Int J Pharm Bio Sci 2014 April ; 5 (2) : (B) 509 - 525

Research Article Biotechnology

International Journal of Pharma and Bio Sciences ISSN 0975-6299

STRATEGIES FOR CLONING AND HIGHER EXPRESSION OF ASPERGILLUS FLAVUS URATE OXIDASE GENE IN E.COLI

SANKARI.N*[1], M.VIJAYALAKSHMI[1], M.DECCARAMAN[1] AND R.BALASUBRAMANIAN[2]

1.Dr.MGR university and research institute, Periyar E.V.R High Road, Maduravoyal, Chennai.
2.University of Madras,Chennai.

ABSTRACT

Evolution of recombinant DNA technology led to the procession of therapeutic fields in oncology, autoimmune, diabetic, cardiovascular, more in diagnostic field to identify a specific cause, and in many sections of industrial platforms. Demand for these recombinant protein products, increasing day by day, and created the need to develop efficient expression techniques to increase the productivity with low production cost. The key factors in the development of therapeutic biologics are, starting the work with authenticated gene sequence and cost effective process development. Urate oxidase, the enzyme from *Aspergillus flavus* cloned in *Escherichia coli*, and used in the prevention and treatment of hyperuricemia associated with lymphoid malignancies. Here we depicted an optimized workflow for the cloning and high volumetric production of recombinant urate oxidase in *E.coli* with systematic optimization of the expression levels. The *E.coli* strain, *Rosetta, plys S* was used as the workhorse for production of recombinant urate oxidase. In the current work, we attained high expression levels (40%) with the highest wet pellet (60g/L).Parameters including confirmation of the gene orientation and other upstream parameter of the suitable OD of hosts, optimal IPTG concentration for induction, and time to harvest were studied systematically to achieve the high volumetric productivity.

KEY WORDS: Urate oxidase, Cloning, Purification, Rasburicase, Gout, tumor lysis syndrome, and Cancer.

SANKARI.N
Dr.MGR university and research institute, Periyar E.V.R High Road, Maduravoyal, Chennai. sankari.n.biotech@gmail.com

***Corresponding author**

INTRODUCTION

Uric acid is the end product of the purine breakdown in birds, reptiles and higher mammals, as they are devoid of functional uricase genes[1]. But in turn bacteria, yeasts break down uric acid into Allantoin with the help of an uricase enzyme[2] (urate oxidase; EC 1.7.3.3). Urate oxidase is a homo-tetrameric enzyme, which catalysis uric acid to 5-hydroxyisourate[3]. Uricase enzyme is used in the treatment of gout and tumor lysis syndrome. It is also used as a diagnostic reagent in the detection of uric acid. Urate oxidase (UOX) is expressed in many organisms ranging from bacteria to mammals. Few genus of microbes producing urate oxidase are P*roteus*[4], *Brevibacterium*[5], *Pseudomonas*[6], *Arthrobacter*[7], *Streptomyces*[8], *Candida*[9], *Bacillus*[10], *and Aspergillus*[11]. Although many sources of enzyme are available, the low natural expression of enzyme has created a space to work on other alternatives which will increase the production of enzyme in large quantities. Rasburicase is produced by genetically modified *Saccharomyces cerevisiae* cloned with *Aspergillus flavus* cDNA[11], and which is available under the brand name of Fasturtec, and Elitek. The cost of the treatment by using innovator drug is high due to its production methodologies, and not affordable by many people from developing and low developed countries, so we sought to produce the Rasburicase in *Escherichia coli* to minimize the production cost, ultimately accessible to all the people. So aim of the present work was to develop a prokaryotic vector containing urate oxidase gene and to optimize the conditions of the expression of recombinant UOX in *E.coli* to meet the demand to produce the enzyme in large magnitudes for various biological purposes. The bacterial expression host is easy to handle and high level of protein recovery can be achieved[12], so recombinant uricase was expressed in *E.Coli*. Recombinant uricase produced from bacterial host also exhibits the same level of activity[13] and stability. In the present work, Recombinant uricase was expressed in the cytoplasm of E. coli in active and soluble form (>40% of the total protein).

MATERIALS AND METHODS

(i) Enzymes, Plasmids

Restriction enzymes were purchased from Fermentas, and other molecular biology chemicals and kits were purchased from Sigma Aldrich, expression host cells and vectors were purchased from Novagen, and glassware and other purification chemicals were of analytical grade purchased from local companies.

(ii) Microorganisms

The strain of *Aspergillus flavus* used in this study was isolated from soil and maintained on Potato dextrose agar slants and sub-cultured for every month.

(iii) Growing of A. flavus

The fungal spore inoculum was prepared by adding 10 mL of the sterile distilled water containing Tween 80 to the PDA slants where culture was maintained. The spores were freed using a sterile inoculation loop under aseptic conditions. 1 ml of spore suspension was used as the inoculum. The inoculum was added into a medium containing 1 g of KH_2PO_4, 2 g of NH_4NO_3, 0.2g of $MgSO_4.7H_2O$, 20 mg of $CaCl_2.2H_2O$, 4 mg of $MnSO_4$, 2 mg of $Na_2MoO_4.2H_2O$, 2.5 mg of $FeSO_4.7H_2O$, 20 g of Uric acid and the volume was made up to 1 liter with R.O water and the pH was adjusted to 5.5.The spore suspension was inoculated into 250 mL conical flask containing 100 mL of the above media. The culture was grown for 96 hours at 35°C in a shaking incubator. Small Ball to filamentous structures was observed after 96 hours.

(iv) Total RNA isolation and cDNA synthesis

The 96 hours grown culture was centrifuged at 10000 g for 20 min at 4ºC. 100 mg of mycelia mat was transferred to the mortar by discarding the supernatant. The mycelia mat was crushed to power by adding liquid nitrogen, and the

crushed powder was used as a source of material to purify total RNA. Total RNA was isolated as per the technical data sheet provided by the supplier[14].

(v) Synthesis of cDNA

The RT-master mix was prepared as per the Table 01 by placing it on ice and then the reaction mixture was transferred to the thermo cycler, programmed at 37^{0}C for one hour for the synthesis of cDNA[15].

Table 1

Master-mix used for the synthesis of cDNA.

