ANNUAL PROGRESS REPORT

BIOSENSOR FOR URIC ACID ESTIMATION

(TILL 5TH MAY, 2010)

ANNUAL REPORT (FROM 6TH MAY 2009 TO 5TH MAY 2010)

PROJECT TITLE: **BIOSENSOR FOR URIC ACID ESTIMATION**

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DATE OF START: 6TH MAY 2009

DURATION: 3 YEARS

OBJECTIVES OF THE PROPOSAL:

- Exploration of sensor material for electrochemical sensing and design of electrodes.
- Immobilization of pure enzymes and analysis of uric acid.
- Collection of suitable microbes.
- Studies on characterization of microbes and their gene sequencing.
- Extraction of enzymes from microbes.
- Immobilization of microbes /extracted enzymes on electrodes.
- Development of microbial and enzyme sensor for Uric acid.

SUMMARY ON PROGRESS INCLUDING FINDINGS IF ANY (DURING THE PERIOD OF REPORT):

Thus the progress during the first six months involves:

a. Purchase of chemicals and enzymes: We have purchased Uricase enzyme, *Candida* sp, recombinant Expressed in E.coli (SIGMA; code: U0880-250UN) and other chemicals useful for uric acid estimation.

b. Appointment of JRF: Ms. Tanushree Ghosh has been appointed as research fellow.

c. Research:

Isolation of Uric Acid consuming Bacteria:

A soil sample was collected in bird-litter contaminated area. Isolation of bacteria was accomplished. Six different bacteria were successfully isolated using uric acid as the only nitrogen source in the growth medium. One of these six microbes was studied to see the rate of uric acid consumption.

Figure 1-Six isolates on Nutrient Agar plates, after three days of incubation at 37°C. Clear zones of different diameters were found around the bacterial colonies. Longest zone diameter given by the bacterium BT-UA was characterized first for uricase production.

Characterization of Bacteria:

The bacterium (named **BT_UA**) was found Gram negative, bacilli. The bacterium was partially characterized. Genome sequencing of the bacteria was done. 16s r DNA sequencing based identification of bacterial sample(BT UA) was found to be Comamonas sp. with 99% identity.

Table 1– Biochemical characterization of the BT-UA

Biochemical Tests	Strain BT-UA
Glucose Utilization	+
Sucrose Utilization	+
Fructose Utilization	+
Maltose Utilization	-
Rhamnose Utilization	-
Glycerol Utilization	-
Starch Utilization	-
Citrate Utilization	+
Cysteine	+
Glycine	+
Phenylalanine	+
Aspartic acid	+
Glutamic acid	+

Table 2– Antibiotic sensitivity of the BT-UA

Tests	Strain BT-UA
Streptomycin (25µg)	Sensitive
Cholramphenicol (50µg)	Sensitive
Neomycin (30µg)	Sensitive
Kanamycin (30µg)	Sensitive
Azithromycin (30µg)	Sensitive
Vancomycin (30µg)	Resistant
Novobiocin (30µg)	Sensitive
Ampicillin (25µg)	Resistant
Gentamicin (30µg)	Sensitive
Tetracycline (30µg)	Sensitive

Rifampicin (30µg)	Sensitive

Induction of enzyme production:

Expression of uricase was accomplished by induction method. Growth on Nutrient Agar-Uric acid plates were observed for uric acid utilization as nitrogen source. Bacteria were supposed to utilize uric acid by converting the water insoluble uric acid to a soluble product and clear zones were observed around the bacterial colonies.

The Bacteria was allowed to grow in enrichment media (0.8% uric acid) at 37°C for over night. Then it was inoculated in Basal Trace medium (0.3% uric acid) and allowed to grow at 37°C at 120 rpm in the orbital shaker.

Monitoring of Biomass & Enzyme production during growth period:

Both the biomass and enzyme production were monitored at regular intervals using spectrophotometer. Consumption of uric acid during growth also was determined by measuring uric acid concentration in culture medium.

Simple colorimetric method was employed to 3.0ml of culture. 1.0ml of 0.6 N NaOH was added and mixed well. The mixture was centrifuged at 5,000 rpm for 5 min. The supernatant was mixed thoroughly with 1.0ml Phosphotungstic acid and allowed to stand for 15 min at room temperature. Absorbance was recorded at 720nm.

