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PURIFICATION &CHARACTRERIZATION OF BENZONITRILE PRODUCED BY *BACILLUS PUMILLUS S7*

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ABSTRACT

Dihalogenate benzonitrile are active compounds in a number of herbicides and poses to have a deleterious health effect. The biocatalyst was applied to the biotransformation of benzonitrile,3-cynopyridine,(R,S)-3-hydroxy -2- ethylene butanenitrile The enzyme involved in the degradation of benzonitrile by bacteria identified as *Bacillus pumilus* S7a strain which utilized benzonitrile as sole source of carbon and nitrogen were partially purified by ammonium sulphate precipitation of (80%). The GC results revealed that benzonitrile in single step pathway was converted to ammonia with the formation of benzoic acid as an intermediate by enzyme benzonitrilase.In the present study nitrilase enzyme was produced and purified using *Bacillus pumilusS7*. The purified enzyme was showing maximum activity at a pH 6(35.32 µmole/min) and maximum benzonitrile degradation with free enzyme was observed at temperature 30^0c (29.27µmole/min).The metal source $FeSo_4$ (40.55 µmole/min) and enzyme stable up to 30 min (45.14 µmole/min) The Vmax is 0.188µg/ml and km is 0.00178µm.The molecular weight of benzonitrilase were found to be 97400 Dalton

Keywords: Biodegradation, Benzonitrilase, Bacteria, Bacillus, Purification.

INTRODUCTION

Nitrilase, which are generally highly toxic due their cyno functional group, can be used by some microorganisms as carbon and nitrogen sources. Nitrilase catalyzes the direct cleavages nitrilase to the corresponding acid and ammonia.(kobayshi 1994 ref 2),whereas nit rile hydratase catalyse the hydration of nitriles to amides(Asano1980,Kobayshi 1992).Both the enzymes are involved in biosynthesis of the plant harmone indol 3 –acetic acid in plants(Barteling 1992,Barteling 1994,Bartel 1994).Nitrilase are widely manufactured and extensively used by the chemical industry, and nitrile herbicides are also widely applied in agriculture.

Thiemann and Mahadevan demonstrated that nitrilase (EC 3,5,5,1) purified the hydrolysis of indolacetonitrile to indolacetic acid and ammonia ,several nitriles found and characterized.(Asano 1982) reported that the formation of nitrile hydretase and amides, and purification and enzymological properties of the former enzyme from Artrobacter Sp.J-1.Only two fungal nitrilase –from Fusarium solani and F.Oxysporum were purified and characterized (Gold lust and Bohak 1989).We improved the production of nitrilase in several species of filamentous fungi by using picolinitrile(Kaplan et al 2006)..(Layh *et al*.1992) isolated several bacterial strains with nitrilase activities from the environment. They were isolated from enrichment cultures using different arylacetonitriles such as 2-methyl- or 2-ethylbenzylcyanide as sole sources of nitrogen. One of these strains, *Pseudomonas fluorescens* EBC191, was able to use different arylacetonitriles (e.g. 2-phenylpropionitrile) as nitrogen sources and converted the nitriles to the corresponding -substituted carboxylic acids. It was demonstrated that strain EBC191 synthesized a nitrilase, which converted *O*-acetoxymandelonitrile preferentially to *R*-acetoxymandelic acid (Layh *et al*., 1992). The enzyme was subsequently partially purified, biochemically characterized, and the N-terminal and some internal amino acid sequences were determined (Moser, 1996; Layh *et al*., 1998). This enzyme seems to possess some potential for the enantioselective production of carboxylic acids from racemic nitriles. In the present work, the nitrilase gene was identified in a genomic library of *P. fluorescens* EBC191, expressed in *Escherichia coli* and the recombinant protein biochemically characterized.

