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Production and over-expression of arginase with enhanced enzyme activity by an efficient recombinant *Escherichia coli* system

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Abstract

Our aim has been to develop an experimental system for the production and overexpression of arginase using genetically engineered recombinant DNA in transfected Escherichia coli cells. mRNA was isolated from the filamentous fungus, Neurospora crassa MTCC-858 [FGSC: 746{77:25C}], and cDNA encoding arginase was produced from it by RT-PCR. The cDNA was modified, ligated to a pARC035 vector, and transfected into E. coli GJ1158 and JM101 cells. Transformed colonies were selected by salt induction and IPTG-X-gal, respectively. Recombinant arginase from E.coli JM101 had 2.2 fold higher activity than the native enzyme, and was produced 3 times as quickly. In terms of both activity and economy, this recombinant arginase can be made available on a scale which could meet the high demand of its use as a potential therapeutic agent for treating a variety of cancers.

Keywords: Overexpression, Arginase, Genetical engineering, Recombinant technology, transfection, Cancer therapy

Abbreviations: mRNA, Messenger ribonucleic acid; RT-PCR, Reverse transcription polymerase chain reaction; cDNA, Complementary deoxyribonucleic acid; IPTG-Isopropylthiogalactoside; X-Gal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

Introduction

Arginase is the terminal enzyme of the urea cycle allowing the disposal of nitrogenous waste from protein catabolism by catalyzing the hydrolysis of arginine in the final stage of the urea cycle. The enzyme exists in 2 forms that have evolved with differing tissue distributions, metabolic functions and subcellular locations in mammals. The cytosolic form, Arginase I, is found predominantly in the liver cells and is important in ureogenesis. Arginase II is a mitochondrial enzyme that occurs mainly in extrahepatic tissues, including kidney, brain and skeletal muscle. Apart from being ubiquitously present in many mammals, there are a wide range of microbial sources of arginase, including bacteria (many *Bacilli, Agrobacterium-Rhizobium* group, cyanobacteria, *Proteus* spp.*)* [1, 2], protozoa *(Entamoeba histolytica*, and *Plasmodium falciparum*) [3, 4], yeast (*Saccharomyces cerevisiae*) [5] and fungi *(Neurospora crassa, Aspergillus nidulans, Agaricus bisporus*) [6-8]. Arginase is also found in plants (*Lathyrus sativus*, *Vitis vinifera*, *Arachis hypogea, Lycopersicon esculatum* etc *)* [9-12] though plant arginases form a separate group in the Arginase superfamily and are not much closely related (in terms of sequence homology) to non-plant (vertebrate, fungi and bacteria) arginases.

The fact that arginine deprivation has been of considerable therapeutic significance in treating different forms of cancer has been reported by many researchers [e.g.13-14]. Unlike normal cells and tissues, many different tumor cells are auxotrophic for arginine, because they cannot synthesize arginine endogenously [15-17]. The mechanism of arginine auxotrophy in these tumors is primarily related to the downregulation of the ASS gene at the transcriptional level by methylation of its promoter sequence [18]. In vitro arginine depletion, either through arginase or use of arginine-free medium, leads to rapid tumor death in a wide range of tumor cell lines because of the high dependency of cells on an adequate supply of this amino acid[19].

The *in vitro* anti-tumor activity of arginase has been well documented since the 1960s [20]. Arginase released from lipopolyssacharide and zymosan-stimulated macrophages can be responsible for the death of V79 Chinese hamster lung cells, L5178Y lymphoma cells, and HSN hooded rat sarcoma in culture [21]. Murine and bovine liver arginases led the total destruction of lymphosarcoma cells when arginine was reduced to <8 μmol over 24 h [22]. Chemically modified arginase II has been employed for treatment of Taper liver tumor and the L5178Y murine leukemia [23]. Single amino acid (arginine) restriction produced by arginase resulted in death of cultured HeLa while human diploid fibroblasts moved in quiescence and were largely protected [24]. The pharmaceutical preparation and method of treatment of human malignancies with arginine deprivation by constructing a recombinant strain of *B. subtilis* LLC101 containing human arginase I gene has been established as a new treatment of liver cancer (hepatocellular carcinoma) [25]. Recombinant *Bacillus subtilis* arginase BCT-100 is cytotoxic to human melanoma cells and thus has become a novel anti-melanoma agent [26]. Inhibition of *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma by pegylated recombinant human arginase (rhArg-peg5,000mw) through arginine depletion has been described in recent reports which represents a major breakthrough in establishing the therapeutic value of arginase [27].