S.No	Name of the component	Volume	Final concentration
1.	10x Buffer RT	2 µl	1x
2.	dNTP Mix (5 mM each dNTP)	2 µl	0.5 mM each dNTP
3.	Oligo-dT primer (10 µM)	2 µl	1 µM
4.	RNase inhibitor (10 units/µl)	1 µl	10 units (per 20 µl reaction)
5.	Omniscript Reverse Transcriptase	1 µl	4 units (per 20 µl reaction)
6.	RNase-free water Variable	8 µl	
7.	Template RNA	4 µl	2 µg
8.	Total volume	20 µl	

(vi) Synthesis of ds DNA

The synthesized cDNA was used as a template to synthesize the ds-DNA by using UOX specific primers. The parameters used in the reaction were, an initial denaturation for 4 minutes at 94^{0}C, then followed by 25 cycles of denaturation for 20 seconds at 94^{0}C, annealing for 30 seconds at 48^{0}C, extension for 20 seconds at 72^{0}C and final extension was done at 72^{0}C for 4 minutes. The forward primer contained Nde I and the reverse primer was with translation termination codon and Hind III sequences.

(vii) Construction of expression vector

a. Preparation of E. coli Top-10 electro Competent Cells

E. coli Top-10 cells were grown in Luria bertani (L.B) media and harvested at optical density (OD) of 0.6. Then the cells were centrifuged at 1600 g for 10 minutes at 4^{0}C, pellet was washed after discarding the supernatant. Centrifugation and washings were repeated, twice with chilled distilled water, 10 % glycerol, 0.1 % glycerol respectively. After the final wash with 0.1 % glycerol, the cells were aliquoted and stored in -80° C for future use.

b. Digestion and Ligation of ds UOX and pET 21b

E. coli Top-10 cells bearing pET21b vector were added to L.B broth with ampicillin and grown for overnight. The plasmid was isolated from this overnight culture. About 2.0 µg of isolated pET21b vector and the PCR amplified DNA were digested with 10Units of Nde I and 10Units of Hind III separately, and incubated at 37^0 C for 2 hours. The digested DNA reaction was electrophoresed in 1% Agarose gel. Digested Urate Oxidase DNA and vector were purified from the agarose gel using Sigma GenElute Gel extraction Kit, as per protocol given in its technical data sheet[16].

c. Ligation of digested samples

Ligation of both double digested insert and vector was done by adding T4 DNA ligase in 2:1 ratio of insert to the vector. The ligation mixture was incubated at 16^0 C, for overnight. After overnight incubation; the reaction mixture was purified by ethanol precipitation method. In ethanol precipitation, 1/20 volume of 3M NaCl and 2.5 volume of ethanol were added to the ligated DNA, then the sample was incubated at -20°C for one hour and the DNA was collected by centrifugation at 10000 RPM for 20 minutes. The sample was re-suspended in ultra-pure water.

d. Transformation of expression vector into Top 10, E.coli cells

The ethanol precipitated ligation mixture was dissolved in 10 µL of ultra-pure water; this sample was mixed with the Top 10 electro competent cells and transformed by Eporator (Eppendorf), as per manufacture's protocol. One mL of L.B media was added to this electroporated sample, incubated at 37° C for one hour. After one hour, the cells were plated on LB- plate supplemented with ampicillin as the selection marker, and incubated at 37° C for overnight.

(viii) Confirmation of clones containing rUOX

The clones were confirmed by colony PCR, restriction digestion, expression checking, and expressed protein confirmation by western blotting.

a. Clone confirmation by colony PCR

Colony PCR was used as first screening step to quickly screen for plasmids containing UOX gene directly from bacterial colonies.This method is easy and quick to confirm the clone, as the tedious plasmid isolation procedure can be avoided in the colony PCR. In the present work, we selected six colonies and each colony was streaked on a culture plate and the same was dissolved in 10 µL of ultrapure wate.The dissolved samples were boiled for 10 minutes and centrifuged at 10000 RPM for 5 minutes and 2 µL of supernatant was taken as a template for PCR. UOX specific primers were used for the initial screening, then to understand the orientation and size of the insert, one of the positive colonies was again screened with T7 promoter primer (as forward primer) and UOX gene reverse primers, the rest of the parameters were same as that used for synthesis of ds-DNA from cDNA. Then the reaction mixtures were analyzed by using 1% agarose gel electrophoresis.

b. Restriction digestion

The positive colonies further screened by the classic method, involved a plasmid isolation followed by restriction digestion. The colonies were picked from the replica pate and transferred to the LB media with ampicillin. The cultures were incubated at 37° C for overnight. The cultures were used for plasmid isolation, and the purified DNA was used for the restriction digestion. Various combinations of restriction enzymes were used to verify the presence of the gene, its size, and orientation of the inserted gene. The planned combination of digestions covers both gene specific and vector specific restriction sites.

c. Transformation of expression vector* into *E. Coli, Rosetta plys S

After the confirmation with colony PCR and restriction digestion, the positive colony was inoculated in LB media with ampicillin and grown at 37°C for the overnight. Expression vector maintained in these Top 10 colonies was isolated by the Sigma miniprep method and used for the transformation into *E.coli*, Rosetta plys S, the expression host. Rosetta pLys S is a well-engineered *E.coli* host cell where majority of the entire gene are expressed, because of its rare codon correction advances. *E. coli*, Rosetta plys S electro-competent cell preparation and electroporation were same as the methods used for Top 10 *E.coli* cells. The transformed cells were grown on LB agar with ampicillin and incubated at 37°C for overnight.

d. Confirmation of the product by expression checking

The confirmed colony with urate oxidase in the expression vector was selected from the replica plate and inoculated in LB media with ampicillin, incubated at 37°C with 200 RPM until the OD reached 1, then induced with 1mM IPTG and harvested after 4 hours. 100 µL of un-induced and induced samples were washed with PBS buffer, centrifuged and sonicated by adding 100 µL of 25mM Tris- HCl (pH 8.5) with 5 mM EDTA. The induced sonicated samples were again centrifuged at 10000 RPM for 10 minutes. Supernatant was separated, added 2X reducing buffer to it. To the pellet 200 µL of 1X SDS-PAGE reducing dye with 8M urea was added. Un-induced, induced pellet, induced supernatant were boiled for 10 minutes, from all these 10 µL samples were loaded on to the reducing SDS-PAGE gel[17] after a pulse spin. The gels were stained by Coomassie Blue staining method.The amount of protein expressed was estimated by densitometry method by comparing the intensity of the protein bands using ImageJ software, a freeware

analysis tool developed at National institute of health (www.imagej.net). .