The enzymes involved are different from the nitrile –degrading nitrile hydretase and nitrilase,and organic nitriles cannot be degrade by these becteria.Benzonitrile was chosen as a substract,because it is the simplest organic nitrile ,widely used as solvent and an imported environmental polluntant.The results indicates the presence of a specialized group of previously unknown haloalkophilic bacteria capable of growing with acetonitrile as sole substract (Dimitry 2007)

MATERIALS AND METHODS

Elective Enrichment and Isolation

1 gm of soil sample was suspended in Basal salt medium containing (KH_2PO_4 1.5 gm; K_2HPO_4 3.5 gm; $Mgso_4.7H_2O$ 0.19 gm; Yeast extract 50 mg; Trace element ; P^H 7.5; Distilled water 1000 ml.) Benzonitrile 0.05% was added aseptically to sterilized and cooled medium. The suspension (100ml) in 250 ml Erlenmeyer flask was incubated at 30^0 C on rotary shaker. After 7 days 2ml of this culture was transferred to 100ml of fresh medium with little rise in benzonitrile concentration. The process was repeated for a total four transfers by step by step raising the concentration of benzonitrile (0.05 to 0.2%). After one month of acclimatization, the last enrichment culture flask was used to isolate microorganisms on basal salt agar containing 0.2% benzonitrile. The colony characterization and gram staining of bacterial cultures were carried out. The pure cultures were maintained on basal salt agar for further studies.

Optimization of cultural condition

All isolates were capable of growing on mineral medium containing benzonitrile as sole source of carbon and nitrogen. Out of these 60 isolates 17 bacterial strains were screened based on maximum production of ammonia. Secondary screening was carried out based on benzonitrile biodegradation at various pH. Three strains were selected showing maximum biodegradation in terms of ammonia production at basic, acidic and alkaline pH. In These strains the enzyme activity was found to be maximum in cell supernatant as compair to cell lyzate.The strain S15 was used further for optimization of growth parameters. The intact cells of S15 was showing maximum benzonitrile biodegradation at pH 4 and temperature 30^0c incubation for 72 hrs.The presence of casein as a nitrogen source and fructose as carbon source were found to enhance the benzonitrile hydrolysis

Development of inoculam

Basal salt broth containing 0.2% benzonitrile was prepared and it is inoculated with selected bacterial strain S 15 This flask was incubated on rotary shaker at 100 rpm for 72 hrs. After 72 hrs cells were harvested by centrifuging the culture flask at 10,000 rpm for 10 min. washing of cell pallet was carried out using saline. These intact cells were suspended in saline and used further to study growth parameters.

Enzyme production

The crude enzyme was submerged fermentation .The Basal salt medium was prepared (pH 7) inoculated the culture of bacterial and incubated at 30^0c for 72 hrs. After incubation, centrifugation the sample at 10000 rpm for 10 min.The cell free supernatant was taken the phosphate buffer and sonicated .Crude enzyme was prepared

Enzyme Assay

The benzonitrilase assay was performed using both cell supernatant as well as cell free extract The standard reaction mixture consisted of 50, umol of potassium phosphate buffer (pH 8.0), 3 ,umol of benzonitrile, and an appropriate amount of enzyme in a total volume of 0.5 ml. The reaction was started by adding the substrate and was carried out at 30°Cfor various times, the activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Enzyme Unit: One unit of Benzonitrilase was defined as the amount of enzyme which catalyzed the formation of 1 micromole of ammonia per min.

GC analysis method

The isolated strains were cultured aerobically at 28^0c for the 3 days on the isolation medium. The cells were centrifuged, washed with physiological saline and suspended in 0.1M potassium phosphate buffer, pH 7.0.The reaction mixture for the screening of benzonitrile producing strains contained 100 µmole of potassium phosphate buffer, 300 µmole of benzonitrile as substract, washed cells from 3 ml of culture broth in a total volume of 1.0 ml. The reaction was carried out at 30^0c for 1 hr. with moderate shaking and terminated by addition of 0.2 ml of 1 N HCL.

The mixture was determined with a Chemito Gas chromatograph, Model GC -7610 equipped with flame ionized detector. The column used was stainless steel silicon 30, packed with porapack Q (80 to 100 mesh) operational conditions were: column temperature,200^0c; injection and detector temperature 151^0c and 201^0c.The carrier gas was N_2 at 40 cm^3/min.

