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# BIOPROCESS OPTIMIZATION AND CHARACTERIZATION OF RECOMBINANT URATE OXIDASE EXPRESSED IN *Escherichia coli*

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## ABSTRACT

Tumor Lysis Syndrome and Gout are the conditions in which uric acid levels in the serum will be increased up to 526 mg/dl but in a healthy human being the uric acid concentration is less than 15mg/dl. In such conditions urate oxidase can be administered parentrally which can solublise the uric acid in to allontoin which has 3 to 5 fold more solubility than uric acid and it can be excreted through urine and maintain the uric acid concentration at normal level. In this article urate oxidase gene was isolated *Aspergillus flavus* and it was cloned in to *E.coli* (BL-21). Urate oxidase gene was expressed in the cytoplasm as soluble and biologically active form. Bioprocess was optimized for 20lts fermenter scale with modified LB medium and purified up to >96% purity using fractionated ammonium sulphate precipitation, diafiltration, anion exchange chromatography, cation exchange chromatography and gel filtration chromatography. The final yield of purified recombinant urate oxidase from the 20lts fermenter was approximately 5 to 6gm of 96% pure and biologically active enzyme.

**KEYWORDS:** **Fermentation, Anion Exchange Chromatography, Cation Exchange Chromatography, Gel Filtration Chromatography and Western Blotting.**

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# INTRODUCTION

Urate oxidase is a peroxisomal enzyme that catalyzes the oxidation of uric acid to allantoin in most of the fish and amphibian. Mechanism of enzyme action involves oxidative degradation of the purine ring during the denovo pathway of purine metabolism[1]. In humans and certain other primates urate oxidase enzyme expression was lost or reduced during the evolution[2]. Urate oxidase is a therapeutic protein used to treat tumor lysis syndrome associated with gout and chemotherapy of most of cancers where uric acid levels will be increased in serum and deposited in the form of crystals in kidney resulted as kidney failure[3]. In such conditions parentral administration of urate oxidase catalyses the enzymatic oxidative degradation of uric acid into a readily excretable metabolite, allantoin can maintain the serum uric acid levels in the normal level. Patients with hematological malignancies, hyperuricemia and related complications especially after chemotherapy initiation are at high risk for tumor lysis syndrome[3]. Urate oxidase is a tetrameric and non-glcosyalated protein with 4 identical subunits of molecular mass 3427daltons, molecular formula is $C_{1523}H_{2383}N_{417}O_{462}S_{7}$. Each monomer contains two instances of this domain. It is functionally active in the form of tetramer without any inter chain and intra chain disulphide bonds[11]. Urate oxidase was naturally expressed in *Aspergillus flavus* but in very minor concentration. In this article bioprocess was optimized to increase the rate of its expression by cloning the gene codes for urate oxidase from *Aspergillus flavus* in to *E.coli* and characterization of the same was done.

# MATERIALS AND METHODS

Iso propial thio galactoside, Dextrose, Peptone, Sodium Hydroxide, Ammonia, Yeast Extract, Sodium Chloride, Potassium Di Hydrogen Phosphate, Ammonium Chloride, Di Potassium Hydrogen Phosphate, Magnesium Sulphate, Silicon Antifoaming, Ampicilin, DEAE-Sepharose fast flow, SP-Sepharose Fast flow SuperdexG-75, Tris Base, Di Sodium Hydrogen Phosphate, Sodium Di Hydrogen Phosphate. All the reagents were highly pure and purchased from the approved vendors.

### *CLONE DEVELOPMENT*

mRNA was isolated from *Aspergillus flavus*, and was used as a source for urate oxidase cDNA[4]. Urate oxidase cDNA was amplified by using the above isolated mRNA by RT-PCR method[6]. Then it was amplified with Forward primer was designed with Nde1site (below under lined) and a complimentary with respective gene.

5' - CG CATATG TCCGCAGTAAAAGCAGCC-3'
*Nde1*

Reverse primer was designed with *ECoR1* site and a complimentary with a target gene.