e. Protein confirmation by western blotting

After confirmation by SDS-PAGE gel, the supernatant was processed and loaded onto the gel in the same way as mentioned above and from the gel, the protein was transferred to the PVDF membrane by semi-dry blot apparatus (Bio-Rad) for 40 min at 10 volts. The transferred membrane was blocked with 3% BSA in 1X PBS and incubated for overnight at 4^0C with primary antibodies raised against the Rasburicase in mouse. Then the membrane was washed thrice with 1X PBS supplemented with 0.1 % Tween, later the membrane was incubated for 2 hours in 1X PBS supplemented with secondary antibody conjugated with alkaline phosphatase. Then the membrane was washed thrice with 1X PBS supplemented with 0.1 % Tween. Finally the membrane was developed by NBT/ BCIP[17,18].

(ix) Preparation of research cell bank

The confirmed clone was inoculated in 100 ml of LB media and incubated at 37°C with 200 RPM till the OD reached to 1 and the sample was centrifuged at 2000 RPM for 10 minutes. Then the pellet was washed with PBS, and centrifuged again. The supernatant was discarded and the pellet was re-suspended in 10% glycerol. Then the samples were aliquot with 1mL volume and stored in -80°C for further use.

(x) Optimization of urate oxidase expression

a. Cultural media and bioreactor conditions

The seed media and the culture media in the bioreactor were composed as per the table 02. All the parameters were optimized in (Sartorius biostat B plus) fermenter with a working volume of 2.5 L. The flow of work includes preparation of seed culture from research cell bank, then inoculation of seed culture into the bioreactor for optimization and expression. In the seed medium, 2 % glucose and in the bioreactor medium, 60 % glucose, 2 % yeast extract were used. The batch mode was converted into fed-batch mode, when the glucose levels were depleted. One vial from the research cell bank was taken; thawed in 37°C water bath, from this 500 µL of the culture was added to 200 mL of the seed media in a one liter conical flask and placed on a rotator shaker at 37°C with 200 RPM and incubated until the OD_{600} reached to 1.5. Usually it takes 11hours to reach the said OD_{600}. 125mL of culture (5 % by V/V) was seeded into the fermenter with 2500mL working volume. pH of the media was controlled at 6.9 by employing 50% liquor ammonia and 30% phosphoric acid. Dissolved Oxygen(DO) was set at 40% of air saturation as the set point. An automatic feedback control system based on the DO was used to control the agitation speed (200-800RPM). In addition to the other components, $MgSO_4$ was added to the culture media with a final concentration of 25 mM, for every 10 units of increased OD_{600}, to support the high cell density growth. Cell growth was monitored spectro-photometrically by measurement of the optical density at 600 nm (OD_{600}). This method was used in all the optimized trial batches.

Table 02
Optimized recipe of seed media and culture media for cultivating E.coli carrying urate oxidase gene.

Component	Concentration
K_2HPO_4	100 mM
$NaH_2PO_4.2H_2O$	20 mM
Na_2SO_4	2.0 mM
$(NH_4)_2SO_4$	2.0 mM
NH_4Cl	1.0 mM
$MgSO_4$	2.0 mM
$Na_2SeO.5H_2O$	700 nM
Na_2MoO_4	125 nM
$Ni(NO_3)_2.6H_2O$	5.0 µM
EDTA	15.0 mM
$FeCl_3.6H_2O$	3.0 mM
$ZnCl_2$	650 µM
$CuCl_2.2H_2O$	100 µM
$CoCl_2.6H_2O$	50.0 µM
H_3BO_3	150 µM
$MnCl_2.6H_2O$	7.0 µM
Thiamine hydrochloride	300 µM

b. Screening for optimal OD_{600} to induce

The fermentation batches were ran as per the method explained above and induced with 1.0 mM IPTG and harvested after 4 hours of the induction. We induced the culture at different OD_{600}, in different batches as tabulated in the table 03. All the batches were harvested at 4 hours of induction, and from all the batches un-induced (before inductions) and induced samples (just before harvesting) were collected and normalized OD_{600} to 2 with PBS buffer. Then the cultures were centrifuged at 10000 RPM for 10 minutes and lysed with the lysis buffer (25 mM Tris- HCl, 5.0mM EDTA, pH 8.0). Then equal volume of 2X reducing gel loading buffer was added to the lysate and boiled at 95° for 5 minutes, from this 10µL of the sample was loaded and analyzed by 12% SDS–PAGE electrophoresis.

Table 03
Sample ID and description of samples, induced at different cell mass.

Id	Description
UD1	Un-induced at OD-20
I1	Induced at OD- 20
UD2	Un-induced at OD- 40
I2	Induced at OD- 40
UD3	Un-induced at OD-60
I3	Induced at OD 60
UD4	Un-induced at OD-80
I4	Induced at OD- 80

c. Screening for the optimal IPTG concentration

From the above experiment, optimal induction OD was determined and further optimization was focused on the concentration of IPTG. The fermentation conditions were maintained as mentioned above and different batches were induced with different concentration of IPTG as mentioned in the table 04. All the un-induced and induced samples were processed as per the method explained above. All the samples were analyzed by 12% SDS-PAGE Electrophoresis.

Table 04
Sample ID and description of samples, induced at different IPTG concentration to find out the optimal IPTG concentration

ID	IPTG concentration in mM
Un	Un-Induced
Ind-1	Induced -1.0
Ind-0.50	Induced-0.50
Ind-0.25	Induced-0.25
Ind-0.125	Induced-0.125
Ind-0.0625	Induced-0.0625
Ind-0.03125	Induced-0.03125
Ind-0.0156	Induced-0.0156
Ind-0.0078	Induced-0.0078

d. Optimizing the best harvesting time point

From the above two experiments, optimal Induction OD_{600} and IPTG concentration were determined, then finally with the above optimized conditions, we ran another batch. In this batch we collected 5 ml of samples after every 1 hour induction and processed separately. The samples were processed in the same way to that of the above method.