Purification of enzyme from bacteria

Step 1 –Ammonium sulphate precipitation

Ammonium sulphate was added to 90% saturation. The crude enzyme prepared was brought to 80% saturation with ammonium sulphate at pH of 6 and kept overnight in cold room. After equilibration, the supernatant was brought to 90% saturation with ammonium sulphate and centrifuged at 8000rpm, at 4^0c for 10 min.Then precipitates were collected separately and dissolved in a 0.05 M phosphate buffer at pH 7 stored at 4^0c for the purification.

Step 2- Dialysis

The precipitation dissolved in 0.05M potassium phosphate buffer and dialysis .after, dialysis the samples were used for protein estimation and enzyme activity

SDS page analysis

Molecular weight and puriety determinations on the nitrilase were performed by electrophoresis on polyacrylamide gel in the presence of SDS by thin layer techanique that enabled up to 1samples to be analysed simultaneously on the same gel slab .This methods used was based on that described by weber et al (1972) for disc gel electrophorasis .Thin layer gels of 0.2%(w/v) SDS dissolved in 100mM-sodium phosphate buffer,pH7.2 were suitable,and were polymerized by using ammonium persulphate as catalyst and NNN'N-tetramethylenendiamine s accelerator in the usal manner.Sample of standards proteins and of nutrias were prepared for application to the gel in 10mM-sodium potassium buffer,pH 7.0containing 1%(w/v)SDS and 1%(v/v) mercaptoethanol at 100^0c.pieces(1.5mm×6mm) of whatmann 3MMchromatography paper were soaked in the sample solution dried to remove superficial moisture and placed vertically in slites along the length of the gel(25cm×12.5cm) on the side adjacent to the cathode.The slite was filled to the surface of the gelwith 10mM-sodium phosphate buffer pH7.2containing 0.1% micropipette .Bromophenol blue was used as the traking dye.The gels were subjectedto transevere electrophorasis on the instrument ,the reserioir buffer,pH 7.2 containing 0.1%(w/v) SDS and 0.1% micro-pipette.Bromophenol blue was used as the traking dye.The gels were subjected to transverse electrophorasis,The reservoir buffer in the cathode compartments beingb 50mM-sodium phosphate buffer,pH 7.2,contaning0.1%(w/v) SDS with voltage of 80-100V and a current of 40-50mA at a tempraturebof 17.5^0c,a running time of about 16 hours was required to complete electrophorasis,ie. For the traking dye approach the anodic side of the gel.The position of the dye was then marked with water proof black ink and the gel stained with coomassie brillent blue.The distance migrated by the stained protein bands and the dye was measured and the relative mobilities of the sample proteins with respect to the traking dye were calculated From a plot of the weights of the polypeptide chains of standered proteins against their electophoretic mobility the molecular weight of the subunits of the isolated nutrias enzyme was determined from their relative mobility.in addition to certain of the standered proteins used in proteins used in gel filtration experiment, the following proteins were also used for callibration

Characterization of purified nitrilase parameter

The partially purified enzyme was used for the characterization of nitrilase and optimization of its activity

Optimization of pH

The pH activity profile of the partially purified enzyme (pH range 3 to 10) was studied citrate buffer (pH range 3.0-4.0), Citrate phosphate buffer (pH range 5.0-6.0), Phosphate buffer (pH range 7.0-8.0), Glacial sodium hydroxide (pH range 9.0-10.0). The standard reaction mixture consisted of 50, umol of potassium phosphate buffer (pH 8.0), 3 ,umol of benzonitrile, and an appropriate amount of enzyme in a total volume of 0.5 ml. The reaction was started by adding the substrate and was carried out at 30°Cfor 20 times, the activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Optimization of Temperature

The activity of enzyme was measured at different temperature ranging from10^0c to 80^0c . The standard reaction mixture consisted of 50, umol of potassium phosphate buffer (pH 8.0), 3 ,umol of benzonitrile, and an appropriate amount of enzyme in a total volume of 0.5 ml. The reaction was started by adding the substrate and was carried out at various temperature for 20mintues, the activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Optimization of Metal ion concentration