Due to its immense potential in clinical applications, the development of recombinant arginase is being researched worldwide. We have designed a system for the production of recombinant arginase by transforming *E. coli* cells with cDNA of *Neurospora crassa* encoding arginase. *Neurospora crassa* is a good source of arginase with two forms of the enzymes being expressed, which is the only reported example of multiple forms of arginase in a microbial organism [28]. When seen in the phylogenetic tree of the arginase superfamily, arginase from *Neurospora crassa* is more closely related to human arginase II than arginase I [12]. Both its arginase proteins are stored in conidia [29] which are spheroid cells, with an average of 2 to 3 identical nuclei and are basically regarded as units of dispersal.

Materials and Methods

Microbial strains: procurement and maintenance

Fungal Strain: The fungal strain *Neurospora crassa* MTCC-858 [FGSC: 746{77:25C}] was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. It was maintained at 25 ^{0} C in the specified Vogel's Minimal Medium with 2% sucrose as the carbon source. It was used for the extraction of mRNA and generation of cDNA from it by employing RT-PCR.

Bacterial Strains: The bacterial strains used were *Escherichia coli* GJ1158 and JM101, obtained from Genei Pvt, Ltd., (Bangalore, India) and maintained at a temperature of 37º C in the specified media.

GJ1158: LB Media minus NaCl, i.e. (LB/ON) Media. (Yeast Extract-5g, Tryptone-10g, pH-7.2, incubation time 24h) for transformation and LB Media (Yeast Extract-5g, Tryptone-10g, NaCl-10g, pH-7.2, incubation time 24h) for growth.

JM101: M9 Medium-5X (30g-Na_2HPO_4, 15g-KH_2PO_4, 5g-NH_4Cl, 2.5g NaCl, 15ml $CaCl_2$, pH-7.4) for revival, YPT Medium-2X [16g-Tryptone, 10g-Yeast Extract, 5g-NaCl, 20g Agar(for plates), 7g-Agar(Soft Agar)] for transformation and LB Media (Yeast Extract-5g, Tryptone-10g, NaCl-10g, pH-7.2, incubation time 24h) for growth.

Isolation of RNA from Neurospora crassa

To conidia harvested from the aerial hyphae, 1 ml sterile water was added and agitated in a vortex mixer for 5 min. Free conidia were collected and counted in a Neubauer Chamber under a compound microscope. Liquid culture medium was placed into 250-ml Erlenmeyer flasks and inoculated with 10^6 conidia per ml.

Isolation of the RNA involved the method of Sokolovsky *et al.* [30] and purity of the preparations was checked by spectrophotometric analysis. The A260/A230 and A260/A280 ratios were 2 or greater, indicating the absence of protein and polysaccharide contaminants. The isolated RNA was used for the generation of cDNA by RT-PCR.

Generation of cDNA through RT-PCR

The generation of double-stranded complementary DNA (cDNA) from crude mRNA by RT-PCR involves 2 successive enzyme reactions using reverse transcriptase and DNA polymerase. The primers used for the RT-PCR step were designed from translated regions of published cDNA gene sequences encoding *Neurospora crassa* arginase:

Sense primer: 5'-atgtcttccccaatcgagtcc-3'

Antisense primer: 5'- tcatagaacgtttcgccggcg -3'

These oligo primers were synthesized by Genei Pvt, Ltd., (Bangalore, India). The generated cDNA was amplified through PCR using the following cycles:

39 cycles	:	1min 94 ^{0}C (denaturation)
		2min 57 ^{0}C (annealing)
		2min 72 ^{0}C (extension)
1 cycle	:	7 min 72 ^{0}C (final extension)

Restriction Digestion of cDNA with Sal I

Amplified cDNA was subjected to restriction analysis by digesting it with Sal I (Genei Pvt, Ltd., Bangalore) that has recognition sequence 5'-*G↓TCGAC*- 3' to check its amplification. Agarose gel electrophoresis visualized the synthesized cDNA as well as the restriction-digested cDNA. The synthesized cDNA was used for subsequent genetic-engineering experiments to develop recombinant *E. coli* cells for the production and over-expression of arginase.