5' - CG GAATTC TTACAATTTAGACTTCAGAGAGAG-3'
*E.coR1*

PCR products were resolved on 0.8% agarose gel electrophoresis. 900kbps band were observed when compared with the marker. Amplified c-DNA was isolated from the agarose gel by ethanol precipitation and pellet was suspended in 100μl of 1X (NEB) buffer and stored at -20ºC for further use[7].

### *ISOLATION OF PLASMID DNA BY ALKALINE LYSIS METHOD*

Vector DNA consists of origin of replication from pBR322, F1 origin, ß-lactamase gene (ß lac) that code for ampicillin resistance, T7 lac promoter that has the lac operator that can bind with lac repressor lac-I which will blocks the transcription initiation under uninduced conditions. Multiple cloning sites (MCS) are

present downstream to the T7 promoter are Nde1, Sma1 & EcoR1restriction sites respectively. Nde1 and EcoR1 restriction sites are used to specifically excise the vector for the insertion of recombinant urate oxidase[4].

### *RESTRICTION DIGESTION OF VECTOR AND URATE OXIDASE GENE*

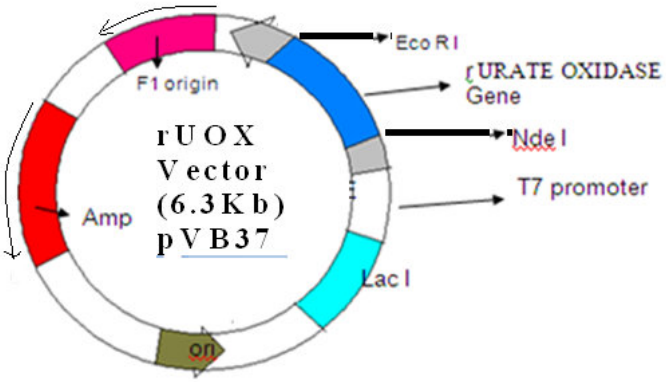
The c-DNA and the vector were digested with *Nde1* and *EcoR1* independently. For vector digestion, 10μg of vector, 3μl of 10X buffer 2 (from NEB), *Nde1* 20Units and *EcoR1* 10 units were used and the volume of the reaction mixture was made up to 30 μl with MilliQ water. For 30μl of c-DNA 4μl of 10X buffer (from NEB), *Nde1* 20 Units and *EcoR1* 10 units were used and makeup the reaction mixture volume to 40 μl with MilliQ water. Reaction mixtures are incubated at 37°C for 2hrs. When digestion was completed the reaction products were loaded on to 1.5% agarose gel with 0.5μg /ml ethidium bromide. The DNA was recovered from gel slice using genei gel extraction kit. To the isolated DNA 2.5 volumes of sodium iodide solution was added and incubated at 45-55$^0$C for 2 to 3 minutes then 15μl of silica beads solution was added and mixed thoroughly It was incubated for 5 minutes at room temperature. The mixture was centrifuged at 12,000 rpm for 30 seconds and the supernatant was discarded. Wash buffer (700 μl) was added, mixed and allowed it to spin at 12,000 rpm for 30 seconds and the supernatant was again discarded. The above steps were repeated for two. 40μl of 1X TE buffer was used for elution of DNA[4].

### *LIGATION*

Recombinant urate oxidase gene was inserted into the vector by ligation. Ligation mixture was prepared in 10μl volume containing ligation buffer 1X, 2μg of the vector, 200ng of insert DNA and 10 Units/μl of T4 DNA Ligase ( from NEB ) and final volume made up with MilliQ water. Ligation was carried out at 16°C for overnight[6]

### *TRANSFORMATION*

BL-21 competent cells were purchased from Novageni. The cells were transformed with the ligation product by heat shock method. Transformed cells were isolated by using specific antibiotics. The transformed colonies were sub cultured in LB media with Ampicilin. From this culture 1.5ml culture was harvested and plasmid DNA was isolated. It was sequenced and confirmed by PCR and restriction digestion methods. Such successfully transformed cells were used for the further study[6].