The standard reaction mixture consisted of 50, umol of potassium phosphate buffer (pH 8.0), 3, umol of benzonitrile, and an appropriate amount of enzyme in a total volume of 0.5 ml as supplementary as metal source like Cr^{2+},$Mg^{2+}Fe^{2+}$,Cu^{2+}. , Mn^{2+},Zn^{2+},Co^{2+},Cu^{2+}. The reaction was started by adding the substrate and was carried out at various temperatures for 20mintues; the activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Optimization of enzyme stability

The benzonitrilase assay was performed using both cell supernatant as well as cell free extract The standard reaction mixture consisted of 50, umol of potassium phosphate buffer (pH 8.0), 3 ,umol of benzonitrile, and an appropriate amount of enzyme in a total volume of 0.5 ml. The reaction was started by adding the substrate and was carried out at 30°Cfor various times; like 10 min, 20 min….. The activity was estimated in terms of ammonia production. Protein was determined by the method of Lowry.

Results and Discussion

By elective enrichment 60 different bacterial cultures were isolated from soil capable of utilizing benzonitrile as a sole source of carbon and nitrogen.(Heper 1977) show that cultures actively growing on benzonitrile as carbon and nitrogen source were tested for their for their ability to oxidized possible intermediate in the degradation of benzonitrile. The production of ammonia was estimated from culture filtrate of all sixty isolates. At primary level 17 isolates were screened out on the basis of maximum production of ammonia as one of the metabolite of degradation.). To study the impact of pH on benzonitrile degradation all 17 isolates were grown at three distinct pH. The results has shown that the strain C8 , S14 and S15 were degrading benzonitrile at high rate in respective pH viz. 7, 9 and 4 (Table 1). Harper (1977) has shown that in Arthobacter sp.J-1 benzonitrile was directly hydrolyzed to benzoic acid and ammonia by nitrilase. In These strains the enzyme activity was found to be maximum in cell supernatant as compair to cell lyzate.The strain S15 was used further for optimization of growth parameters. The intact cells of S15 was showing maximum benzonitrile biodegradation at pH 4 and temperature 30^0c incubation for 72 hrs.

In course of time benzonitrile was degreaded by accumulating benzoic acid and ammonia but benzamide was not detected throughout the cultivation of all three selected strains. Tentatively all three strains are showing the direct degredation of benzonitrile to benzoic acid and ammonia by enzyme nitrilase which was confirmed by Gas chromatography. (Asano 1982) reported that a new enzyme aliphatic nit rile hydretase catalysed the hydration of nitriles to amides in Arthrobacter sp.J -1. The expression of nitrilase was studied by observing enzyme activity in cell supernatant as well as in cell lysates. all these three strains the enzyme activity was more in cell supernatant as compare to cell lysates. Further the strain S15 was showing highest activity which was selected for study on purification and characterization of enzyme.

The strain S15 has shown maximum partially Purified enzyme at 80% by ammonium sulphate method .(Dias 2000) reported that different encapsulated matrics were tested for purified cells of *candida guilliermondii* UFMG- Y65 used for acetonitrile degradation.Acetonitrile degradation by free cells and cells immobilized in Ba-alginate,k-carrageenan. The partially purified enzyme was used for the characterization of nitrilase and optimization of its activity.The enzyme activity found at pH 7of phosphate buffer (35.65µmole/min) . Similarly the enzyme activity was maximum at mesophilic temperature 30 ^{o}c (29.27µmole/min). The enzyme is found to be stable up 30 min(45.14 µmole/min).The metal source $FeSo_4$ (40.55 µmole/min) and The Vmax is 0.188µg/ml and km is 0.00178µm

