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BIOTECHNOLOGY

INTRACELLULAR EXPRESSION AND PURIFICATION OF A RECOMBINANT ENZYME INVOLVED IN BIOCONVERSION OF ARTEANNUIN B TO ARTEMISININ IN *ESCHERICHIA COLI* EXPRESSION SYSTEM

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Abstract

Streptomyces pactum produces an enzyme that converts Arteannuin B to Artemisinin that is an important antimalarial. The gene encoding this novel enzyme from *Streptomyces pactum* MTCC 3664 has been cloned and expressed in *Escherichia coli* BL21 (DE3). The enzyme is expressed in the form of inclusion bodies at 37^{0}C or in a soluble form at 28^{0}C. The recombinant enzyme was purified by Immobilized Metal Ion Affinity Chromatography (IMAC). The purified enzyme produced by constitutive expression was found to be functionally active as it showed the conversion of arteannuin B to artemisinin as detected by TLC and HPLC.

Keywords: *Escherichia coli* BL21 (DE3); Cloning; Constitutive expression; Purification; enzyme; Arteannuin B; Artemisinin

Introduction

Malaria is the world's most important parasitic infection. Malaria is caused by infection of red blood cells with protozoan parasites of genus *Plasmodium.* In humans, the infection is caused by one or more of four species of the intracellular protozoan parasite. *P. falciparum, P. vivax, P. ovale* and *P. malariae.* The most critical problem currently limiting malaria treatment is the emergence and spread of parasite resistance to the majority of antimalrial drugs in use (Woodrow *et al*., 2005). The frequent failure of the conventional treatments using drugs such as chloroquine and sulphadoxine-pyrimethamine (Trape, 2001, Olliaro and Taylor, 2003) have led to the emergence of combination therapies (CTs) particularly, those formulations containing artemisinin or its derivatives (ACTs) as a more reliable treatment option (Duffy and Mutabingwa, 2004; Woodrow *et al*., 2005). The use of ACTs in the treatment of malaria has been recognized as a long term measure to control spread of the disease by the World Health Organization (WHO) under its RBM program (WHO, Access to antimalarial medicines, 2003). Artemisinin exhibits a quick onset of action and high efficacy against the blood stages of *Plasmodium* (ter Kuile *et al*., 1993). They have also been shown to reduce the number and infectivity of gametocytes in the blood (Kumar and Zheng, 1990; Kombila *et al*., 1997; Chen *et al*., 1994 and Targett *et al*., 2001).

Artemisinin (Fig.1) is a sesquiterpene lactone produced from *Artemisia annua* Artemisinin was isolated in pure form in the year 1972 from *Artemisia annua* (Christen and Veuthey, 2001) and in 1979 its structure was elucidated by X-ray analysis (van Agtmael, *et al*., 1999). The structure of this compound is rare among secondary plant metabolites in that it harbors a peroxide bond in 1,2,4-trioxane heterocycle (Avery et al., 1992). *Artemisia annua* is the only practical source of artemisinin but the concentration of artemisinin in *A, annua* is very low, in the range of 0.01% – 0.8%. A relatively low yield of artemisinin is a serious limitation to the commercialization of the drug (Laughlin, 1994). The structure of artemisinin molecule is highly complex and the total chemical synthesis of artemisinin is uneconomical (Yadav *et al*., 2003). Among the various attempts that have been made to enhance the production of artemisinin, biotransformation approach has been identified as a potential alternative. Arteannuin-B is a biogenetic precursor of artemisinin in certain cultivars and is found in higher amounts than the end product in *Artemisia annua* (Nair and Basile, 1993, Singh, 1986 and Akhila *et al*., 1990). Arteannuin-B (Fig. 2) is a sesquiterpene with an unusual a-methylene-γ-lactone (Woodrow *et al*., 2005). Biotransformation of arteannuin B to artemisinin has been carried out with crude leaf homogenates of *Artemisia annua* (Nair and Basile, 1993; Vikas Dhingra, *et al*., 2000). In our laboratory we have adopted the microbial biotransformation approach to enhance the production of artemisinin, as microorganisms are more versatile and easy to handle. We have isolated a *Streptomyces* sp. With the ability to convert arteannuin

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B to artemisinin by screening various soil samples. The enzyme responsible for this bioconversion activity has been purified to homogeneity. The purified enzyme has shown a 78.8% bioconversion of arteannuin B to artemisinin on molar basis. Our studies have shown that microbial biotransformation approach is a very viable and potential alternative to enhance the production of artemisinin.