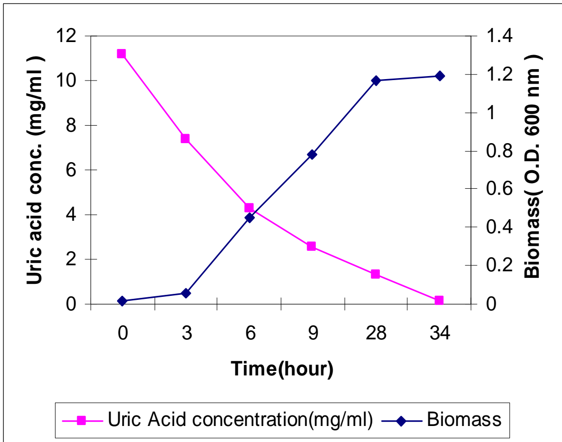

Figure 2- Time course of cell growth and Uric acid consumption by the strain

BT-UA in shake flask culture

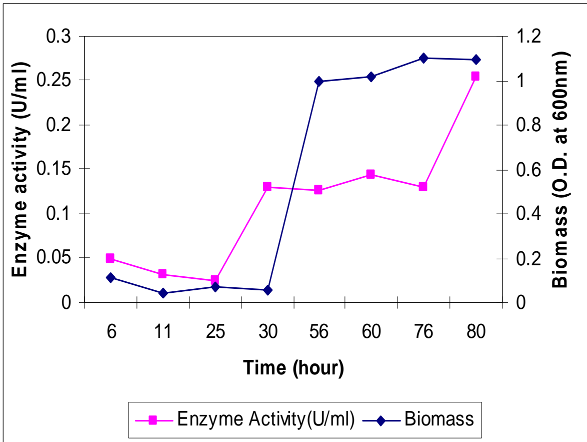

Figure 3-Time course of cell growth and extracellular uricase production of the strain

BT-UA in shake flask culture.

Enzyme extraction and assay:

At every interval 2ml of culture was taken out and centrifuged at 10,000rpm for 10 min. The supernatant was collected and used to assay for enzyme activity. We used the Urate oxidase-peroxidase method for enzyme assay.150µl of 4- Aminoantipyrine (30mM) was added to 600µl of 0.1M Sodium borate buffer (pH 8.5) containing 2mM Uric acid, mixed well and 100µl of 1.5% Phenol was added to this. Then 50µl of 15U/ml HRP and 100µl of crude enzyme was added and incubated for 30min at 37°C.Optical Density was measured at 540nm.Enzyme activity then determined in U/ml and U/mg units. **1unit of enzyme = The amount of enzyme that produces 1.0 µ moles of H_2O_2 per min under the standard assay condition.**

The Km value of the free enzyme (cell free extract) was 3.24mM and Vmax value was 0.0339mM/min.

Table 3-Values obtained from Line weaver-Burke Plot

Km	3.244583 mM
Vmax	0.033922 mM min^{-1}

Table 4-Crude Enzyme Activity

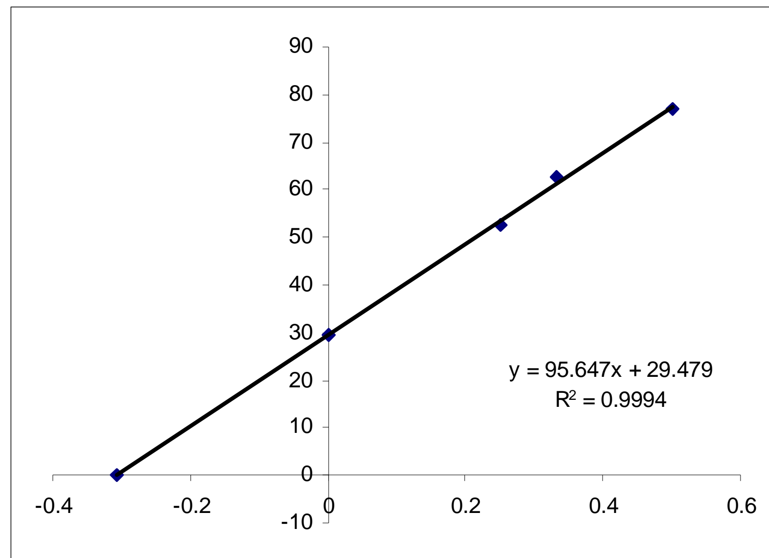

Figure 4- Line weaver-Burke Plot of crude enzyme extract from the strain BT-UA

Factors affecting uricase production:

