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Screening, production, purification and characterization of beta-lactamase from uropathogenic *E.coli*

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Abstract

Beta-Lactamase activity was detected in 15 uropathogenic isolates belonging to family Enterobacteriaceae by iodometric method. E. coli Strain Y3 showing multiple antibiotic resistance (MDR) was used to optimize the conditions for production and assay of the enzyme. The major location for the enzyme amongst all uropathogens was found to be extracellular / Periplasmic (72%) including the selected isolate Y3. The enzyme is mainly cell associated and was released from the cell surface by sonication. During periodic monitoring 8 hours of incubation produces maximum cell mass with optimal enzyme activity at pH 7 and temperature of 35^0C. The beta lactamase was partialy purified by ammonium sulphate precipitation followed by desalting and dialysis. The pooled ammonium sulphate activity was found to be 5.2 EU (1.5 fold purification). Further dialysed extract gave 2.2 fold purification followed by 4 fold purification in case of desalted concentrated enzyme. The enzyme was found to be active and stable at pH 8 for 48 hrs. and at temperature 35^0C for 48 hrs. The enzymes have penicillanase activity, get induced by inducers like penicillin, ampicillin and amoxicillin and are inhibited by Clavulunic acid.

Keywords: Beta lactamases, *E.coli*, uropathogen, MDR,amoxicillin,clavulinic acid

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INTRODUCTION

Infections like urinary tract infection have been the major cause of disease throughout the history of human population. With the introduction of antibiotics, it was thought that this problem will disappear. However, bacteria have been able to evolve to become resistant to antibiotics. The growing threat from resistant organisms calls for concerted action to prevent the emergence of new resistant strains and the spread of existing ones (Senka džidić *et al*. 2008). The variability of structural and functional aspects of antibiotics from all generations is incapable of managing the severe problem of drug resistance due to various drug resistance mechanisms acquired by microorganisms.

Bacterial resistance to beta-lactam antibiotics is often associated with the production of beta-lactamase (penicillin amidohydrolase; EC 3.5.2.6), which hydrolyses the beta-lactam ring at C4 position. Most important part of all penicillins is the β- lactam- ring (4 atom cyclic amide rings) azetidinone, thiazolidine ring fused with to lactam ring bicyclic heterocyclic nucleus of 6 APA. The enzyme is not only present in prokaryotic microrganisms viz. Gram

negative and gram positive but is also present in eukaryotic cells like *Candida albicans*, blue green algae, mammalian liver cell, rat brain etc.[8]. The enzymes produced by gram-positive organisms are frequently extracellular and inducible. They have greater affinity for the penicillins than for the cephalosporins. Those of gram negative organisms are constitutive; more varied in nature, cellular or periplasmic, but may be classified on the basis of substrate profile, inhibition studies, molecular weight, and electrophoretic mobility[15]. Whereas the 'Extended spectrum Beta lactamases" (ESBL) are the enzymes that can hydrolyse third generation penicillins and cephalosporins. These are encoded on a large plasmid that can be horizontally transferred to different genera of bacteria. Ambler has classified beta lactamases on the basis of structural information available of beta lactamases.

UTI is most commonly caused by various species of *Escherichia, Klebsiella, Bacillus, Enterobacter, Pseudomonas, Enterococcus faecalis and Candida albicans* etc (Abigail and Dixie, 2005; Rai et al, 2008). Several reports have indicated that strains of gram negative bacteria, *E. coli*, become massively resistant to the even the Extended Spectrum Beta-lactam antibiotics which are the key drugs to treat the infections especially UTI [10, 3, 12].

The enzymes were first noted in 1940 by Abraham and Chan in the issue of Nature as extracts from various bacteria can destroy penicillins. The term *Penicillinase* was first appear in Quaternary cumulated Index Medicus in 1944.

All comparative data so far presented can be determined with crude cell-free extracts may be subject to error; since inhibiting factors in the crude preparations might cause interference hence the purified enzyme was used. It has not yet been clearly demonstrated that a single enzyme is responsible for the destruction of the various substrates in any one strain or whether the enzymes are the same or different throughout the species. The enzyme responsible for destruction of antibiotic/ antibiotics may be one or more and is considered as set of beta lactamases and ESBLs. The present report describes the extraction and characterization of the crude and purified *E. coli* beta-lactamases enzyme produced by the highly resistant strains are described under optimal conditions of enzyme induction, inhibition, production, maintenance and assay. This enzyme has a specific activity several fold greater than any, beta-lactamases produced by more susceptible strains, even when crude.