Transformation of E. coli cells

Experiments were initially conducted with *E. coli* GJ1158 as a host and subsequent attempts involved JM101 cells. For the development of recombinant cells, 10µl EcoRI phosphorylated linker (8-mer) [New England Biolabs, Massachussets, USA] was ligated [31] to 10µl blunt-ended cDNA with the help of 0.2 U ligase, in a fresh Eppendorf tube containing 2µl 10x ligase buffer). The contents of the tube were mixed and kept for 12 h at 4ºC. To generate EcoRI overhangs, 10 µl of the constructed sample were hydrolyzed with 5 U EcoRI restriction enzyme in 2µl of 10x restriction buffer and incubated for 1 h at 37 ºC. The digestion was stopped by heating at 65 ºC for 10 min. The pARC 035 vector (10 µl) was digested with EcoRI

restriction enzyme as above, followed by its dephosphorylation with 1U alkaline phosphatase in 5 µl 10x alkaline phosphate buffer followed by incubation at 65ºC for 1 h.

The next step included the ligation and cloning of the modified cDNA (10 µl) into 10 µl pARC 035 vector linearized with 5 µl EcoRI in 10x ligation buffer by typical ligation method using 10 U T_4 DNA ligase followed by incubation at 12 ºC for 12 h. For bacterial transformation, techniques developed previously involving the transformation of $CaCl_2$-treated *E .coli* with plasmid DNA were employed [32, 33].

The two strains differ in the induction and selection of recombinant colonies.

GJ1158: Escherichia coli GJ1158 cells were grown in Luria Bertani media overnight. 1 day-old cells were reinoculated into more of the same medium lacking NaCl (LB/ON), the pH being maintained at 7.2. Cells were harvested in sterile centrifuge tubes after 24 h and made competent with $CaCl_2$ solution. Transformation of $CaCl_2$-treated cells with recombinant material was done by previously reported methods [34]. Ampicillin (100 g/ml) and sterile NaCl at a final concentration of 250 mM were used for induction, and the plates were incubated at 37^0C. Colonies appearing after 2-3 days were selected and inoculated into LB media.

JM101: $CaCl_2$-treated JM101 cells were prepared by first growing them in M9 medium for 24 h and reinoculating them in YPT medium [35]. cDNA and pARC 035 vector were added to these cells, which were kept on ice but subsequently heat shocked at 42^0C for 90 sec. To this transformation mixture 25 mg/ml of IPTG-XGal were added. This was quickly vortexing and poured onto the surface of prewarmed YPT agar plates, which were incubated at 37˚ C. Colonies that appeared after 2-3 days were checked for transformants [36-38].

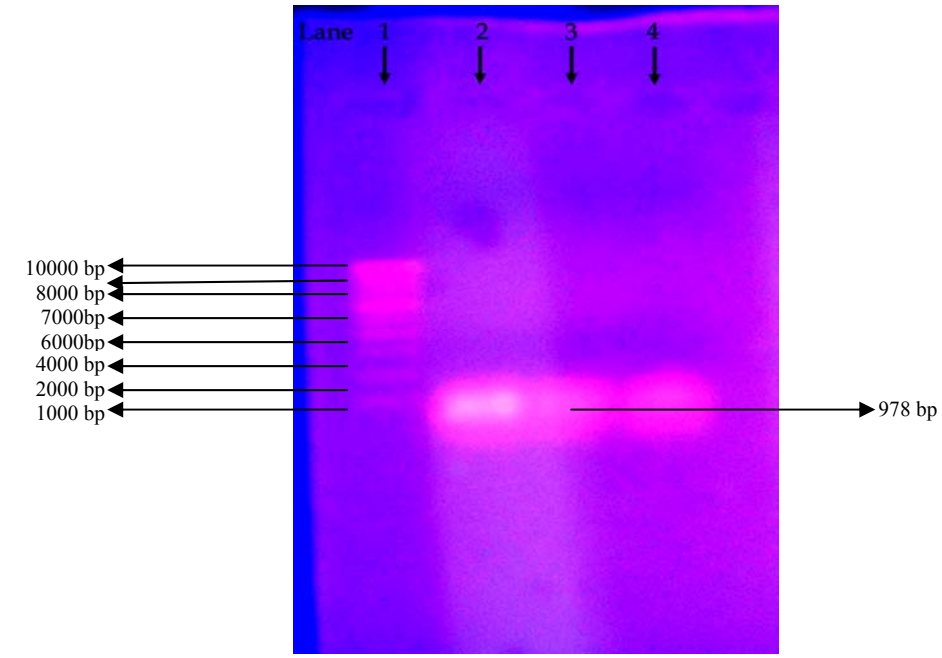
The transformed colonies were grown in LB media before being collected in 0.9% NaCl at 4^oC and sonicated at 50% intensity for 2 pulses of 25 s and 10s. After centrifugation at 5,000 rpm for 20 min at 4^0C), supernatants were retained on ice or frozen at -20˚C until arginase activity was assayed. The pellets contained no arginase activity.