### *BIOPROCESS OPTIMIZATION*

### *SEED PREPARATION*

Media composition for 1Liter

1. Peptone – 9grams
2. Yeast Extract – 4.5grams
3. Sodium chloride – 9.0 grams

The above components were dissolved in 700ml of purified water and volume made up to 1000ml with purified water. The pH was adjusted to 7.0 with 0.5M sodium hydroxide. The media was distributed in to three conical flasks of 1liter capacity each with 300ml. They are autoclaved at 121$^o$C for 30 minutes. Sterile flasks are transferred into the laminar air flow unit and allowed to cool. The flasks were labeled as “Test-1”, “Test-2” and “Control” and

kept under UV lamp for 20 minutes. Later UV lamp was turned off and "Test flask-1" and "Test flask-2" were inoculated with the recombinant urate oxidase clones. All the flasks were incubated in orbital shaker at 37°C, 200RPM for 8 hours[5]. After the incubation period 5ml sample was collected from each flask under the laminar air flow unit. Samples were examined for the OD at 600nm (not less than 15), pH (6.7 to 6.) and microscopy. Test flasks were free from contamination only *E.coli* cells were present, and control flask is free from contamination. Then broth was collected from "Test flask-1" and "Test flask-2" in to a sterile bottle and was used as inoculum for fermenter.

### *FERMENTATION*

**Table 1**
***Media Composition***

| S.No | Name of the Material | Quantity in Grams |
|---|---|---|
| 1 | Peptone | 150 |
| 2 | Yeast Extract | 125 |
| 3 | Potassium Di Hydrogen Phosphate | 45 |
| 4 | Di Potassium Hydrogen Phosphate | 90 |
| 5 | Magnesium Sulphate | 10 |
| 6 | Sodium Chloride | 150 |
| 7 | Ammonium Chloride | 30 |
| 8 | Silicon Antifoaming Agent | 15 |

The above components were dissolved in 8 liters of purified water and volume made up to 12 liters with purified water[5].

### *DEXTROSE SOLUTION-I*

15 grams of dextrose was dissolved in 70mL of purified water in feed bottle and volume made up to 100ml with purified water[5].

### *DEXTROSE SOLUTION-I*

350 grams of dextrose was dissolved in 600mL of purified water in feed bottle and volume made up to 600ml with purified water[5]. Both dextrose solution-I and dextrose solution-II bottles were sterilized with siphon sets.

### *ISO PROPIAL THIO GALACTOSIDE*

3.57 grams of iso propial thio galactoside was dissolved in 15 ml of purified water and filtered through 0.22 μm filters aseptically into sterile falcon tubes and stored at $20^0$C[5].

### *AMMONIA SOLUTION*

50% V/V ammonia solution was prepared with purified water in a sterile feed bottle.

### *FERMENTATION PROCESS*

Fermenter was washed thoroughly with 0.5 N sodium hydroxide and purified water until pH came to 5.5 to 7.0 then sterilized by steam sterilization. pH and dissolved oxygen probes were calibrated and fixed in to the fermentor[9]. Above prepared 12 liters media was aseptically transferred in to the fermenter with Peristaltic pump under 200RPM agitation pH was adjusted to 7.4 with 20% sodium hydroxide solution. Media was sterilized at $121^0$C for 30 min and cooled to $35^0$C. Dextrose solution I and II, 50% ammonia solution and inoculum bottles were aseptically connected to the fermenter[5].

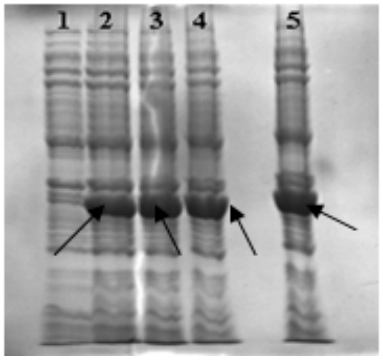
**Table 2**
***Fermentation Parameters***

| S.No | Parameter | Set Point |
|---|---|---|
| 1 | Temperature | $35^0$C |
| 2 | Air | 0.5VVM |
| 3 | Dissolved Oxygen | 100% |
| 4 | Back Pressure | 0.8Bar |
| 5 | Agitator Speed | 300RPM |
| 6 | pH | 7.15 to 7.2 |