MATERIALS AND METHODS

Bacterial strains, media, antibiotics, chemicals and culture conditions:
Fifteen urinary pathogens were isolated from non repetative clinical specimens. They are characterized biochemically as described in the Bergey's manual of Determinative Bacteriology. Other strains like ATCC 25922 (reference strain for AST) and NCIM 2391 (standard beta lactamases producing *Bacillus* culture) were supplied by NCIM, NCL, Pune. All the chemicals used in the preparation of reagents were of analytical-grade and were ordered from SRL India, MERK India; Himedia Pvt. Ltd. Mumbai, India. All media were autoclaved as per the specific media consideration and checked for sterility before inoculation.

Assay of beta-lactamase:
Iodometric method used to assay enzyme Beta Lactamase was as follows: Benzyl penicillin (K-phosphate buffer 0.1 M, pH 5.9) degradation was measured by considering the residual iodine (Iodine reagent 0.0166N in sodium acetate buffer) which reacts with the acid produced due to enzymatic degradation. This iodine is then back estimated using equivalent of sodium thiosulphate (0.0166 N) by titrimetric method where starch (1%) is an indicator. Reactions were performed at 37^0C in phosphate buffer, pH 5.9, for *E. coli* cultures. The enzyme activity = µmol of penicillin degraded/ml of enzyme/Hr.

Screening and enzyme location analysis for beta lactamase activity:
Fifteen urinary pathogens isolated from non repeatative clinical specimens. They are screened for beta lactamase activity. Enzyme activity was determined in cell supernatant and cell lyzate for all isolates in order to analyse the enzyme location. The highest enzyme producing isolate was found to be multiple drug resistant *E. coli* Y3 and is used for further study.

Beta lactamase production at different phases of growth:
Cells of clinical isolate Y3, were grown in nutrient broth at 37^0C with shaking. Samples (5 ml) were taken every half an hour. Cell density and enzyme activity were measured simultaneously. Cell density was measured at 500 nm. Beta lactamase activity was determined after ultrasonic disruption of cells (10 Hz for 8 min) as enzyme location was found to be periplasmic.

Production of crude enzyme under optimized conditions:
E. coli culture was grown from a single colony inoculum. The nutrient broth having pH 8 is used for the higher production of enzyme. It contains ampicillin (20µg/ml) as inducer for enzyme production. The incubation was done aerobically at 35^0C. These cells are then harvested by centrifugation (7,000 x g, 20 min at 4°C), and washed twice in phosphate buffer (0.01 M, pH 7.0) at 4°C. Cells were resuspended to obtain the O.D. adjusted cell density. The cells were disrupted using an ultrasonic disintegrator, with 5 min sonic disintegration at 4°C/ till complete visible cell disintegration. Cell debris was first removed by centrifugation (30,000 x g, 20 min at 4^0C). The cell lyzate is referred to throughout as periplasmic crude, Beta lactamase preparation.

Enzyme purification:
The purification was carried out at 4^0C on the crude extract, the cell lyzate obtained after sonication. with some modifications in the method given by Distasio[6].

Ammonium sulphate precipitation
Powdered ammonium sulphate AR was added up to 80% saturation. The crude enzyme obtained as per the enzyme location was brought to 60 percent saturation with ammonium sulphate at pH 8 for Y3 and kept overnight in cold room. After equilibration, the supernatant was brought to 80 percent saturation with ammonium sulphate and centrifuged at 8000 rpm, at 4^0C for 10 min. Then the precipitates were collected separately and dissolved in a 0.1 M phosphate buffer at pH 8 for Y3 stored at 4^0C for further purification.

Dialysis:
The pre-treated dialysis tubes were used for dialysis of the precipitates collected after ammonium sulphate precipitation. The precipitate was dissolved in 0.1*M* phosphate buffer (pH 8 for Y3) and dialyzed. After dialysis, the samples were used for protein estimation and enzyme assay.

Desalting:
The dialyzed enzyme was applied to Sephadex G-25 column that was pre- equilibrated with 0.1 *M* Phosphate buffer (pH 8). The protein elution was done with the same buffer at a flow rate collect 3 mL/1 min. The fractions were collected and assayed for protein at 280 nm as well as for enzyme activity Iodometrically. The active fractions were pooled, dialyzed against the 0.1 M phosphate buffer *p*H 8, and concentrated.

