration determination in normal and leukemic serum samples. Detection limit with calcium alginate immobilized method proved to be superior by [2]. *Present biosensor had made certain improvements over existing biosensors e.g. miniaturization of sample volume.*

MATERIALS AND METHODS

Microorganism and its growth

Isolated bacterial *Staphylococcus sp*. was screened from sewage water sample using Rapid plate assay method [25]and identified by gram staining; for further identifications it was sent to IMTECH, Chandigarh. After every 30 days culture was maintained by sub-culturing and stored at 4^0C.

Enzyme activity and Kinetic characterization

Growth pattern and L-asparaginase activity was assessed after every 3 hrs up to 36 hrs. Crude extract of cells after sonication was used for assessing enzyme activity through nessler's method [26]. Culture(O.D. - 2.231) was centrifuged at 5000 rpm and pellet was washed with 0.1 M PBS and supernatant formed the enzyme sample. Crude enzyme sample was purified through acetone precipitation and further used in the present study [27]. K_m&V_{max} of asparaginase was evaluated in both free and immobilized state using different solvents system (TEOS, TMOS, TMOS: TEOS in ratio of 1:1)[28, 29].

2.3 Fabrication of Biosensor for Asparagine

Bio-component was co-immobilized with indicator phenol red upon plastic chips using TMOS (tetra methyl ortho silicate) as gelling agent. Enzyme (1.72 IU/ml) was suspended in 500µl of Phosphate buffer (pH-8.0). Cocktail preparation consisted of 50µl of extracted enzyme, 600µl alcohol, 50µl (TMOS), 10µl NaOH (5mM) and 20µl phenol red. Above mentioned preparation was introduced upon transparent plastic chips for hydro sol-gel formation and kept for 1hr gelation at 4^0C.Immobilized chips were used for asparagine analysis using fiber optic tip(Fig.5). Asparagine concentration up to 1nm was analyzed by means of 5.0µlsubstrate;absorption spectrum was constantly recorded for 10 minutes and standard chart was prepared [30].

Clinical Application of developed biosensor

Fabricated biosensor analyzed asparagines concentration in leukemic and normal blood samples collected from Behgal Hospital Mohali (Punjab); total nine different leukemic serum samples of different ages (Throat , blood) cancer andtwo healthy individuals were monitored for asparagine concentration determination and *color change of membranes could be observed*(Fig.4).The work was carried out as per Ethical and Biosafety Committee guidelines with Institutional clinical *Ethical committee no. ICEC/10/2011*.Plastic chips were checked at regular interval for storage stability.

RESULTS AND DISCUSSION

Isolate was found to be gram positive with cream colored circular colonies with smooth surface; cocci shaped cell arranged in clusters, was confirmed to be *Staphylococcus sp*. by IMTEC, Chandigarh(Fig.1).

Enzyme activity and kinetic characterization

Isolate showed maximum growth and asparaginase activity (1.72 IU/ml) at 15hrs of log phase (Fig.2)and purification achieved through acetone precipitation with specific activity increased from 113.95 to 335.27 IU/mg; 77% recovery and 2.94 purification fold. Kinetic characterization of enzyme in both free and immobilized state was evaluated. Km and Vmax in free State was found to be 0.232mMand6.66umol.min-1respectively. Km and Vmax in different solvents (TMOS, TEOS, TMOS: TEOS in ratio of 1: 1) were found to be - 2.32, 5.26, 3.57mM; 3.70, 3.33

& 2.63 umol.min-1 respectively(Fig.3). Kinetic characterization with TMOS was found to be suitable immobilizing matrix.

Asparagine Biosensor and its application

Absorption spectrum of phenol red indicator was screened from 500nm to 600nm with 538.06 nm selected for further spectra studies. Absorption at 538.06 nm was used for computation of further research due to highest absorption observed and application along with reference chart constructed as shown in (Fig.6&7).Immobilized plastic discs were used for analysis using fiber optic spectrofluorimeter; Response time was optimized to be 7 min with lower detection limit –0.1nMand linear range of 1nM. Asparagine concentration determined by the developed biosensor in patients of different ages (Throat, blood cancer) shown in (Fig.8) and normal healthy individuals is shown in Table 1 & 2 respectively. Leukemic samples proved to have high asparagine concentration in comparison to normal healthy individuals[31].