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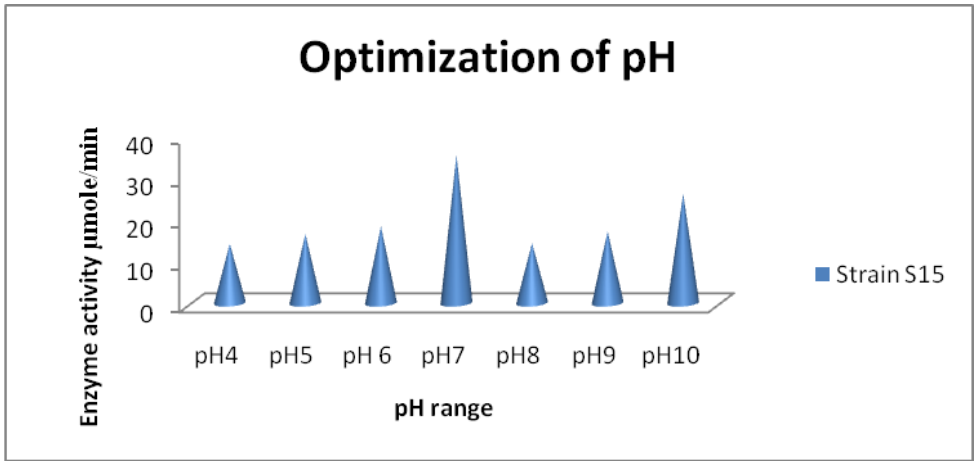
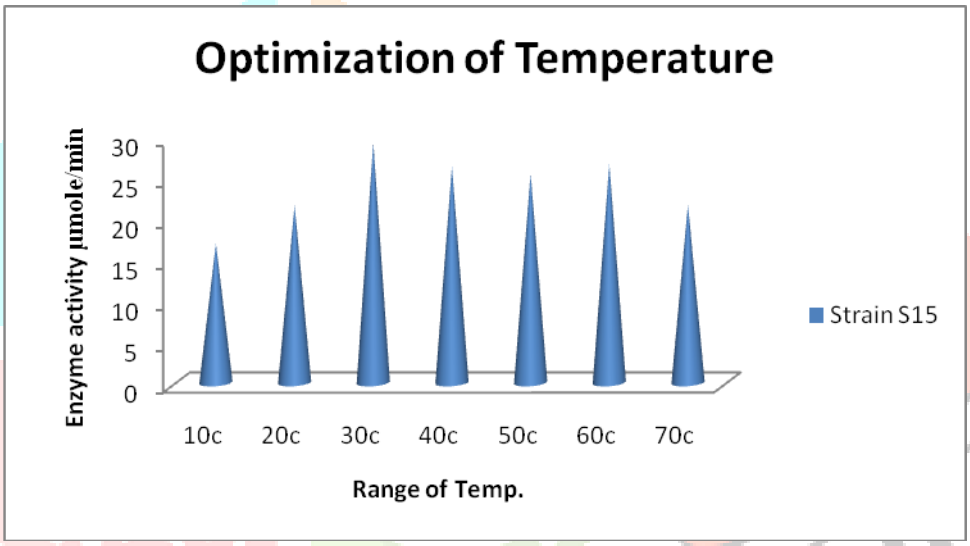