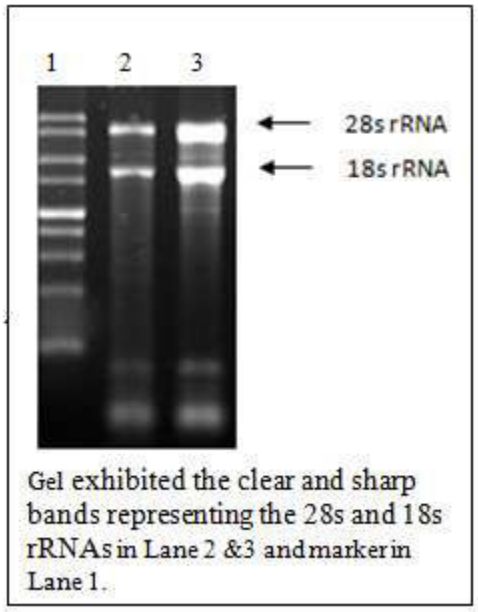
RESULTS

1. Construction of pUOX vector

1.1. Isolation of RNA

The isolated RNA samples were run on the denatured agarose gel with ethidium bromide. The gel pattern showed the clear and sharp bands with more intensity representing the 28s and 18s rRNAs, which clearly revealed the presence of intact total RNA (Fig: 01).

Figure 01
Analysis of total RNA isolation.

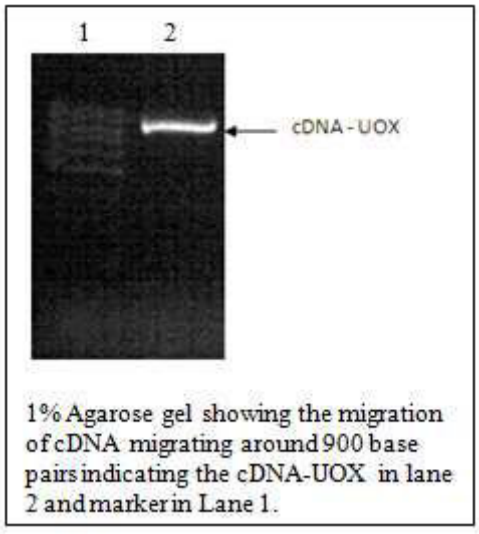


Gel exhibited the clear and sharp bands representing the 28s and 18s rRNAs in Lane 2 &3 and marker in Lane 1.

1.2. Synthesis of cDNA

The isolated total RNA, used as the template for the synthesis of cDNA- UOX. After the PCR by reverse transcriptase, the PCR sample was loaded onto 1% agarose gel. Analysis showed the presence of sharp intact cDNA- UOX gene band at 900 base pairs. (Fig: 02).

Figure 02
Agarose gel showing the cDNA-UOX

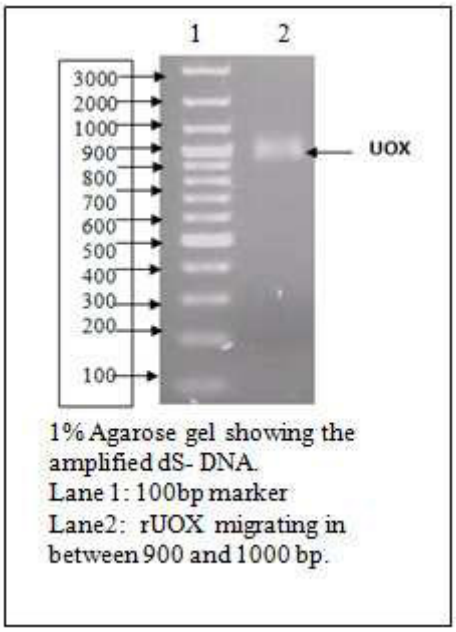


1% Agarose gel showing the migration of cDNA migrating around 900 base pairs indicating the cDNA-UOX in lane 2 and marker in Lane 1.

1.3. Amplification of dsDNA

The PCR product was electrophoresed on 1% Agarose gel and showed the migration of UOX gene at 900 base pairs indicating the correct size of the DNA (Fig: 03).

Figure 03
Agarose gel showing the amplified dS- DNA



1% Agarose gel showing the amplified dS- DNA.
Lane 1: 100bp marker
Lane2: rUOX migrating in between 900 and 1000 bp.

2. Construction of expression vector

Then schematic representation of the expression vector containing UOX gene is represented in figure 04 and the clone is designated as pET21b-UOX.

Figure 04
Schematic diagram of expression vector

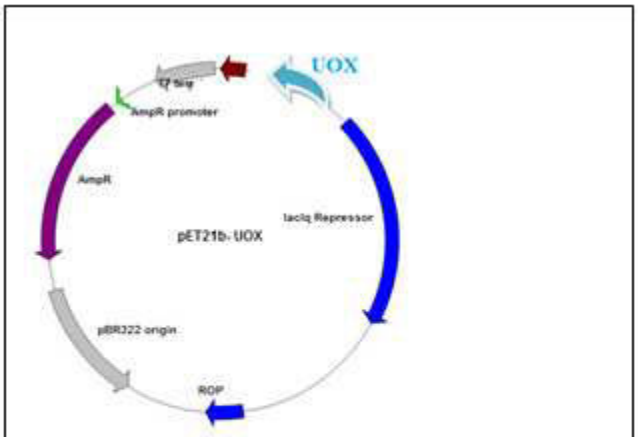


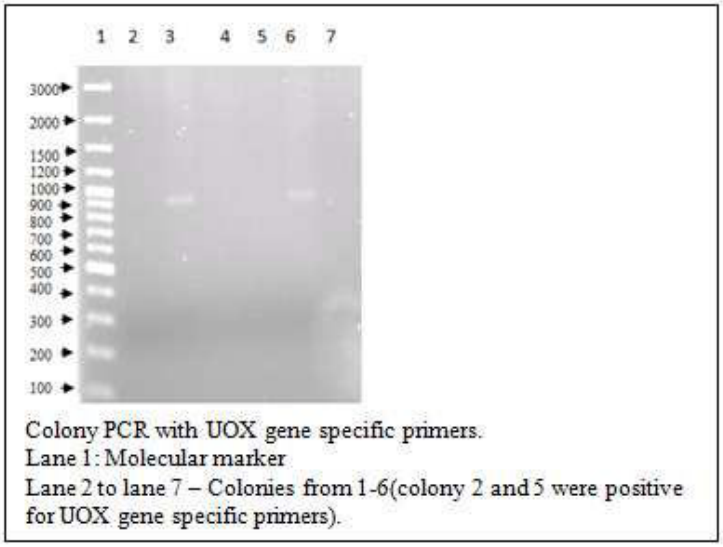
Fig: 04 Schematic diagram of expression vector - PCR product was digested with Nde I and Hind III and ligated into vector digested with the same sites.