Carbon sources

The Basal-Trace medium was supplemented by 0.35% of each dextrose, fructose, sucrose, starch, glycerol and cellulose. Fig-5 show that the highest uricase activity, we observed in the medium containing fructose. However the control (without carbon source) shows higher uricase activity than fructose supplement. Carbon source may not be essential for uricase production.

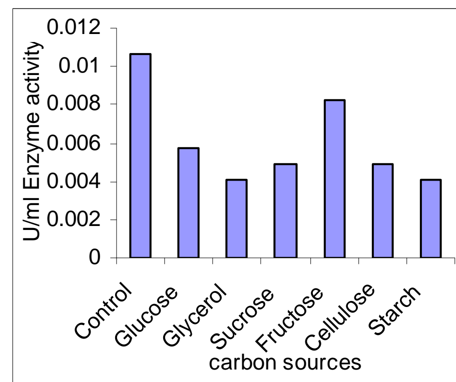

Fig-5: Effect of different carbon sources on uricase enzyme production

Nitrogen sources

Uric acid was omitted from basal-trace medium and supplemented by 0.3% of $NaNO_3$, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, peptone and tryptone as a sole source of nitrogen. In presence of nitrogen sources other than uric acid, uricase production was reduced. Peptone and tryptone showed similar effect and nearly half uricase production as in uric acid supplemented medium. Whereas -NH_4^{+1} showed inhibitory effect on uricase production(Fig-6).

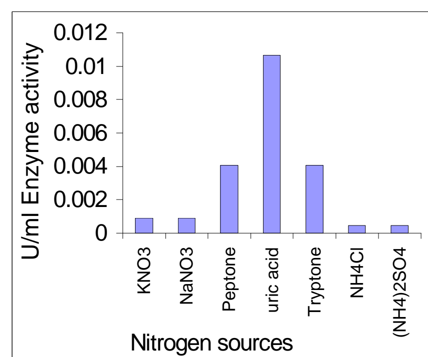

Fig-6: Effect of different nitrogen sources on uricase enzyme production

Phosphate sources

Different phosphate sources such as K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, K_3PO_4 have been added to the fermentation medium at a concentration equimolar to the concentration of the basal phosphate source (K_2HPO_4 + KH_2PO_4). Results show that the control (combination of 0.2% K_2HPO_4 and 0.05% KH_2PO_4) produced highest uricase enzyme. Whereas, for the individual phosphate sources, K_3PO_4 showed 1/3rd of activity in comparison to control (Fig-7)

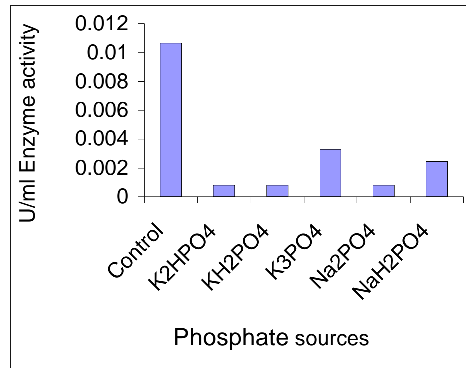

Fig-7: Effect of different nitrogen sources on uricase enzyme production

Amino Acids

The Basal-Trace medium was supplemented by 0.05% of each cystein, glycine, glutamic acid, aspartic acid and phenylalanine. Among the five different amino acid supplements, only phenylalanine (Fig-8) showed positive effect on uricase production and higher uricase activity than in control (without any amino acids).