Enzyme activity of recombinant arginase was determined by the colorimetric assay method of Archibald [39] of the urea product released from the arginine substrate along with ornithine, and compared with that of the native enzyme of *Neurospora crassa*.

Results and Discussion

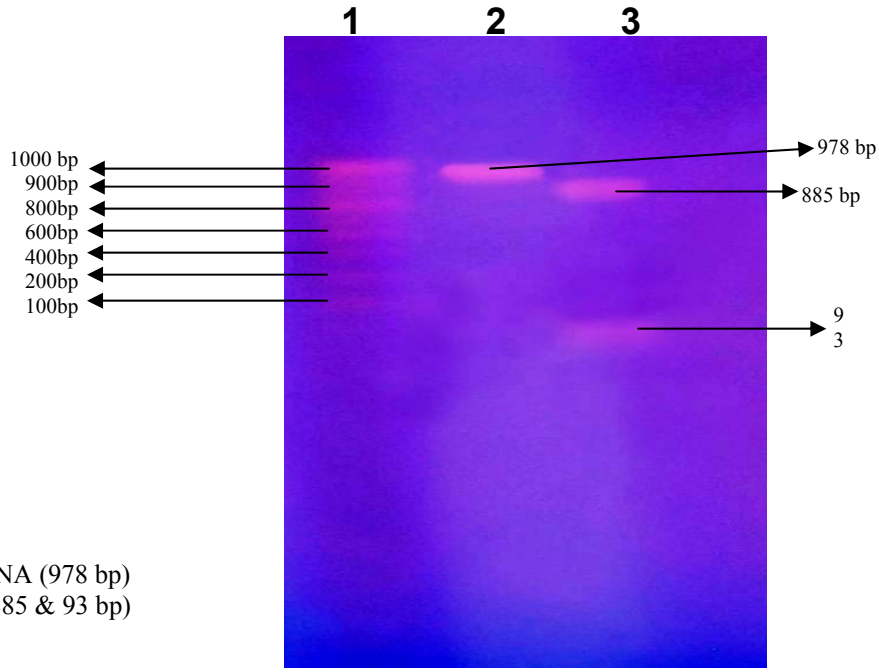
We have established an experimental system based on recombinant DNA for the biosynthesis of recombinant L-arginase derived from *N. crassa* in *E. coli.* The following summarises the findings:

1. The cDNA synthesised by RT-PCR was ~978 bp. It was used for the transformation of *E. coli* strains GJ1158 and JM101.
2. It was cut during restriction digestion by Sal-I into 2 fragments of ~885 and ~93 bp.
3. When recombinant arginase obtained from GJ1158 and JM101 was assayed for activity, the enzyme from JM101 was ~2.2 fold greater than that of the native enzyme from *N. crassa*. The activity of the GJ1158 enzyme was closer to that of the native enzyme.



Lane 1- Marker (1000bp)
Lane 2, 3&4- PCR Amplified cDNA (978 bp)

Figure 1. Visualization of cDNA generated from mRNA through RT-PCR.



Lane.1-Marker(100bp)
Lane 2 - PCR Amplified cDNA (978 bp)
Lane 3 - cDNA fragments (885 & 93 bp)

Figure 2. Visualization of different cDNA fragments resolved after Sal I digestion.

The compatibility of the vector pARC035 used in the experimental procedure to transform JM101 has been reported in a patent application (entitled "Plasmid vector in *E. coli*" [40]. One reason for the better transformation in JM101 than GJ1158 may be the superior compatibility of the vector in the former strain. Intracellular urea formation data as a

function of time in JM101, induced with IPTG-X-gal and incubated at varying time s following incubation, are shown in Figure 3.