3. Confirmation of the clone

Colony PCR with gene specific primers, restriction digestion, and western blot were performed to confirm the UOX gene presence, position and size as mentioned in the methods. The PCR samples with gene specific primers were electrophoresed on 1% agarose gel analysis (Fig: 05), showed that the PCR

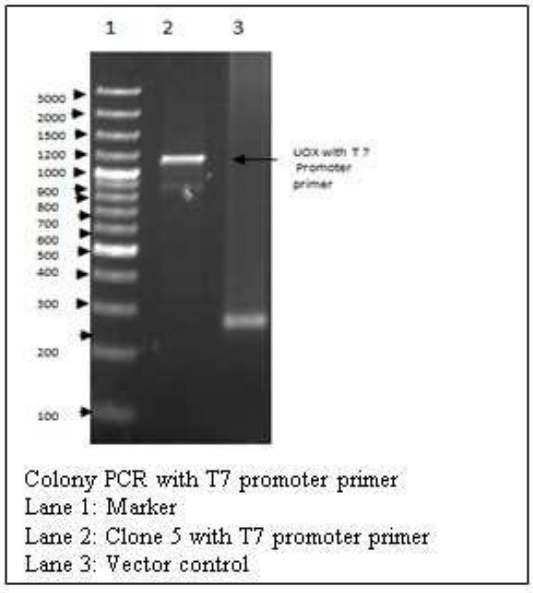
product from colonies 2 and 5 were migrating at 900 base pairs representing expression vector with urate oxidase gene in the host. PCR with the combination of T7 promoter primer, and UOX reverse primers showed the migration of amplified band at 1100 base pairs, the extra 200 base pairs were amplified from the vector indicating the correct orientation of the gene in the expression vector (Fig. 06). After confirmation by colony PCR, confirmed Top 10 cells with the expression vector was inoculated in LB media supplemented with ampicillin and used for the isolation of plasmid DNA for further analysis by restriction digestion, and for the transformation of the pET21b-UOX into the expression host, *E. Coli*, Rosetta plys S.

Figure 05
Colony PCR with gene specific primers.



Colony PCR with UOX gene specific primers.
Lane 1: Molecular marker
Lane 2 to lane 7 – Colonies from 1-6(colony 2 and 5 were positive for UOX gene specific primers).

Figure 06
Colony PCR with T7 promoter primer



Colony PCR with T7 promoter primer
Lane 1: Marker
Lane 2: Clone 5 with T7 promoter primer
Lane 3: Vector control

4. Expression of Urate oxidase

The expression vector was digested with different combination of enzyme to verify the orientation of the gene as per the Table 04.The analysis of the Agarose gel electrophoresis of the digested samples (Fig. 07) evidenced the right orientation of the coding region, and made us to move for expression of urate oxidase. The expression gel clearly showed the migration of well-expressed band at around 35 kDa, correspond to urate oxidase. Expression analysis by 12% SDS-PAGE gel showed that the uricase was expressing as cytoplasmic soluble protein, we could not notice the protein other than cytoplasmic soluble fraction(Fig: 08).

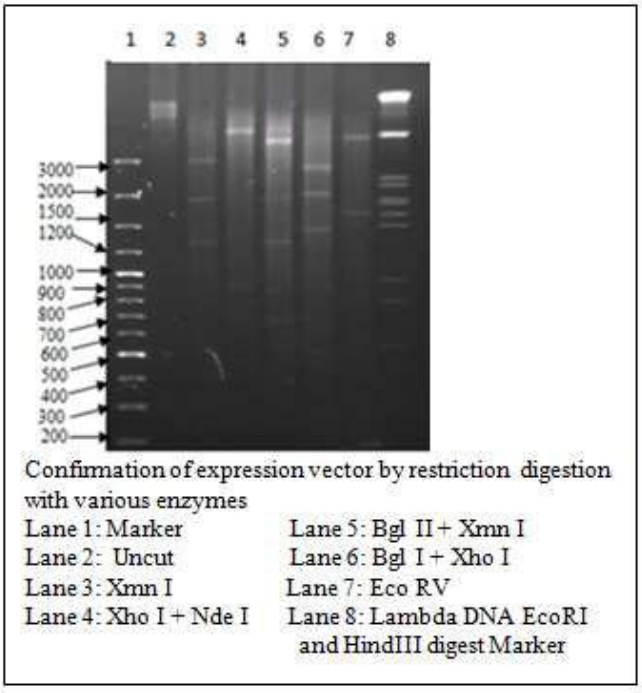
Table 04

Result of the restriction digestion of expression host with various combinations

Restriction sites used for digestion	Expected band pattern	Observed band pattern
Xmn I	3055 1934 1302	~ 3055 ~1934 ~1302
Xho I + Nde I	5364 927	~5364 ~927
Bgl II + Xmn I	2377 1934 1302 678	~2377 ~1934 ~1302 ~678
Bgl I + Xho I	2812 2050 1429	~2812 ~2050 ~1429
Eco RV	3590 1701	~3590 ~1701

Figure 07

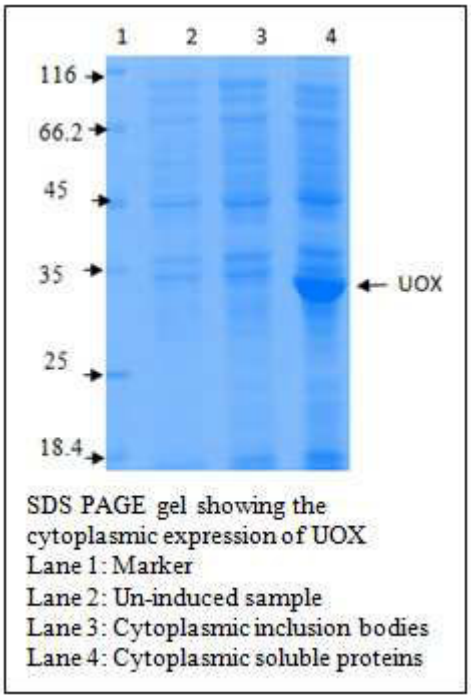
Confirmation of expression vector by restriction digestion with various enzymes