Fig.1 Artemisinin

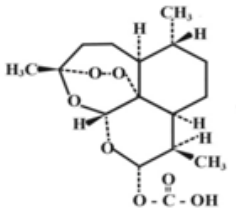
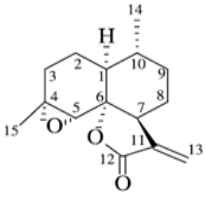


Fig.2 Arteannuin B



The strain, BL21 (DE3), is one of the popular strains for the protein expression. BL21 (DE3) contains the λDE3 lysogen, which expresses T7 RNA polymerase under the control of the lacUV5 promoter1). Upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG), T7 RNA polymerase is expressed and it induces a high-level protein expression from T7 promoter driven expression vectors (e.g., pRSET A). *E. Coli* BL21 (DE3) strain is a derivative of *E. coli* B strain and lacks both the lon protease and the ompT membrane protease which may degrade expressed proteins (Andreishcheva *et al*., 2006).

The present perspective of the study is to express and purification of the enzyme which involved in the bioconversion of arteannuin B to Artemisinin.

Materials and Methods

Strain and Reagents

Streptomyces pactum MTCC 3664 strain was isolated in our lab and subsequently deposited in Microbial type culture collection (MTCC). All Media and analytical grade chemicals were purchased from Hi media and sigma.

Purification of the enzyme involved in Bioconversion

Purification of the enzyme involved in bioconversion was attempted using different methods such as ammonium sulphate precipitation, ion exchange chromatography, affinity chromatography and gel-filtration chromatography. The fraction of proteins precipitating between 0-50% ammonium sulfate saturation was further purified by passing through Q-sepharose, an anion-exchange column using 50mM Tris buffer (pH-7.2). Active fraction eluting at 0.3 M Sodium chloride was further purified by gel-filtration chromatography on Sephacryl-S-100 HR column. Proteins were eluted with 50mM Tris-HCl (pH 7.2) with a flow rate of 12 ml/h. SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) was performed according to Laemmeli (1970), using 10% polyacrylamide gels in amersham gel electrophoresis unit (Amersham Biosciences, Minnesota USA). The active fraction obtained from gel-filtration chromatography was subjected to SDS-PAGE analysis. Electrophoresis was carried out at a constant voltage of 75 v/cm at room temperature. Electrophoresis was carried out until the tracking dye reached the end of the gel. Standard medium range molecular weight markers (Medium range Genei, Bangalore, India) were also electrophoresed along side for reference. Comassie brilliant blue R250 staining was used to visualize protein bands on the gels (Morrissey 1981). The molecular mass of the purified enzyme was determined by comparison of its electrophoretic mobility with that of marker proteins (Fig. 3).

N- Terminal and MALDI TOF analysis of the purified enzyme

The band of interest was transferred from the SDS-PAGE gel to PVDF membrane by using Western blotting. The protein sample derived from PVDF membrane was subjected to chemical modifications (reduction and alkylation). The protein sample was digested with trypsin. The MALDI probe was inserted into the Mass Spectrometer (Bruker & Ultraflex) and mass spectrum was acquired using flex control™ 2.2 software (Webster, 2005). Peptide mass finger printing of the purified enzyme by MALDI-TOF has showed it in Fig.4 & 5.

Design of suitable primers

The forward primer was designed by using N-Terminal sequence (MANDAST), it is corresponding to nucleotides 1 to 21 and the restriction cleavage sites for *Xho1* as under lined were included. The reverse primer was designed by using MALDI-TOF sequence it is complementary to the nucleotides 1258 to 1278 in gene. The restriction cleavage sites for *Nco1* as shown in bold were included in reverse primer.

5'-ATCGCTCGAGACCGTTTTCGACCTAGTGA-3'

5'-ATCGCCATGGCTAGTCGTGGGCGTGCAG-3'

The length and melting temperature of the oligonucleotide have been kept sufficiently high of 62^0C to avoid any non-specific amplification.