Enzyme characterization studies:
Physiological character analysis:
The partially purified enzyme obtained after desalting was used for the characterization of beta lactamase and to optimize its activity. Testing of the activity of the enzyme was carried out initially at the range of *p*H (6 to 8) and temperatures (25^0 to 55^0C). The *p*H activity profile of the partially purified enzyme (*p*H 6 to 8) was studied. Phosphate buffer (0.1 M, *p*H range 6.0-8.0), was used for this purpose. The assay was carried out using 1 ml of approximately diluted enzyme and 0.0166N substrate (penicillin). Enzyme activity was calculated by iodometric analysis. Similarly, the activity of the enzyme was measured at different temperatures ranging from 25^0C to 55^0C, and at the optimum *p*H using the assay procedure above. The partially purified enzyme was used for the characterization of Beta -Lactamase and to optimize its activity. Testing the activity of the enzyme was carried out at wide range of *p*H (6, 7 and 8) and temperatures (25, 35 and 55^0C). The *p*H activity profile of the partially purified enzyme (6, 7 and 8) was studied. Phosphate buffer (0.1 M, *p*H range 6, 7 and 8). The assay was carried out using 0.1 ml of approximately diluted enzyme. Specific activity was calculated iodometrically. Similarly, the activity of the enzyme was measured at different temperatures ranging from 25^0C, 35^0C and 55^0C, and at the optimum *p*H using the assay procedure above.

Inducer profile study:
The substrate profile study was done using penicillin G (0.0166N), Ampicillin 30µg/ml and Amoxicillin 30µg/ml. The enzyme is allowed to interact with substrate and is determined Iodometrically.

Inhibitor analysis:
Clavulunic acid is used to study enzyme inhibition. The enzyme is studied for inhibition at various concentrations of

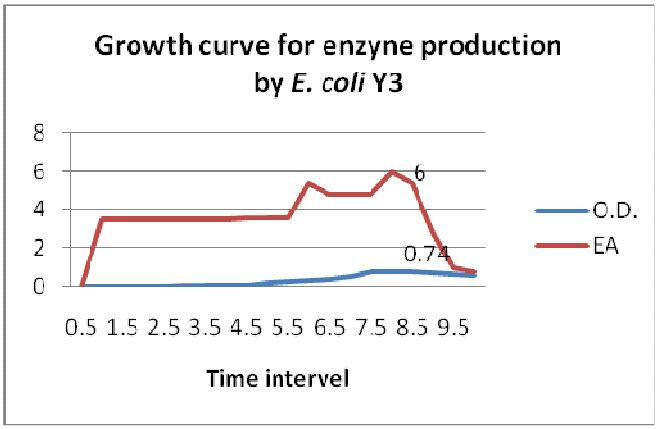
RESULTS

Identification of clinical isolates:
All the clinical isolates were isolated on Mac-conkeys agar show pinpoint pink coloniesindicating their Lactose fermentative nature. All bacteria were confirmed to be *E. coli* by using IMViC test, CLED agar with Andrade indicater (bright pink color), EMB agar (blue black metallic sheen), Endo agar (red rose metallic sheen), [17].

Table: I: Location of enzyme

Sr. No.	Culture No.	Cell density (24) hrs (at 500 nm)	Activity in cell supernatant	Activity in cell lysate	Location of Enzyme
1	00043	0.435	0.4	0.2	Extra cellular
2	00192	0.352	1.6	1.4	Extra cellular
3	00560	0.108	1.4	2.4	Periplasmic
4	00625	0.467	1.2	2.4	Periplasmic
5	00801	0.043	1.2	2.4	Periplasmic
6	00908	1.008	2.6	2.8	Periplasmic
7	02973	1.564	1.6	1.8	Periplasmic
8	03793	1.126	1.8	1.6	Extra cellular
9	05718	0.817	2.4	3.0	Periplasmic
10	33717	0.476	1.4	1.8	Periplasmic
11	33807	0.993	1.8	1.4	Extra cellular
12	34690	1.608	1.6	2.0	Periplasmic
13	34987	0.995	1.4	1.6	Periplasmic
14	34967	0.857	1.4	1.6	Periplasmic
15	34990	1.249	2.0	2.2	Periplasmic

Graph I: Growth curve



Preliminary screening for Beta-lactamase activity and analysis about enzyme location:
The extracellular and intracellular penicillinase activity was determined by using cell supernatant and cell lyzate respectively using the iodometric method. The major location for the enzyme amongst all uropathogens was found to be extracellular / Periplasmic (72%). (table No. I). The clinical isolate Y3, exhausted for further study was showing maximum intracellular enzyme activity (3 EU). *E. coli* Y3 was found to be Multiple Drug Resistant culture, showing resistance to Ampicillin, Amoxicillin, Pipracillin, Carbancillin, Mithicillin, Cloxacillin, Oxacillin, Cefotaxime, Ceftazime, Cefazolin, Ceftriaxone and was sensitive to Amikacin, Amoxicillin with Clavulunic acid and Nitrofurantoin.