Table 1 Asparagine Analysis in various samples (Throat and Blood leukemic samples)

S. No	Patients name	Age/Sex	Treatment Undertaken	Asparagine conc. (M)
1	Navdeepkaur	28/M	Chemotherapy	0.146×10^{-2}
2	Roursingh	70/ M	Chemotherapy + Radiotherapy	0.561×10^{-3}
3	Chandermaya	45/ F	_	0.606×10^{-3}
4	Jinderdass	45/M	_	0.090×10^{-2}
5	Kulwantkaur	57/ F	Chemotherapy	0.128×10^{-2}
6	Paramvirkaur	44/F	Chemotherapy	0.130×10^{-2}
7	KulwantKaur	35 /F	Chemotherapy + Radiotherapy	1.165×10^{-2}
8	Kitabo	45 /F	Chemotherapy	1.139×10^{-2}
9	Mahipal	45 /M	Chemotherapy	0.928×10^{-2}

Table 2 Asparagine analysis in normal blood samples

S.No	Donors name	Age/ Sex	Blood Group	Asparagine conc.(M)
1.	Manpreet	23/F	B^+	1.38×10^{-4}
2.	Sonika	22/F	A	0.67×10^{-4}

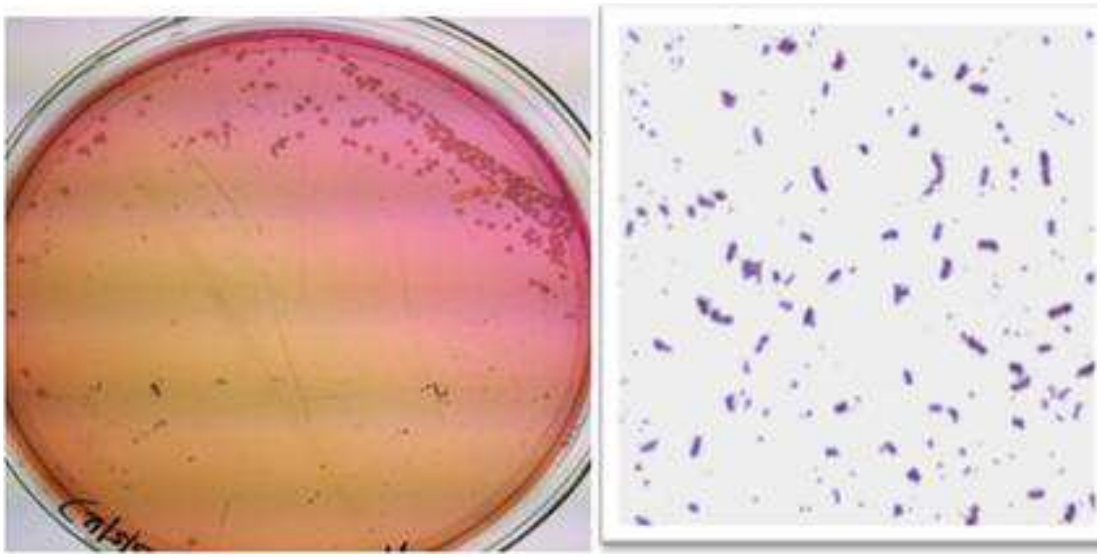

Fig. 1: Rapid Plate assay method and Gram staining slide

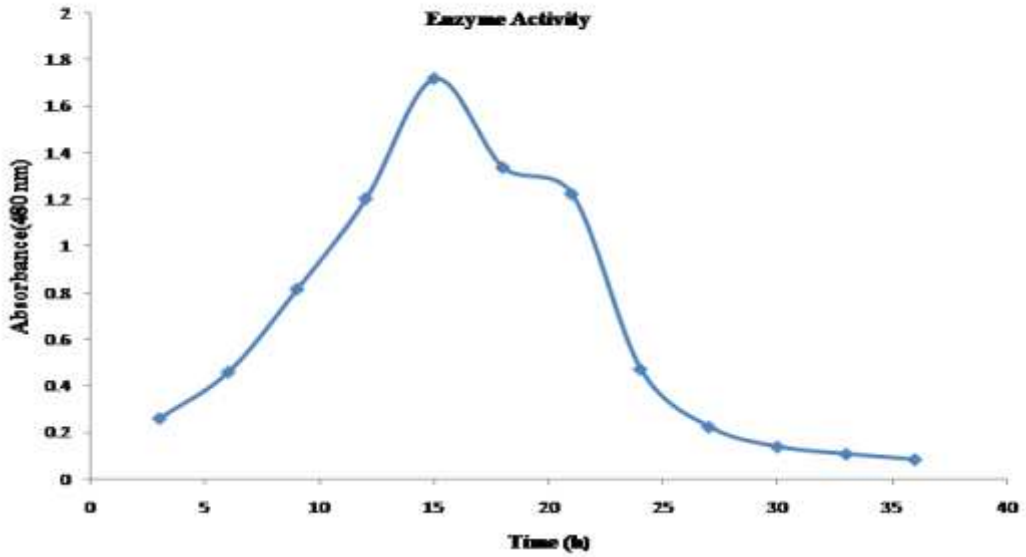

Fig.2: Standard curve of enzyme activity

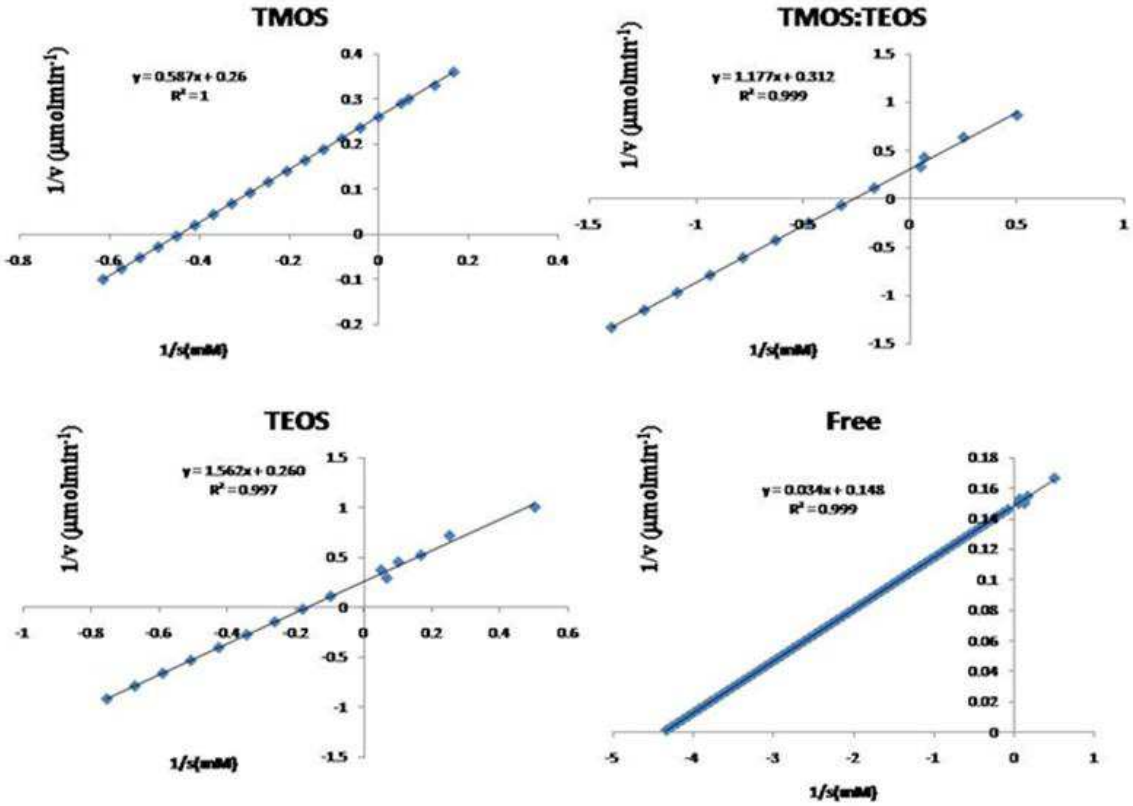

Fig. 3: Kinetic characterization graph depicting K_m&V_{max} in free and immobilized state