Dextrose solution-I and inoculum were transferred aseptically to the fermentor. Fermentation process was continued out as per the parameters mentioned in table-2[9]. Whenever dissolved oxygen went down to 40% or below parameters like agitator speed was increased from 300 to 850R, aeration increased from 0.5 to 1.2 VVM in stepwise of 50RPM and 0.2 VVM were adjusted respectively. Dextrose solution-II was charged to the fermenter as per the process requirements, based on dissolved oxygen (40 to 50%). pH was maintained in the range of 7.15 to 7.20 with 50% ammonia solution. Fermentation process was carried out for 8 hrs. Then 10mL sample was collected and labeled as '0' hour and following in process tests were performed[5].

1. Plasmid Stability – Not less than 80%.
2. Optical Density at 600nm - Not Less than15
3. Microscopic observation – Free from contamination

Then the culture was induced by feeding with 15 ml of sterile iso propial thio galactoside solution and the induction process was run for 4 hours. 10mL of in process sample was collected for every one hour labeled as follows ('1'hr, '2'hr,'3'hr & '4'hr). These in process samples were analyzed for rate of expression Then broth was harvested from the fermenter into a clean container by reducing the fermenter temperature to $5^0C$[6].




***SDS PAGE analyis of rate of expression samples Lane 1- 5 are'0' to 4th hours expression sample.***

### *DOWN STREAM PROCESS*

1000ml of broth was distributed into each centrifuge bottle. Bottles were centrifuged at 2 to 4°C and 4000RPM for 20min. Biomass was collected into a sterile container and supernatant was .discarded.

### *CELL LYSIS*

Cell Lysis was done by using high speed homogenizer (Panda) equilibrated with 20 mM Tris pH 9.3. Biomass was prepared by suspending the cell pellet in 20mM Tris pH 9.3 at 1:5 ratio between cell pellet and buffer in homogenous and uniform condition. Cell suspension was loaded at 10 to 12 ml per minute. The Acceptable rate of cell lysis was 90%[4].

### *AMMONIUM SULPHATE PRECIPITATION*

Ammonium sulphate solution was added at 15% concentration to above said cell lysate and incubated for 1 hour then it was centrifuged at 12000RPM for 30 minutes at 2 to 8°C. To the supernatant ammonium sulphate solution was added at 50% concentration and incubated for 10 to 12 hours at 2 to 8 °C under constant stirring. Then it was centrifuged at 12000 RPM for 30minits. Pellet was suspended in 10mM Tris pH 9.3 at 1:5 ratio between pellet and buffer[4].

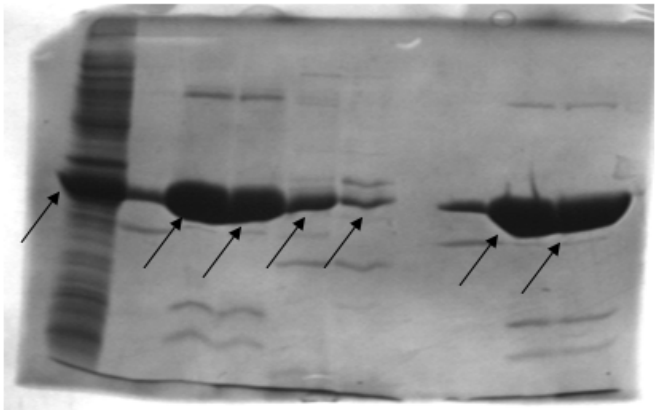
### *DIAFILTRATION-I*

Millipore Tangential flow filtration system with two 10 KDA cutoff filter cassettes equilibrated with 10mM Tris pH 9.3 was used. Diafiltration-I was done with the above suspension using 10mM Tris pH7.0 at 10ml per minute flow rate until conductivity reaches to less than 0.2mS/cm[4].