Confirmation of expression vector by restriction digestion with various enzymes

Lane 1: Marker
Lane 2: Uncut
Lane 3: Xmn I
Lane 4: Xho I + Nde I
Lane 5: Bgl II + Xmn I
Lane 6: Bgl I + Xho I
Lane 7: Eco RV
Lane 8: Lambda DNA EcoRI and HindIII digest Marker

Figure 08
SDS PAGE gel showing the cytoplasmic expression of UOX

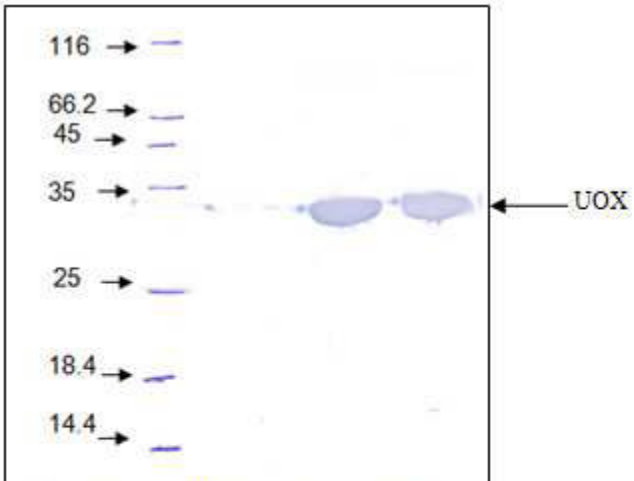


SDS PAGE gel showing the cytoplasmic expression of UOX
Lane 1: Marker
Lane 2: Un-induced sample
Lane 3: Cytoplasmic inclusion bodies
Lane 4: Cytoplasmic soluble proteins

5. Confirmation by western blotting

The western blot also asserted the expressing protein at around 35 kDa is urate oxidase (Fig**: 09**).

Figure 09
Confirmation of urate oxidase protein by western blotting



Confirmation of urate oxidase protein by western blotting
Lane 1 Protein molecular marker
Lane 2 - ---
Lane 3 - Standard
Lane 4 - UOX

6. *Optimization of urate oxidase expression in E. Coli, Rosetta plys S*

SDS-PAGE analysis of the expression gel at different biomass (Fig 10) showed that the optimal expression OD_{600} was 40, and, we noticed an abrupt drop in the expression of protein from 50 OD_{600}. Biomass at induction at 40 OD_{600} was 60 grams per liter with an expression of 40 percent. Studies from the optimal IPTG concentration (Fig 11 and 12) showed that the expression upto 0.03125 mM IPTG concentration showed the same expression levels and we finalized the induction with 0.00315 mM IPTG concentration was optimal for the induction. SDS-PAGE analysis of the expression gel for best harvesting time point (Fig 13) showed that the optimal expression was at 6^{th} hour. The samples were not collected after 7 hours of induction, since the OD_{600} of the cells was going down. Thus the optimal expression parameters for urate oxidase expression in *E. coli*, Rosetta plys S was at 40 OD_{600} induced with 0.03125 mM IPTG which was harvested after 6 hours of induction.

Figure 10

SDS-PAGE gel analysis showing optimal OD for induction.

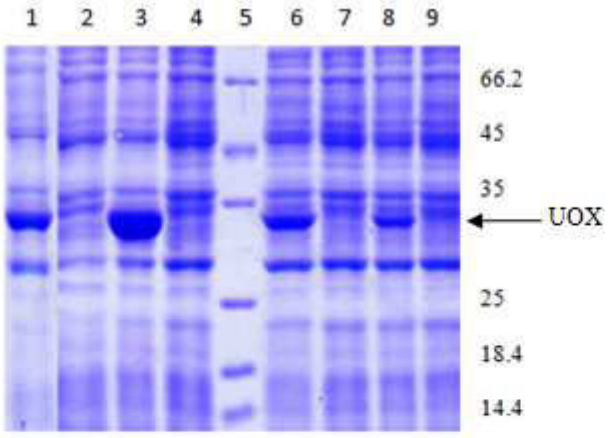
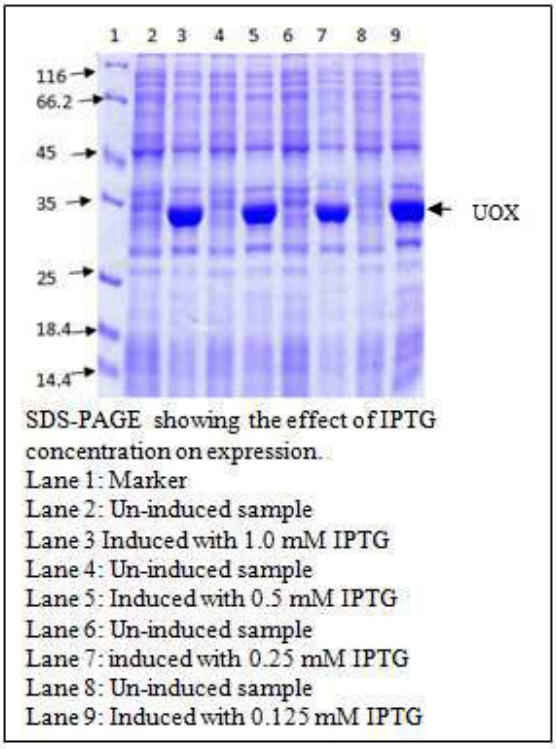


Fig 10: SDS-PAGE gel analysis showing optimal OD for induction.