Cloning and expression

Polymerase Chain Reaction (PCR)

Preparative PCR was carried out in 500 µl reaction (10 × 50 µl) for 25 cycles using Taq DNA polymerase and Pfu™ DNA polymerase (Stratagene, USA, which has 3'-5' exo nuclease proof reading activity) in a ratio of 24:1, in a GeneAmp 9700 thermal cycler (Perkin-Elmer Applied Biosystems). The reaction was set up as shown in Table 1 and 50 µl of the reaction mixture was distributed into ten PCR tubes.

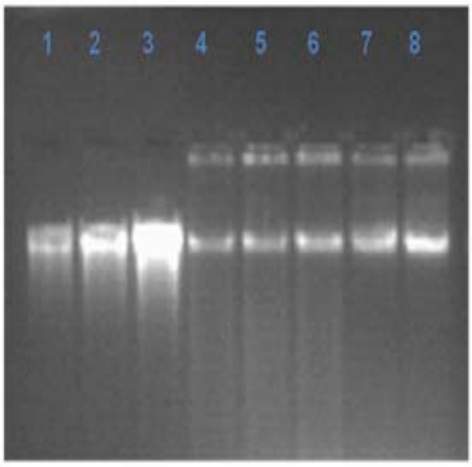
Table 1: PCR reaction for amplifying JIC gene

Master mix components	Volume in µl for 1 reaction	Final volume in µl
Template DNA (50 ng)	1.0	10.0
Forward primer (10 pmoles /µl)	2.0	20.0
Reverse primer (10 pmoles /µl)	2.0	20.0
dNTP's Mix (2 mM)	5.0	50.0
Pfu Polymerase Buffer	5.0	50.0
Taq and Pfu Polymerase (24:1)	1.0	10.0
Distilled water	34.0	340.0
TOTAL	50.0	500.0

The initial denaturation at 94^{0}C for 3 min was followed by 25 cycles of PCR involving denaturation of the template DNA at 94^{0}C for 30 sec, annealing of the primers to the template DNA at 62^{0}C for 40 sec and extension of the annealed primer at 72^{0}C for 60 sec. For final extension the block temperature was kept at 72^{0}C for 10 min. The block was finally allowed to cool down at 4^{0}C.

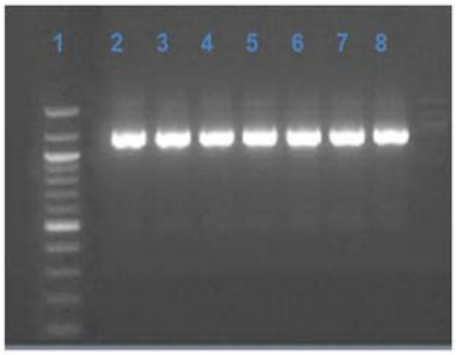
A proof reading thermo stable enzyme Pfu ™ DNA polymerase was used in association with Taq DNA polymerase for PCR, which helped in avoiding any misincorporations. The primer could successfully amplify the gene as evidenced by agarose gel electrophoretic (Sambrook., 2001) analysis of PCR amplified product (Fig.7) .

Fig. 6 Genomic DNA



Lanes 1-3 Lambda DNA (50ng/µl);
Lanes 4-8 Genomic DNA

Fig. 7 PCR amplification of the desired gene



Lane 1 NEB Ladder (100bp);
Lanes 2-8 PCR amplified product

Genomic DNA (Fig.6) from Streptomyces *pactum* was isolated by the improved high salt method and was subjected to polymerase chain reaction (PCR) by forward and reverse primers based on the N-Terminal and MALDI-TOF analysis a set of specific primers were designed by using primer 3 software. After purification by running 1.2% agarose gel electrophoresis for 1 h at 10 V/cm, the PCR amplified product was digested with *Xho1* and *Nco1*(Invitrogen) and cloned into pRSET A vector (Novagen, Madison, WI)

The expression plasmid pRSET A- containing the gene was transformed into competent *E. coli* BL21 (DE3) (Novagen, Madison, WI). Multiple hosts containing appropriate construct were first grown on LB plates supplemented with 100 mg/ml Ampicillin at 37^{0}C overnight. Several single colonies were seeded into 5 ml liquid LB (Ampicillin 100 mg/ml) and cultured at 37^{0}C overnight. Then the strain expressing the gene insert was inoculated (1:100) into 500 ml shake flask with 100 ml of LB medium containing 100 mg/ml Ampicillin, which was placed on a incubated shaker at (180 rpm) at 37^{0}C till the OD_{600} reached 0.6. Later the

cells were induced by 0.5mM and 1.0mM IPTG and cells were grown at 37, 30, 28, and 23^{0}C. Aliquots of the culture were withdrawn at different time intervals (1,2,3,4,5,6 and 10 h) and collected by centrifugation. The pellets were either prepared immediately for SDS-PAGE or stored at -80^{0}C for the analysis of protein expression.