### *ANION EXCHANGE CHROMATOGRAPHY*

XK50/30 column packed with DEAE Sepharose was used. Column specifications were as follows column diameter 5cms, resign height 12 to 13cms height equalent to theoretical plates should be not less than 3000 plates per meter and asymmetry should be 0.8

to 1.5. Column was regenerated with 1M sodium hydroxide and 1M sodium chloride and equilibrated with 10mM tris pH9.3[11]. Then above diafiltered sample was loaded at 20ml per minute. If there was any rise in UV at 280 nm flow rate was reduced to 15ml per minute. After loading column was washed with 10mM tris pH 9.3 until absorbance at 280nm was less than 0.1AU. Then protein was eluted with 20mM phosphate buffer with 50mM sodium chloride (pH 8.0). Fractions were analyzed for the presence of Recombinant Urate Oxidase using 12% SDS PAGE and positive fractions were pooled[4].



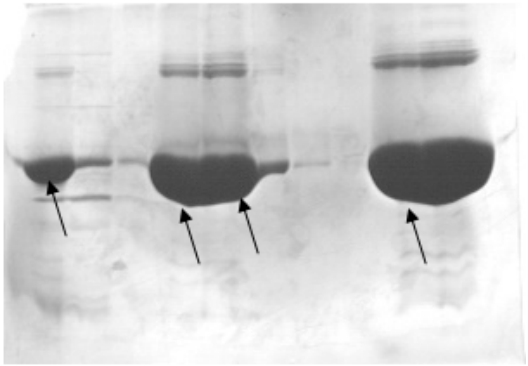
***SDS PAGE analysis of recombinant urate oxidase DEAE Sepharose Fractions Lane 1 to 9- Before Load, Flow Through, Fraction- 1 to fraction 4, Equilibration wash, Fraction- 2 9(20µl), Fraction- 3(20µl).***

### *DIAFILTRATION – II*

Millipore Tangential Flow Filtration system fixed with two 10Kda filter cassettes and equilibrated with 10mM tris pH7.0 was used Diafiltration–II was done with the above pooled positive fractions using 10mM tris pH7.0 at 10ml per minute flow rate until the conductivity reaches to less than 0.3mS/cm[4].

### *CATION EXCHANGE CHROMATOGRAPHY*

XK50/30 column packed with SP Sepharose was used. Column specifications were as follows column diameter 5cm's, resign height 12 to 13cm's height equalent to theoretical plates should be not less than 3000 plates per meter and asymmetry should be 0.8 to 1.5. Column was regenerated with 1M sodium hydroxide and 1M sodium chloride and equilibrated with 10mM tris pH7.0[10]. The above diafiltered protein sample was loaded at 20ml per minute. If there was any rise in absorbance at 280 nm flow rate was reduced to 15ml per minute. After loading column was washed with 10mM tris pH 7.0 until absorbance at 280nm should be less than 0.1AU. Then product was eluted with 20mM phosphate buffer with 400mM sodium chloride (pH 7.0). Fractions were analyzed for the presence of recombinant urate oxidase using 12% SDS PAGE and positive fractions were pooled[4].



***SDS PAGE analysis of recombinant Urate Oxidase SP-Sepharose Fractions. Lane 1- 10 is as follows respectively (10µl) Before Load, Flow Through, Equilibration wash, Fraction- 1 to fraction 5, gap, Fraction- 2(20µl), Fraction- 3(20µl).***

### *GEL FILTRATION CHROMATOGRAPHY*

XK50/950 column packed with Superdex G-75 was used. column specifications were as follows column diameter 5cms, resin height 76 to 78cms height equalent to theoretical plates should be not less than 10000 plates per meter, asymmetry should be 0.8 to 1.2 column volume 1512ml of resin and load volume should be not more than the 3% of the column volume[11]. Column was regenerated with 0.3M sodium hydroxide and equilibrated with 20mM phosphate buffer 75mM sodium chloride pH7.0. Then cation exchange chromatography eluate was loaded at 5ml per minute. Then 10mM phosphate buffer 75mM sodium chloride pH7.0 was keep on passing until UV at 280nm starts to rise. Initially aggregated protein was eluated then product of interest as a separate peak. Both are collected as separate fractions. They are analyzed for the presence of recombinant urate oxidase using 12% SDS PAGE which was free from the aggregates[4].