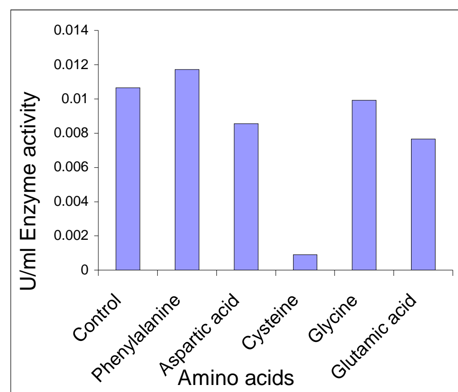

Fig-8: Effect of different amino acids on uricase enzyme production

Elements

Different elements such as Ca^{2+}($CaCl_2$), Co^{2+}($CoCl_2$), Mn^{2+}($MnCl_2$), Mg^{2+}($MgSO_4$), Cu^{2+}($CuSO_4$), Fe^{2+}($FeSO_4$), Fe^{3+}($FeCl_3$), Zn^{2+}($ZnCl_2$) were added to the fermentation medium at a concentration equimolar to the concentration of the trace elements of basal-trace medium.

Individual supplement of different elements showed negative effect on uricase production. Whereas in control medium, the combination of Ca^{2+}($CaCl_2$), Mg^{2+}($MgSO_4$), Fe^{2+}($FeSO_4$) and Cu^{2+}($CuSO_4$) was found to be highly productive for uricase (Fig-9)

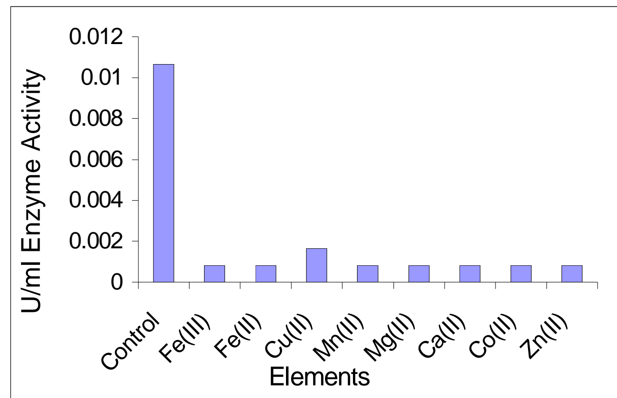

Fig-9: Effect of different elements on uricase enzyme production

Vitamins

The Basal-Trace medium was supplemented by 0.02% of each ascorbic acid, folic acid, nicotinic acid, riboflavin and yeast extract. Here, Yeast extract was used as the standard source of vitamin. Ascorbic acid having structural similarity with uric acid, showed profound effect on uricase production. The Fig-10 shows highest uricase activity in the medium containing ascorbic acid than control (with uric acid, without any vitamin sources).

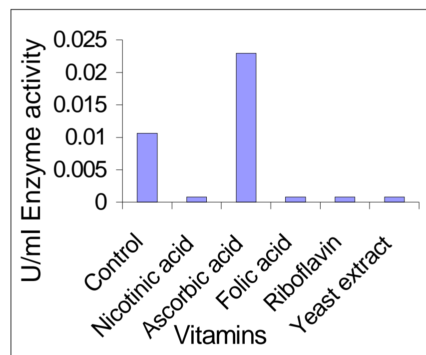

Fig-10: Effect of different vitamins on uricase enzyme production

Initial pH of the medium

To determine the optimum pH for uricase production, the bacteria was cultured in the pH media, the initial pH was adjusted to 5.2, 6.2, 7.0, 7.5, 8.0, 8.5 and 9.0. It was found that the initial pH of the medium affected the uricase activity profoundly. At pH 7.0, the uricase production increased up to 84 hours and suddenly dropped at 96 hours as the bacteria died (Fig-11).For other pH mediums, uricase activity increased up to 72 hours and dropped at 84 hours. Through out the course of growth, the activity was high at pH 7.0. Thus the pH 7.0 was found to be optimum pH for the strain BT UA.