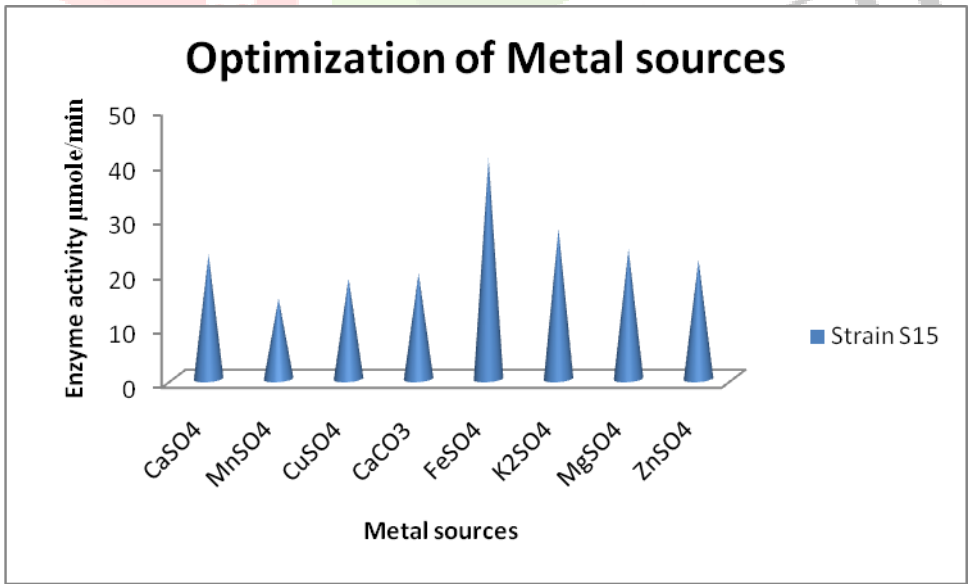
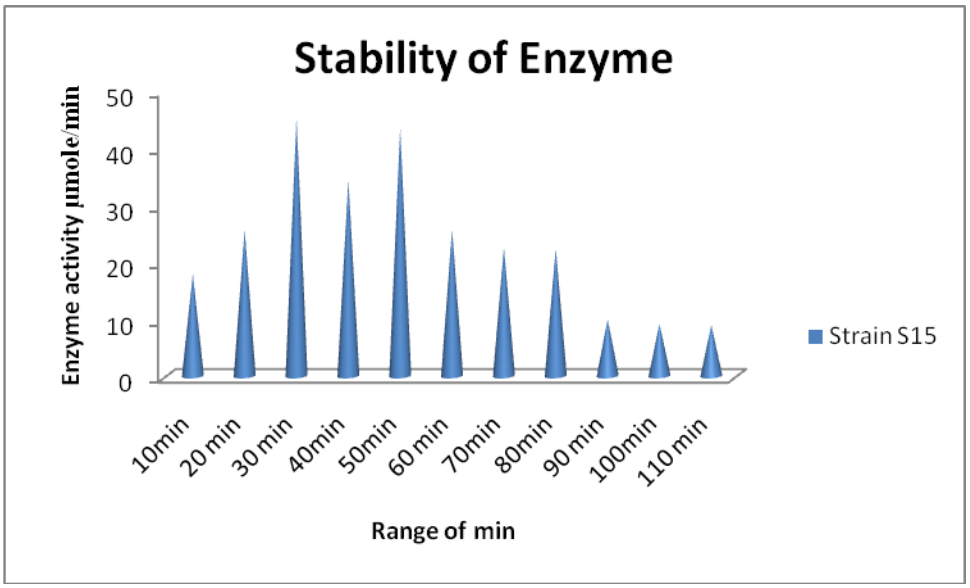
Characterization of purified nitrilase parameter