### *URATE OXIDASE CHARACTERIZATION*
### *PHYSICAL CHARACTERIZATION*

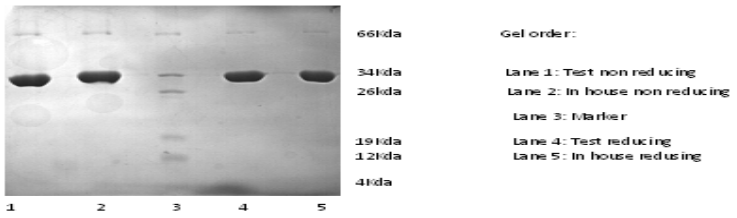
Final protein sample was clear colorless solution and its pH 6.8 to 7.2.

### *CONCENTRATION ANALYSIS*

Protein concentration at each step was calculated as per the following formulae
OD at 280nm / 7.5 * Dilution factor[13.]

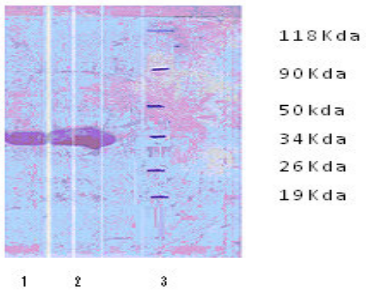
### *SDS PAGE ANALYSIS*

Gel Filtration Chromatography Eluate of interest was loaded on to12% poly acryl amide gel. After the run the gel was stained with Comassie brilliant blue G-250. It was showing a principle band at 34Kda when compared with the marker[4].




### *WESTERN BLOTTING*

Final gel filtration chromatography eluate was loaded on to12% polyacrylamide gel. After the run gel was blotted on to the poly vinyl di fluoride membrane. The principle band at 34Kda was observed with the urate oxidase antibodies expressed in mouse[4].




*Western blot of recombinant urate oxidase Lane-1 & 2 are Test sample 1 & 2, Lane 3 is Marker*

### *N-TERMINAL SEQUENCING*

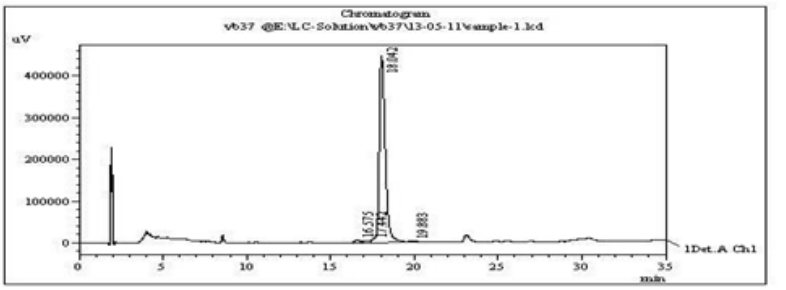
N-Terminal sequencing was as per Edman degradation method Amino acid sequence of 1st 13 from N-terminal end was as follows[4]. "SAVKAARYGK/LDNV"

### *ACTIVITY ASSAY*

The activity of the recombinant urate oxidase was assayed by following the disappearance of uric acid detected by a decrease in the absorbance at 293nm[12]. The assay mixture with 1µg of purified recombinant urate oxidase in Tris EDTA Acetate buffer and 36mM uric acid in a final volume of 3ml and specific activity was 33IU/mg[13]. Here one unit is defined as the amount of the enzyme needed to transform 1 µmol of uric acid to allantoin in 1min at $30^0$C and pH 9.0.

### *RP HPLC ANALYSIS*

Final protein at 1mg/ml was loaded on to on to the C-18 column with Shimazdu HPLC 2010[11]. The mobile phase composition is as follows Buffer A: - 0.1% Tri fluoro Acetic acid in HPLC grade water. Buffer B: - 0.1% Tri fluoro Acetic acid in HPLC grade Acitonitrile.