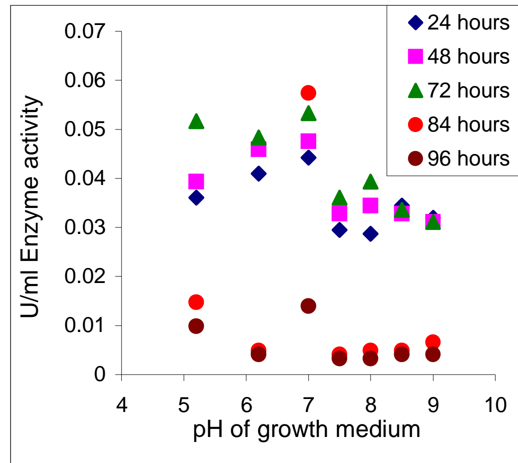

Fig-11: Effect of initial pH of the growth medium on uricase enzyme production

Development of Biosensor:

Amperometric experiments were performed with an autolab (Ecochemie B.V. The Netherlands) electrochemical analyzer (PGSTAT 12). The terminals of the working (WE), reference (RE) and counter (CE) electrodes of the Autolab analyzer were connected to the respective terminals of the disposable screen printed electrode via standard connectors and experiments were driven were connected to the autolab analyzer via the GPES software installed on a computer interfaced with the Autolab analyzer.

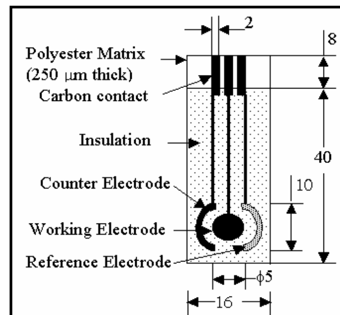

Fig-12: Schematic diagram of the transducer (screen printed electrode).

Reagents:

- Sodium Borate buffer-0.1M, pH 8.5
- 80 mM KCl solution
- Uric acid (HIMEDIA)

Immobilization of enzyme:

The lyophilized uricase (crude enzyme) was immobilized on the working electrode by glutaraldehyde cross-linking to Gelatin. The electrodes were then kept in refrigerator at 4°C until further use.

Biosensor preparation:

Uricase catalyses the oxidation of uric acid in presence of oxygen, producing allantion and hydrogen peroxide. Formation of hydrogen peroxide was detected by the amperometric method.

Working potential:

The biosensor potential was set at 0.4 V as found in the cyclic voltammogram analyzer.

Amperometric response and calibration curve:

Uric acid solution was prepared in 0.1M Sodium borate buffer (pH 8.5). The transducers were connected with respective terminals of the Autolab electrochemical analyzer (PG STAT 12) and different concentrations of uric acid were used to determine response current in 200 µl of reaction mixture (80mM KCl solution). Calibration curves were prepared for higher and lower ranges of uric acid (mg/dl).

Biosensor for uric acid Estimation:

The relationship between the response current and uric acid concentration at 0.4V has been shown. The response current was increased with increasing concentration of uric acid. The linearity of the curve was highly satisfactory and ranging between 0-6 mg/dl. The pH optimum for maximum response was found to be 8.5 at 2mM Uric acid in 0.1 M Sodium borate buffer at 25°C (Fig-12).Calibration curves at concentrations of serum uric acid range0-60 µg/ml (Fig-13) was constructed .The limit of detection of the biosensor was found to be 12µg/ml of uric acid.

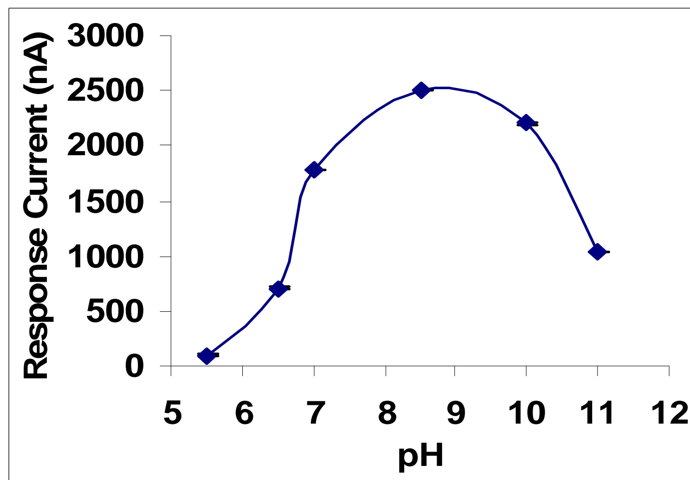

Fig-12: Effect of pH on response current at 2mM Uric acid in 0.1 M Sodium borate buffer at 25°C