Enzyme characterization

Physiological characters analysis

Graph II: pH optimization

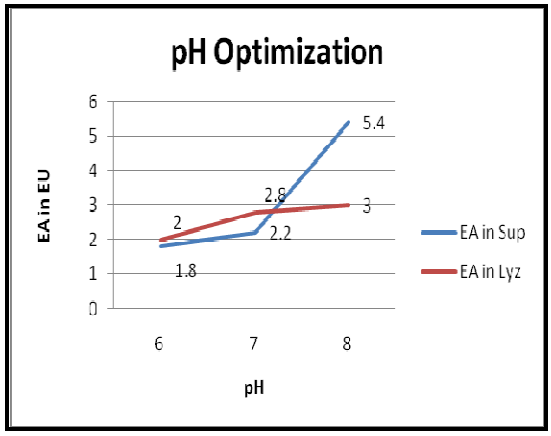


Table IIai: Statistical analysis for optimization of pH

Hrs	**6**	**7**	**8**	Total
24	3.4	4.6	6	14
48	3.4	3.4	4.2	11
72	2.4	2.8	3.6	8.8
96	1.8	1.2	2.8	5.8
Total	11	12	16.6	**39.6**

Table IIaii: Calculations:

C.F.	130.68	**Source**	**df**	**ss**	**Mss**	**F**	
pH SS	4.46	**pH**	**2**	**4.46**	**2.23**	**9.511848**	*
Hrs SS	12.01333	**Hrs**	**3**	**12.01333**	**4.004444**	**17.08057**	**
Total SS	17.88	**Error**	**6**	**1.406667**	**0.234444**		
Error SS	1.406667	**Total**	**11**	**17.88**			

Graph III: Temp. Optimization

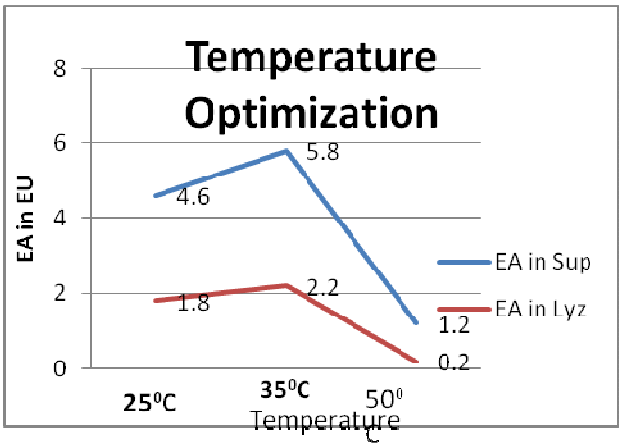


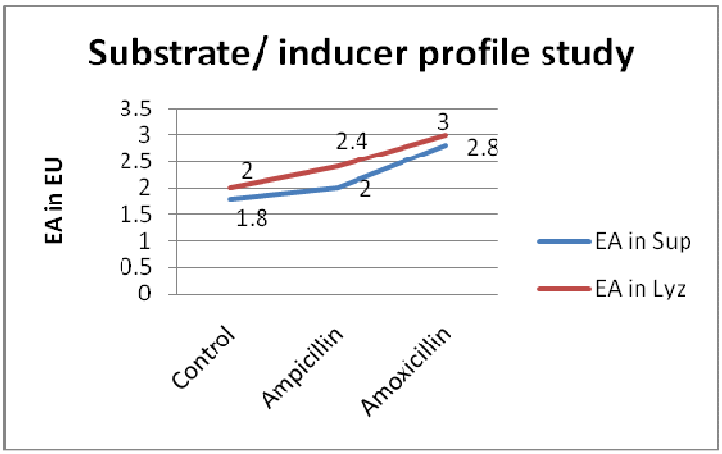
Table IIbi: Statistical analysis for optimization of temperature:

Hrs	**25**	**35**	**45**	**55**	Total
24	3.8	5.2	3.4	2.4	**14.8**
48	3.8	3.2	3.2	1.6	**11.8**
72	3.8	3.2	2.6	1.4	**11**
96	2.6	1.4	1.8	0.8	**6.6**
Total	**14**	**13**	**11**	**6.2**	**44.2**
TotasSS	50.08	49.48	31.8	10.92	**142.28**
CF	122.1025				

Table IIbii: Calculations

CF	122.1025		**Source**	**df**	**SS**	**MSS**	**F**	
Temp SS	131.11	9.0075	**Temp.**	**3**	**9.0075**	**3.0025**	**10.54537**	**
Hrs SS	130.71	8.6075	**Hrs**	**3**	**8.6075**	**2.869167**	**10.07707**	**
Total SS	142.28	20.1775	**Error**	**9**	**2.5625**	**0.284722**		
Error SS		2.5625	**Total**	**15**	**20.1775**			

Graph IV: Inducer profile study



Graph V: Inhibitor profile study

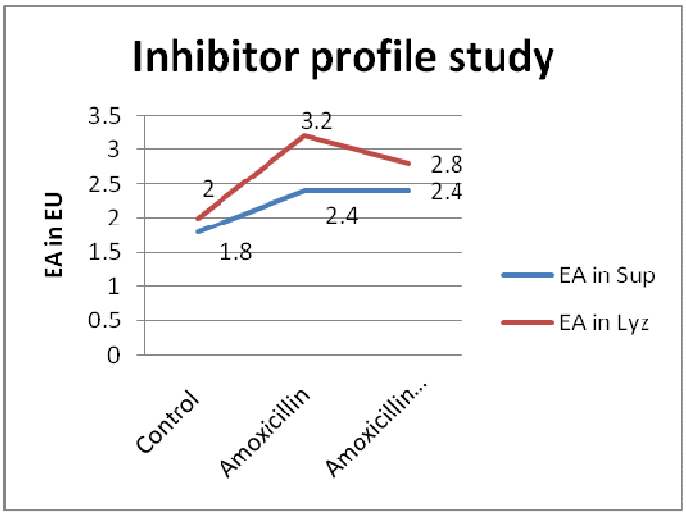


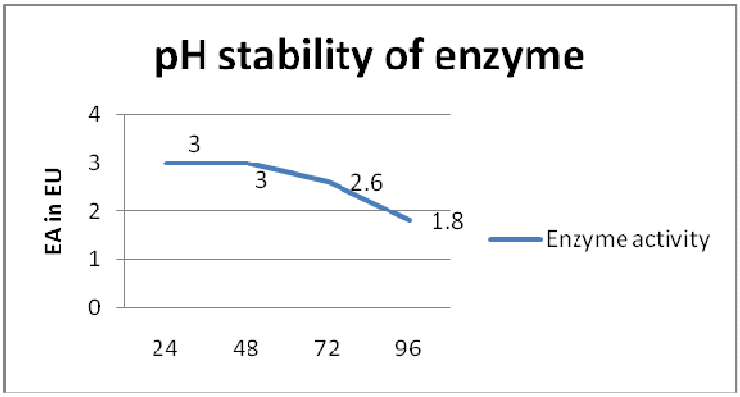
Table III: Enzyme production
Table IIIa: Cell disintegration for obtaining crude enzyme:

Sr. no.	Method	Intracellular EA	Extracellular EA	Location of enzyme
1	Sonication	2.4	1.8	**Intra cellular/ Peripalsmic**
2	Lysozyme	1.4	0.8	**Intra cellular/ Peripalsmic**

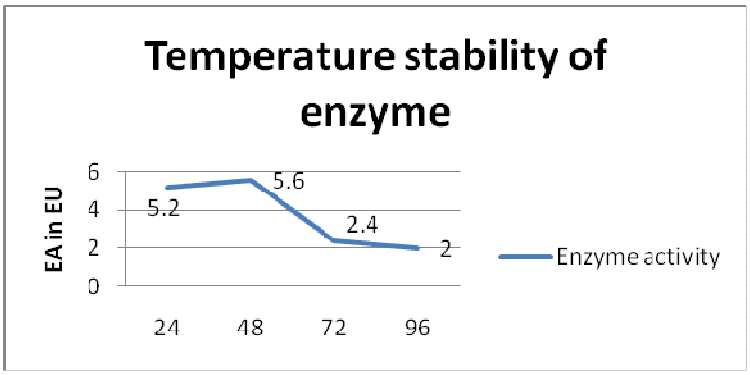
Table IIIb: Enzyme purification

Summary of Enzyme Purification			
Sr. no.	Step of Purification	Enzyme activity (EU)	Specific activity (EU/mg)
1	Crude extract	3.0	3.74
2	Pooled enzyme after Ammonium sulphate precipitation	5.2	6.50
3	Post dialysis	5.6	6.97
4	Post Desalting	5.8	7.22