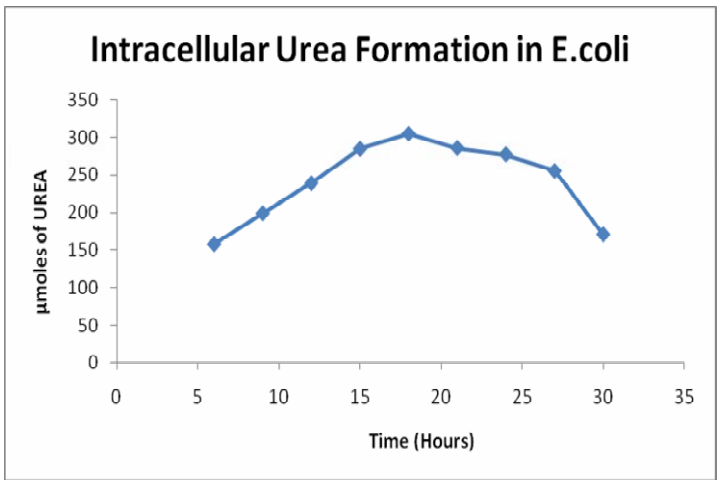


Figure 3. Intracellular urea formation as a function of time in *E. coli* JM101

A comparison of the time-frame for arginase production in native and recombinant strains is shown in Figure 4.

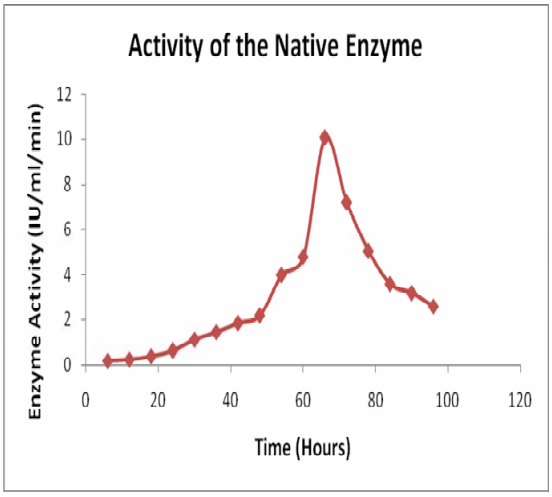


Figure 4 (a). In native

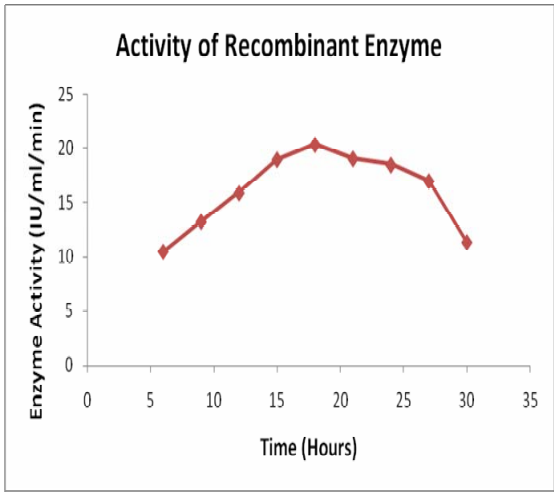


Figure 4 (b). In recombinant strains

Figure 4. Comparison of the time-frame for arginase production.

Arginase has become a particularly promising chemotherapeutic agent that reduces the essential requirement of this amino acid in cells that lack the endogenous elements of the urea cycle allowing the conversion of citrulline into arginine. Tumor cells are especially vulnerable, and this is the basis of the selective action of this protocol. Furthermore, no further action needs to be taken for the tumour cells to succumb to the effects of deprivation, the majority dying within several days of arginine restriction [27], In particular tumor defective in their ‘‘R’’ checkpoint in G1 or other cell cycle aberrations continue to cycle in the absence of arginine, and the gross imbalance induced contributes to cell death [41]. The need for recombinant arginase products as this protocol becomes increasingly of therapeutic

value has been the driving force behind the design of the present study aimed at over-expressing arginase.

In conclusion, since the recombinant arginase produced by transforming *E. coli* JM101 cells with cDNA of Arginase I of *N. crassa* has ≥ 2-fold more activity than the native enzyme and can be produced in a third of the time required for native enzyme production, we see this as an efficient and economic means of obtaining the product by a means that can be scaled up in suitable bioreactors for its rapid commercialization as a effective anti-cancer agent.

Table 1. Comparison of Enzyme Activity of native and recombinant arginase

Strain	O.D. $_{540}$	Enzyme activity (U/ml/min)	% Increase in enzyme activity
***Neurospora crassa* [FGSC:746{77:25C}]**	0.391	10.06	----------
***E. coli* GJ1158**	0.451	11.56	15
***E. coli* JM101**	0.791	20.33	102

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