PeakTable @E:\LC-Solution\Vb37\13-05-11\sample-1.lcd

Detector A Ch1 220nm

| PeakN | Ret. Time | Area | Height | Area % | Height % |
|---|---|---|---|---|---|
| 1 | 16.575 | 163089 | 6704 | 1.433 | 1.455 |
| 2 | 17.442 | 151367 | 5866 | 1.330 | 1.273 |
| 3 | 18.042 | 10966818 | 445825 | 96.344 | 96.752 |
| 4 | 19.883 | 101668 | 2394 | 0.893 | 0.520 |
| Total | | 11382942 | 460790 | 100.000 | 100.000 |

After the analysis the peak was integrated for the purity it was showing 96% purity

| S.No | Process step | Recovery in Grams |
|---|---|---|
| 1 | Cell lysate | 28 |
| 2 | Diafiltration - I | 26 |
| 3 | Anion Exchange Chromatography | 11 |
| 4 | Diafiltration - F | 10.6 |
| 5 | Cation Exchange Chromatography | 8 |
| 6 | Gel filtration Chromatography | 6.5 |

## CONCLUSION

The fermentation process parameters were optimized so that rate of expression of recombinant urate oxidase gradually increased from '0' hour to four hours after the induction. Two hundred and fifty grams of wet cell pellet was obtained from the fermenter. In the optimization of down stream process parameters protein purity gradually increased up to 96%. Where in anion exchange chromatography number of impurity bands were high, in cation exchange chromatography protein was having aggregates which were resolved in gel filtration chromatography. 96% pure and biologically active protein resulted from the process. The final yield of purified Recombinant urate oxidase from each liter of the culture was about 270mg. The summary of each purification step is presented in below table.

## REFERENCES


1. Keilin, J. (1959) The biological signafence of uric acid and guanine excretion. Biol. Rev 34, 265 – 296.
2. Oda, M., Satta, Y., Tekenaka, O. and Takahata, N. (2002) loss of urate Oxidase activity in hominoids and its evolutionary implications J. Biochem 19, (5) 640-653.
3. Conger, J.D. (1980) Acute uric acid nephropathy, med. Clin. North Am. 74, 859–871.
4. J.Sambrook, E.F. Fritsch& T.Maniatis Molecular cloning A Laboratory Manual.
5. Strategies for Achieving High Level Expression of Genes in *Escherichia coli*, Microbiological Reviews, Sept 1996, p.512-538.
6. Koyama, Y., Ichikawa, T., and Nakano, E. (1996) Cloning, sequence analysis and expression in *Escheria coli* of the gene encoding the *Candida utilis* urate oxidase (uricase), J. Biochem 120, 969–973.
7. Yeldandis, A.V,. Yeldandi, V,. Kumar, S,. Murthy, C.V,. Wang, X.D, Alvares, K. Rao, M.S. and Reddy J.K. (1991) Molecular cloning evolution of the urate Oxidase encoding gene in hominoid primates: nonsense mutations, gene 109, 281-284.
8. Sheng wu Gong Cheng Xue Bao 19 Zhu, X.J., Liu, J.G. and Li, G.X. (2001) Cloning and expression of urate Oxidase and its applications in serum uric acid analysis 17, 173-182.
9. Nakagawa, S., Oda, H. and Anazawa, H. (1995) high cell density cultivation and high recombinant protein production of *Escheriachia coli* strain expressing uricase, Biosci. Biotechnol. Biochem. 59, 2263–2267.
10. Leplatosis, P., Le Douarin, B. and Loison, G. (1992) High- level production of a peroxisomal enzyme: *Aspergillus flavus* uricase accumulates intracellularaly and is active in *Saccharomyces cervisiae,* Gene . *Anal Biochem* 122, 139-145.S
11. Conley, T.G. and Priest, D.G. (1990) Purification of uricase from mammalian tissue .*Prep* Biochem 9, 197 – 203.-12.
12. Bergmeyer, H.U. (1963) meathods of enzymatic analysis, p. 500–504. Verlag chemie Weinheim, Academic press New York and London.
13. Priest, D.G. and Pitts, O.M. (1972) Reaction intermediate effects on the spectrophotometric uricase assay. *Anal Biochem* 50, 195-205.
14. Dr. P. Josephine Latha and Dr. S. Ganesan Evaluation of Serum Uric Acid and Lipid Profile in Hypertension (ijpbs 2013 496 – 502).