Graph VI: pH stability of enzyme



Graph VII: Temperature stability of enzyme

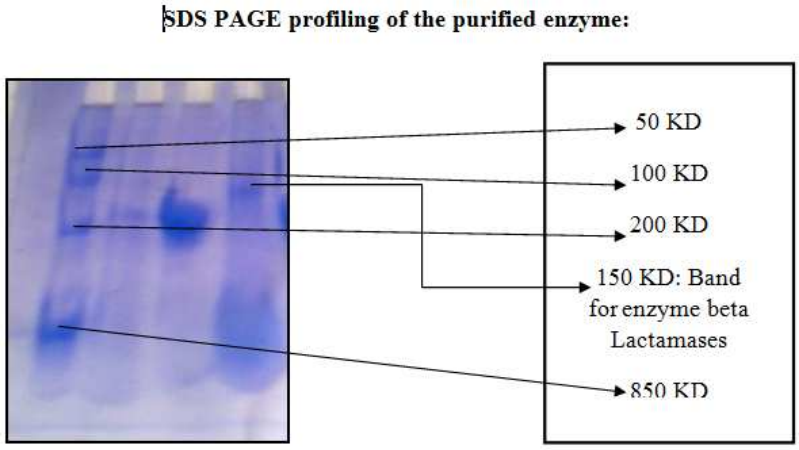


Beta Lactamase production at different phases of growth:
Strain Y3 was used to study the formation of Beta -Lactamase enzyme during growth in nutrient broth (Graph I). Its maximum activity (6.0 EU) occurred at about 8 hours, during stationary phase after initiation of growth. During late stationary phase the activity was found to be near about constant (5.4 EU) but at the lower side till 9.5 hours. After 10 hours there was a sudden decrease in beta lactamase activity.

Optimized enzyme Production
The selected clinical isolate have produced the enzyme Beta lactamases with enzyme activity of 3.6 EU/ml in aerobic condition after 24 hrs obtained in cell lyzate. The optimum pH for production was found to be 7(Graph II) and optimum temperature was found to be 35^0C. These veriables were also confirmed using stastistical analysis (tableIIai,ii and table IIbi,ii). After the inducer and inhibitor profiling, amoxicillin at concentration of 10 mcg/ml , was found to be the ,more efficient inducer than ampicillin (10 mcg/ml) (Graph IV) whereas amoxicillin along with

Clavulunic acid (20mcg/ml) the enzyme production was found to be less (GraphV). Both pH (table no. IIai, IIaii) and time (in Hrs.) (table no. IIIai, IIIaii) are significantly affecting enzyme production when analysed using ANOVA, however, the effect of time was more influential, as its F value was significant at p=0.01.

Plate I: SDS PAGE profiling of the purified enzyme



Enzyme Purification:

Enzyme activity was determined during downstreaming process. For extracting the periplasmic enzyme of Y3 the most efficient cellular disintegration was obtained by sonication (EA= 2.4) than by the use of lysozyme (EA= 1.4, Table no. IIIa). This pooled enzyme was further purified using ammonium sulphate precipitation which was found to be 5.8 at 70% saturation of ammonium sulphate. The pooled ammonium sulphate activity was found to be 5.2 EU (1.5 fold purification). Further after dialysis (EA= 5.6) (2.2 fold purification), desalting and vacuum concentration steps the enzyme activity was found to be 5.8 EU (4 fold purification) (Table no IIIb).

The purified enzyme was analysed for its physicochemical stability. The enzyme was found to be active at pH 8 for 48 hrs (Graph VI) and at temperature 35^0C for 48 hrs. (Graph VII.). Molecular weight of isolated enzyme was determined by SDS PAGE and found to be in between 100 to 150 KD (Plate I).