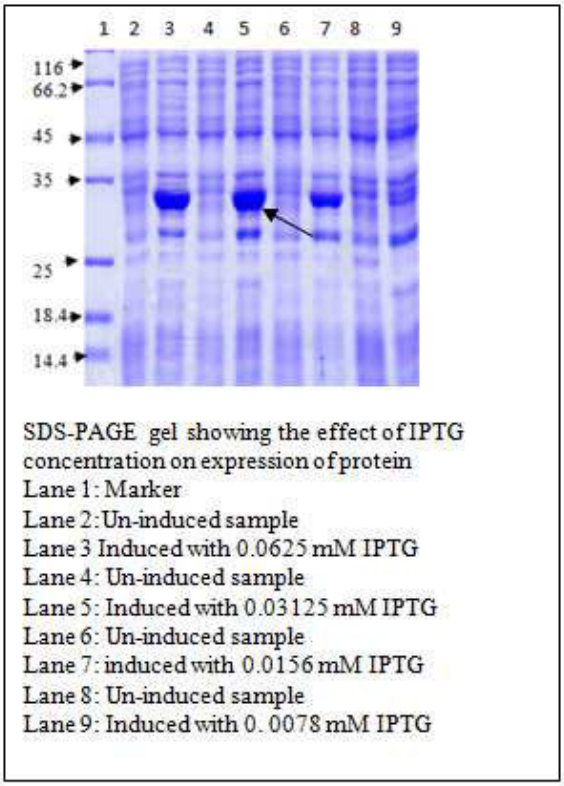
Lane 1: Induced at OD 20
Lane 2: Un-induced at OD 20
Lane 3: Induced at OD 40
Lane 4: Un- Induced at OD 40
Lane 5: Marker
Lane 6: Induced at OD 60
Lane 7: Un-Induced at OD 60
Lane 8: Induced at OD 80
Lane 9: Un-Induced at OD 80

Figure 11
SDS-PAGE showing the effect of IPTG concentration on expression.



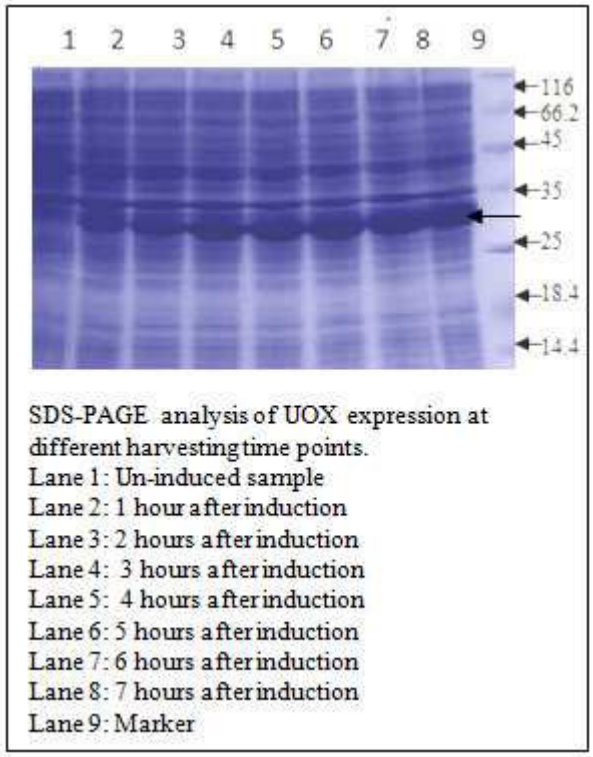
SDS-PAGE showing the effect of IPTG concentration on expression.
Lane 1: Marker
Lane 2: Un-induced sample
Lane 3 Induced with 1.0 mM IPTG
Lane 4: Un-induced sample
Lane 5: Induced with 0.5 mM IPTG
Lane 6: Un-induced sample
Lane 7: induced with 0.25 mM IPTG
Lane 8: Un-induced sample
Lane 9: Induced with 0.125 mM IPTG

Figure 12
SDS-PAGE gel showing the effect of IPTG concentration on expression



SDS-PAGE gel showing the effect of IPTG concentration on expression of protein
Lane 1: Marker
Lane 2: Un-induced sample
Lane 3 Induced with 0.0625 mM IPTG
Lane 4: Un-induced sample
Lane 5: Induced with 0.03125 mM IPTG
Lane 6: Un-induced sample
Lane 7: induced with 0.0156 mM IPTG
Lane 8: Un-induced sample
Lane 9: Induced with 0. 0078 mM IPTG

Figure 13
SDS PAGE analysis of UOX expression at different harvesting time points



SDS-PAGE analysis of UOX expression at different harvesting time points.
Lane 1: Un-induced sample
Lane 2: 1 hour after induction
Lane 3: 2 hours after induction
Lane 4: 3 hours after induction
Lane 5: 4 hours after induction
Lane 6: 5 hours after induction
Lane 7: 6 hours after induction
Lane 8: 7 hours after induction
Lane 9: Marker

DISCUSSION

Currently number of biologics used for therapeutic applications increased with an impressive manner, out of various expression hosts available for their expression, *E. coli* extends an excellent scope for the rapid and economical production of many recombinant proteins, which are intended to cure various indications in the field of diabetes, cancer, auto-immune, cardiovascular and ophthalmic areas. Cloning with the correct gene sequence and protein expression are the important event in the bio-therapeutic industry. We confirmed the clone by colony PCR, restriction digestion, expression analysis and by western blotting of the protein. All these proved the authentication of the urate oxidase, later we moved to work on the optimization of fermentation. As fed batch gives high volumetric productivities, we optimized the fermentation with respect to induction OD, concentration of IPTG, and time to harvest. The main focus to optimize all these parameters was to reduce the cost of the final product. As the production cost of the final drug product is essentially depend on the expression levels and biomass of the expression host. The fermentation was designed to run in the aerobic mode and the components of the seed and culture media were selected carefully and trace elements were also added to the media, as these elements are required for the formation of Formate hydrogen lyase complex (FHL complex). FLS complex is necessary for the conversion of toxic formic acid to CO_2 and elemental hydrogen[20]. Formic acid is produced during the oxygen limited condition in the fermenter in the high cell density fermentations. In the present study, we used Glucose as the carbon source, since it is inexpensive and readily available and in the optimized conditions[21], we achieved 60 grams of biomass with around 40 % of expression in the lab scale fermenter. The present work even

describes the common workflow to be followed during the development of a biologics at the industry. Many researchers around the world,[22,23,24] worked on the cloning and expression of urate oxidase in *E. coli* with a different source of gene, very limited number of researchers[25,26,27] had worked with *Aspergillus flavus* as the source of gene. Jianmin Li *et al.,*[26] achieved 43% of expression levels with 8.8 g/ L and Murali Krishna *et al.,*[27] achieved 20g/L of wet pellet with an unspecified amount of expression levels. In this present work, we achieved high expression levels (40%) with the highest wet pellet (60g/L) by considering all the key parameters including the composition of fermentation media and conditions. The application of recombinant technology and the fermentation media components optimization was exploited to achieve high productivity of recombinant urate oxidase.