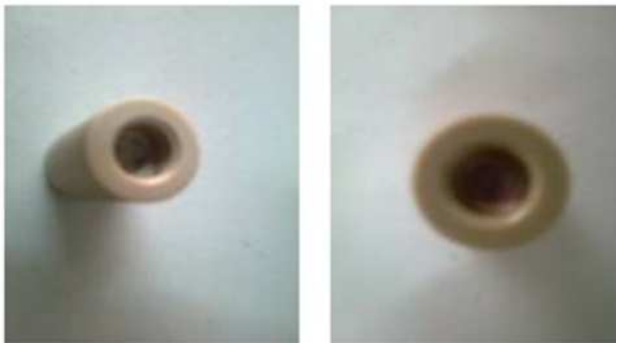

Fig. 4: Color change of membranes

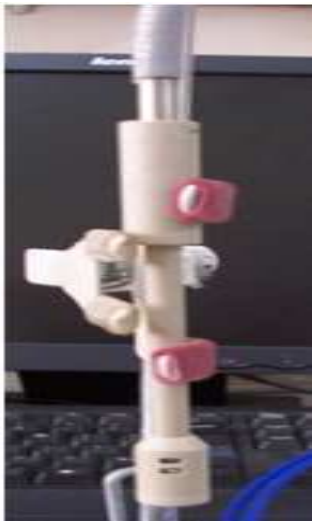

Fig. 5 Closer view of fiber optic tip

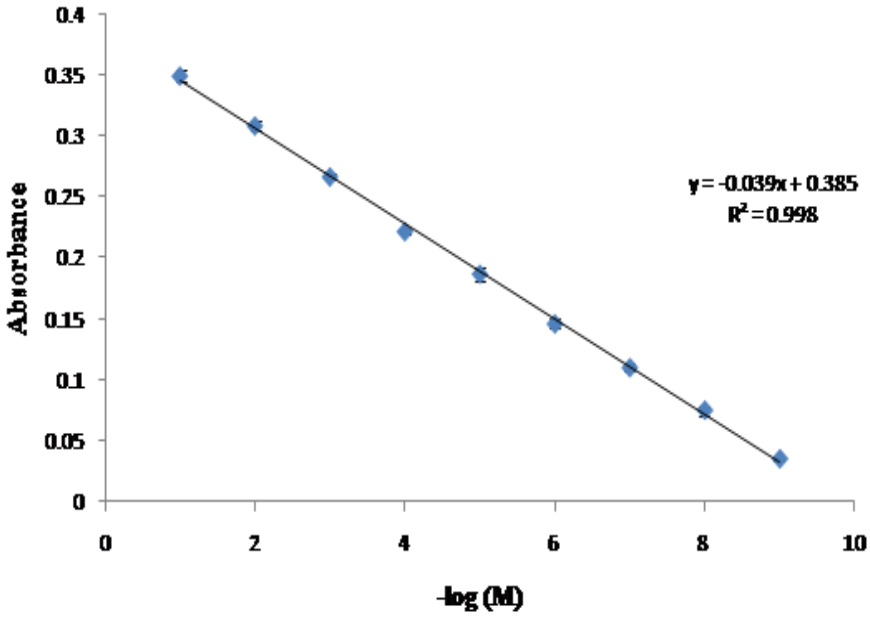

Fig. 6: Standard reference chart for different asparagine concentrations (10^{-9} - 10^{-1} M)