DISCUSSION

Vrábelová had worked on the nosocomial plasmids responsible for multiresistance of bacterial isolates from inpatient cases. In his study nitrocefin was used for the detection of beta lactamases enzyme. All isolates has shown production of beta lactamases in present study whereas *E. coli* strain Y3, compared to other urinary pathogens, consistently produced high levels of beta-Lactamases activity.

Sawai et al. (1978) has worked on the iodometric Assay Method for Beta-Lactamases detection. In their study they have used various beta lactam (6APA and 7APA) antibiotics for enzyme Quantitation using both enzyme and alkali and have obtained thedependency of F value for hydrolysis of cephalothin depended upon the hydrolysis procedure and also concluded that the both processes led to formation of different products whereas enzymatic degredation of 7APA was done in our study.

Very less work has been done on beta lactamases in *E. coli* where as only the increased production under anaerobic conditions was studied by Rashtchian in 1979. The analysis for beta lactamases produced by *Bacillus fragilis* AM78 was analysed by Britz and Wilkinson in 1978. The specific activity was found to be 3,424 U/mg i.e. about 3,000-fold that of the crude cell-associated enzyme. In present investigation *E.coli* Y3 produces 4 fold rise in enzyme activity(5.8 U) after desalting and concentrating using vaccum. Yoshiaki Fujii-Kuriyama while working on *Proteus morganii* has observed a single polypeptide of molecular weight in between 38,000 to 40,000 D using SDS PAGE with isoelectric point as pH 7.2. The optimal pH was about 8.5 which was found to be 8 in case of our enzyme. Mayers and Shaw in 1989 has worked on New methods for the production of consistently high levels of metal-

dependent/3-1actamases (/3-1actamhydrolase, (EC 3.5.2.6) from strains $69/H/9 and $/B/6 of *Bacillus cereus* are described which have significant advantages over those reported previously.

CONCLUSION

Being a war against microorganisms, it is found to know more about their molecular and fiunctional organizations so that new methadologies for combating them can be made up. The more and more structural and functional illucidation which was done in this study was thus be able to mask the effect of enzymetic shild being offered by the ESBL producing bacteria. The enzyme kinetic studies carried out will be significant in future while formulating or modifying existing antibiotics used in UTI.

REFERENCES

[1] A. Abigail, W. Dixie; Bacterial Pathogenesis: A Molecular approach, Washington DC ASM Press, **2005**.
[2] A. Rashtchian, G. Nouravarsani, E. Miller, Gerlach, *Am Agents & chemother*., **1979**, 16, 772
[3] A.Sharma, M. Prakash, M. Veena. R. Eshwar Singh, K. Basavaraj, G. Vishwanath , Inte JABPT, 3 , 9.
[4] E. Abraham, E. Chain, *Nature*, **1940**, 146: 837.
[5] G. Rai, H. Upretin, S. Rai, K. Saha, R. Shrestha, *Nepal Med Coll J*., **2008**,10, 86.
[6] J. Distasio, A. Niedennan, *J Biol.. Chem.*, **1976**, 6929.
[7] J. Myers, R. Shaw, *Elsevier Biochimica et Biophysica Acta*., **1989**, 995, 264.
[8] J. Smith, M. Hamilton; Beta Lactamases, Academic press, **1979**.
[9] M. Britz, R. Wilkinson, *Am Agents and chemo.*, **1978**, 13, 373.
[10] M. Sinha, H. Shrinivasa, R. Macaden, **2007**, *IJMR* 126, 63.
[11] M. Vrábelová, K. Kollárova, D. Michálková-Papajová, J. Hanzen, P. Milošovič, M. kettner, *The Jl of App Res.*, **2004**, 4, 312.
[12] M. Vardi, T. Kochavi, Y. Denekamp, H. Bitterman, **2012**, *IMAJ*, 14.
[13] S. Dzidic, J. Suskovic, B. Kos, *Food Technolo. Biotechnolo*., **2008**, 46, 11.
[14] T. Sawai, I. Takahashi, S. Yamagashi, *Am agents & chemother*., **1978**, 13, 910.
[15] Williams, Wilkins; Bergey's manual of Determinative Bacteriology, Baltimore MD, **1994**.
[16] Y. Fujii-Kuriyama, Y. Yamamoto, S. Sugawara, *J of Bacteriolo*., **1977** 131, 726.
[17] Y. Mulay, Wadegaonkar, P. and Dharmadhikari S., *Bionanofrontier*, **2011**, 4, 43.