ABBREVIATIONS

Isopropyl-thio-β-galactoside (IPTG), Tumor Lysis syndrome (TLS)

REFERENCES

1. Lee CC, X Wu, RA Giggs, RG Cook, DM Muzny, and T Caskey., Generation of cDNA probes directed by amino acids sequence: cloning of urate oxidase. Science. (293): 1288-1291, (1988).
2. Keilin,J., The biological significance of uric acid and guanine excretion. Biol. Rev. (34):265–296,(1959).
3. Motojima K, Goto S .,Organization of rat uricase chromosomal gene differs greatly from that of the corresponding plant gene. *FEBS Lett.* 264 (1): 156–158 ,(1990).
4. Rando D, Steglitz U, Morsdorf G & Kaltwasser H., Nickel availability and urease expression in Proteus mirabilis. Archives of Microbiology (154): 428-432, (1990).
5. Kida J, Kunihisa M., Studies on bacterial uricase—part I: isolation of uricase producing bacteria and some cultural conditions for production. *Journal of Fermentation Technology*. (44):789–796, (1966).
6. Frank W, Hahn GE., Uricase chungen zum bakteriellen purin uber den abbau von amino-, oxy-, and methylpurinen durch *Pseudomonas aerogenousa* (*B. pyocyaneum*) *Zeitschrift für Physiologische Chemie*. (301):90–106,(1955).
7. Suzuki K, Sakasegawa S-I, Misaki H, Sugiyama M.,Molecular cloning and expression of uricase gene from *Arthrobacter globiformis* in *Escherichia coli* and characterization of the gene product. *Journal of Bioscience and Bioengineering*. 98(3):153–158. (2004)
8. Watanabe Y, Yano M, Fukumoto J., Studies on the formation of uricase by *Streptomyces*—part I: the effect of purine bases on the induced formation of uricase by the cultured cells. *Agricultural & Biological Chemistry*. 33(2):1282–1290. (1969)
9. Tanaka A, Yamamura M, Kawamoto S, Fukui S., Production of uricase by *Candida tropicalis* using*n*-alkane as a substrate. *Applied and Environmental Microbiology*. 34(4):342–346,(1977).
10. Bongaerts GPA, Vogels GD., Uric acid degradation by Bacillus fastidious strains. Journal of Bacteriology. 125(2):689–697,(1976).
11. Alain Bayol, Joe Capdevielle, Pascal Malazzi, ArmelleBuzy, Marie Claude Bonnet, Nathalie Colloc'h, Jean-Paul Mornon, Denis Loyaux and Pascual Ferrara., Modification of a reactive cysteine explains differences between rasburicase and UricozymeR, a natural *Aspergillus flavus* uricase. Biotechnology and Applied Biochemistry 36(1): 21-31,(2002).
12. Lesley, S. A.,High-throughput proteomics: Protein expression and purification in the

post genomic world. Protein Expr. Purif. 22: 159–164,(2001)
13. Jianmin Li, , Zhao Chen, Lihua Hou, Hongyan Fan, Shaojie Weng, Chun'eXu, Jun Ren, Bing Li, Wei Che.,High-level expression, purification, and characterization of non-tagged Aspergillus flavus urate oxidase in Escherichia coli" Protein Expr. Purif. 49(1):55-59. (2006)
14. http://www.qiagen.com/products/catalog/sample-technologies/rna-sample-technologies/total-rna/rneasy-plant-mini-kit.
15. http://www.qiagen.com/products/catalog/assay-technologies/end-point-pcr-and-rt-pcr-reagents/omniscript-rt-kit
16. http://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma/Bulletin/na1111bul.pdf.
17. Laemmli, U.K.,Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, vol. 227 (5259): 680-685, (1970).
18. Alice Alegria-Schaffer, Andrew Lodge, Krishna Vattem.,Performing and optimizing Western blots with an emphasis on chemiluminescent detection. Methods in enzymology, 463:573-99,(2009).
19. Tahrin Mahmood, Ping-Chang Yang, "Western blot: technique, theory, and troubleshooting". North American journal of medical sciences. 4(9):429-34,(2012).
20. Bagramyan K, Trchounian A. Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from Escherichia coli. Biochemistry (Mosc); 68 (11):1159-70, (2003).
21. Lee SY. High cell density culture of Escherichia coli. Trends Biotechnol; 14:98-105. (1996).
22. Chen Z, Wang Z, He X, Guo X, Li W, Zhang B. Uricase production by a recombinant Hansenula polymorpha strain harboring Candida utilis uricase gene. Appl Microbiol Biotechnol. Jun;79 (4):545-54,(2008).
23. Lotfy WA. Production of a thermostable uricase by a novel Bacillus thermocatenulatus strain. Bioresour. Technol. 99: 699-702,(2008).
24. Yuichi Hongoh , Tetsuhiko Sasaki, Hajime Ishikawa.Cloning, sequence analysis and expression in Escherichia coli of the gene encoding a uricase from the yeast-like symbiont of the brown planthopper, Nilaparvata lugens .Insect Biochemistry and Molecular Biology ,173–182, (2000).
25. Legoux, R., Delpech, B., Dumont, X., Guillemot, J.C., Ramond, P.,Shire, D., Caput, D., Ferrara, P., Loison, G., Cloning and expression in E. coli of the gene encoding Aspergillus flavus urate oxidase. J. Biol. Chem. 267, 8565–8570, (1992).
26. Jianmin Li, , Zhao Chen, Lihua Hou, Hongyan Fan, Shaojie Weng, Chun'eXu, Jun Ren, Bing Li, Wei Che. High-level expression, purification, and characterization of non-tagged *Aspergillus flavus* urate oxidase in Escherichia coli. Protein Expr. Purif. 49(1):55-59, (2006).
27. Murali Krishna. Kalugonda, T. C. Venkateswarulu, Ravikanth Reddy Kosana, Chitra Bajjji,Venkat R. Konda,Anil. G, Kanumuri Radha Madhavi,Rajesh R. Tummuru And T. Srivalli. Bioprocess optimization and characterization of recombinant urate oxidase expressed in Escherichia coli. Int J Pharm Bio Sci; 4(4): (B) 608 – 616(2013).