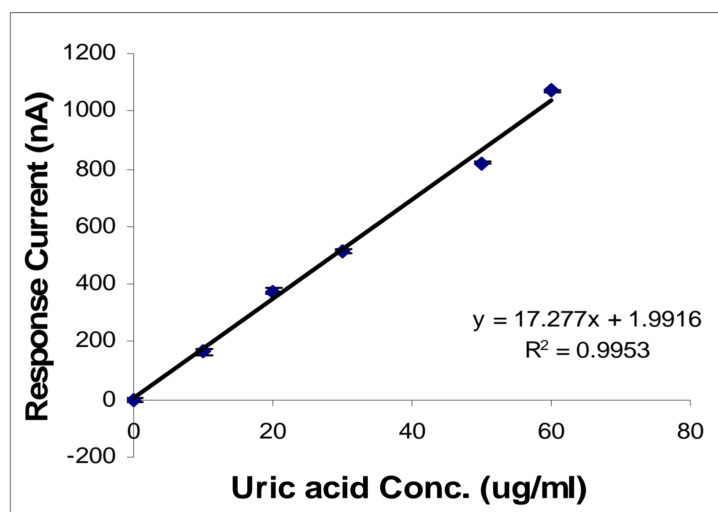

Fig-13: Calibration curve of uric acid ranges from 0 to 6.0 µg/ml

Since the electrode responses were kinetic, the apparent Michaelis-Menten constant (K'_m) was calculated for the immobilized crude enzyme by amperometric method. The plot of 1/current against 1/[S] was obtained (Fig-15). The maximum current response i_{max} and apparent Michaelis-menten constant (K'_m) were calculated from the intercept and slope. The maximum current response i_{max} was 1666 nA, with K'_m = 42.99 µg/ml. The apparent Michaelis-menten constant (K'_m) sometimes reported as sensor affinity.

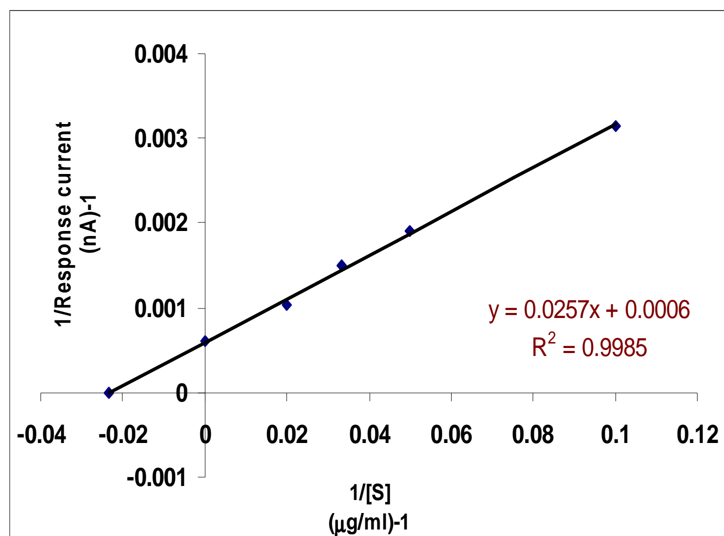

Fig-15: Effect of uric acid concentration upon the amperometric response of immobilized crude enzyme(Line Weaver-Burke Plot)

RESEARCH WORK WHICH REMAINS TO BE DONE UNDER THIS PROJECT:

a. Pure enzyme sensor for uric acid.
b. Microbial sensor for uric acid.
c. Purification of enzyme from selected microbes.
d. Sample analysis.

ANY PUBLICATION:

1. **Amperometric Biosensor For Uric Acid Estimation Using Microbial Enzyme Source,** T Ghosh, P Sarkar, National Seminar On Frontiers In Nanomaterials and Biosensors (NSFNMB'10), Karaikudi, 4-5 March, 2010**(AWARDED BEST PAPER).**
2. **Optimization of Uricase Production from Microbial Source and Development of Amperometric Biosensor for Uric acid,** T Ghosh, P Sarkar, National Seminar On recent Trends In Biotechnology, Department of Biotechnology, The University of Burdwan, Burdwan, 11-12 March, 2010.
3. **Crude enzyme based amperometric biosensor for uric acid-** under preparation.