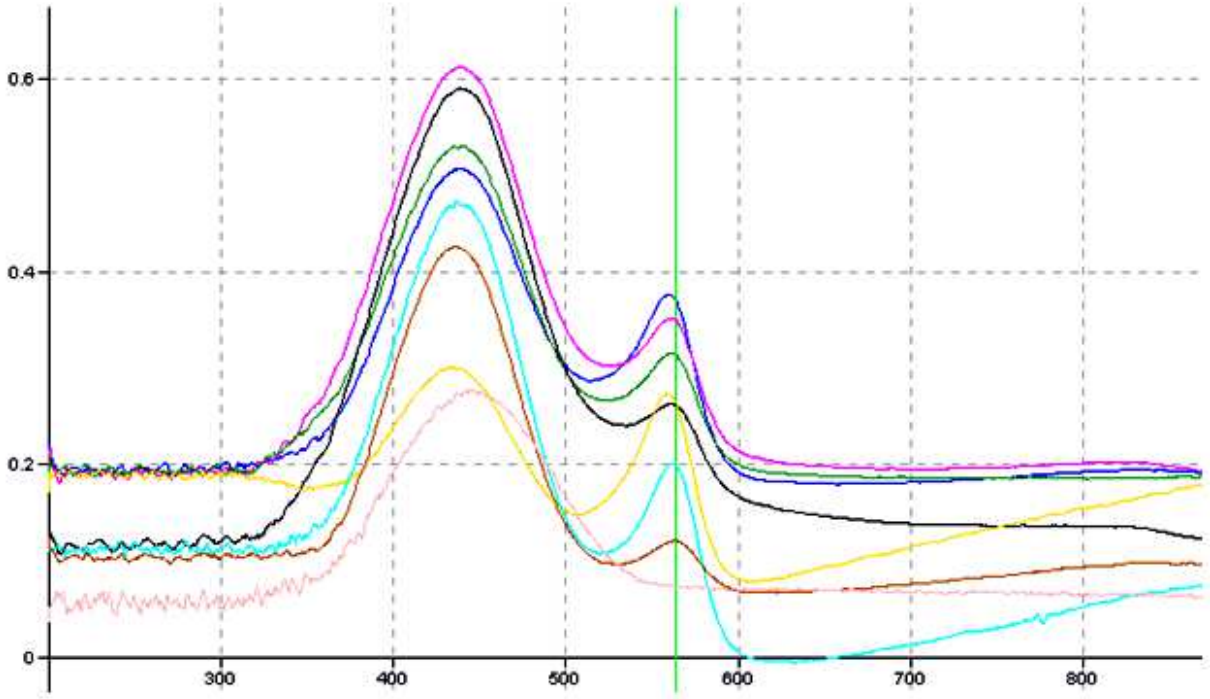

Fig. 7 Standard reference chart in graphical form (a to h) represents 10^{-8} to 10^{-1} M asparagine concentrations

Fig. 8: Leukemic samples collected

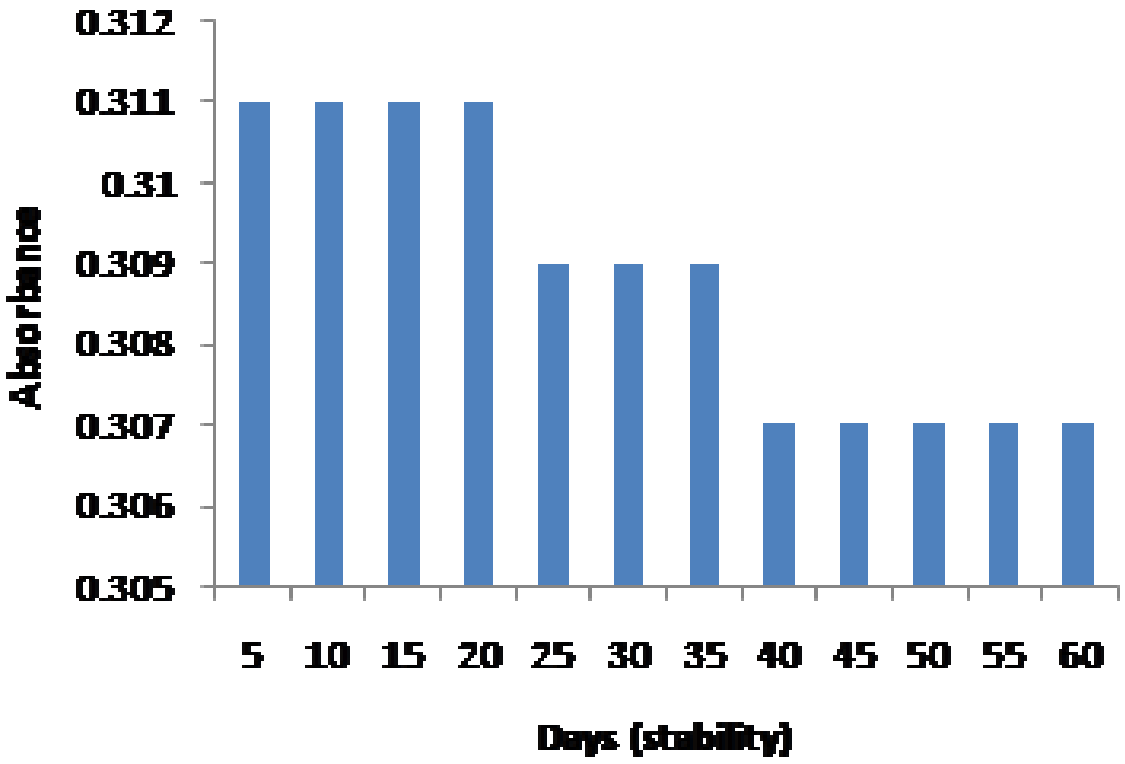

Fig. 9: Stability graph of bio-component chips in terms of absorbance

CONCLUSION

L-asparaginase producing *Staphylococcus* sp. was successfully isolated from sewage waste water. Kinetic characterization with tetramethylorthosilicate was found to be suitable solvent for further study. Standard chart was prepared depicting response time of 7 min; miniaturization volume-5ul; lower detection limit –0.1nM and linear range of 1nM. Application of developed Biosensor in clinical and normal healthy individuals was studied. Immobilized plastic chips were stable for 40 days at 4ºC with further reduction in activity as shown in (Fig. 9).

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