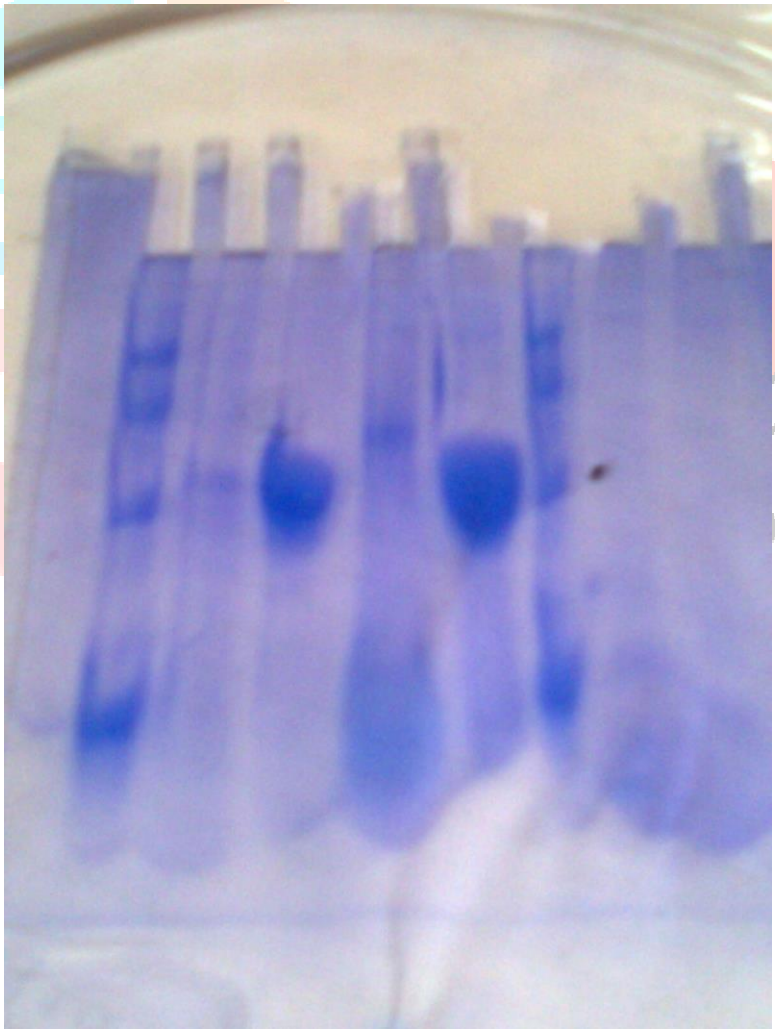








Determination of Vmax and km



Gas chromatography

Tue, 29th Jun, 2010 22:11:03 S15 Page 1

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ISTD Amount : 0
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Analyst : p
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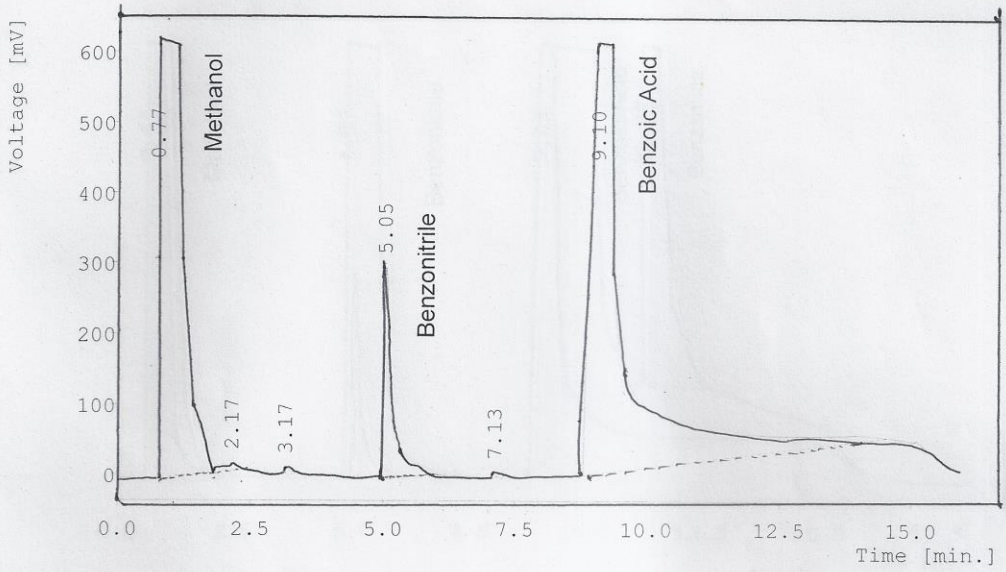
Style : report



Result Table - Calculation Method Uncal

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1	0.767	20202.5151	622.6774	0.4900	37.4728	39.9809
2	2.167	194.8162	10.3093	0.1533	0.3614	0.6619
3	3.173	80.3208	8.3291	0.1333	0.1490	0.5348
4	5.050	3315.0363	296.6329	0.1333	6.1489	19.0462
5	7.133	56.9882	5.9107	0.1733	0.1057	0.3795
6	9.097	30062.7477	613.5793	0.4867	55.7622	39.3967
-	Total	53912.4244	1557.4387			