Purification of Recombinant protein

Cells were harvested from 500 ml LB broth culture by centrifugation (8000 rpm, 15 min) and the pelleted bacteria were resuspended in 100 ml cold lysis buffer (50 mM NaH_2PO_4, 300 mM Nacl, 10 mM imidazole, pH 8.0; one tablet of protease inhibitor complete EDTA-free (Roche);10 µg/ml RNase A (Sigma); 5 µg/ml DNase 1 (Sigma),0.1 mg/ml lysozyme (Sigma)). The cell lysate was disrupted by sonication on ice for 10 minutes with the following parameters i) sonication power of 750 watts; ii) pulse duration 9 sec with 5 seconds gap between pulses and cell debris was removed by centrifugation (9000 rpm for 20 min at 4^{0}C). The soluble lysate supernatant and insoluble cell debris were analyzed by SDS-PAGE.

For purification of the soluble enzyme, an Immobilized metal ion affinity chromatography (IMAC) purification method was followed. Firstly, the soluble lysate supernatant was transformed to a new sterilized Falcon tube by filter (0.45 µm) and mixed gently with 5 ml of Ni-NTA His bind equilibrated with lysis buffer (described above) by shaking at 4^{0}C for 1 h. then the supernatant/resin mixture was located to chromatography columns equilibrated with five column volumes lysis buffer, allowing bound resin to pack under gravity flow.

The column bed was washed with 10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.0 using NaOH; one tablet of protease inhibitor complete EDTA-free (Roche); 10 µg/ml RNase A (Sigma); 5 µg/ml DNase 1 (Sigma); 0.1 mg.ml lysozyme (Sigma)) to remove hybrid proteins. Subsequently, the fusion protein was eluted with an imidazole gradient (50-250 mM) in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The eluted protein was analyzed by SDS-PAGE and dialyzed against buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) to remove imidazole at 4^{0}C overnight.

Determination of Protein activity

Bioconversion Assay:

Incubation system: The enzyme activity was assayed by incubating a mixture of 3 ml of purified JIC enzyme with 100 µg of arteannuin B, and the co-factors: 0.1mM ATP, 1 mM Mg^{+2} , 1 mM Mn^{+2} , and 0.1 mM NADPH in 50 mM Tris-HCL buffer (pH 7.2) for a period of 3 h at 30^{0}C. Parallel to this, a control was run with all other than components except the substrate (Nair & Basile, 1993).

Extraction of artemisinin: On completion of incubation time, the reaction was stopped by adding ethanol and chloroform in 1:1 proportion. Artemisinin was extracted twice from the reaction mixture with hexane in a separating funnel. The hexane fraction was collected in a separate collection beaker and was allowed to evaporate.

Detection of artemisinin by TLC: The residue was dissolved in methanol and was tested for the presence of artemisinin by performing thin layer chromatography (TLC) on silica gel 60 plates (Merck) with a mobile phase of ethyl acetate and hexane in a ratio of 2:8. The authentic sample of arteannuin B and artemisinin were spotted on TLC plate together with control and experiment. The plates were kept in vertical position in solvent system in a closed chamber saturated with solvent vapors. After allowing the solvent system to run about ¾ on the plate, the plate was removed and dried. The plate was sprayed with iodine vapors for visualization of spots.

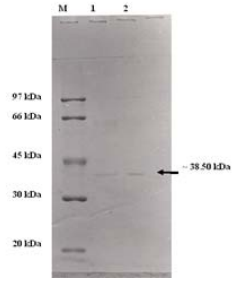
Quantification: Artemisinin produced by bioconversion of arteannuin B using crude enzyme of *E. coli* BL21 (DE3) was quantified by HPLC (Gupta *et al*., 1997). HPLC was performed on Shimadzu Prominent series using a Kromasil 100, C 18 column (150 X 4.6 mm, ODS with particle size of 5 µm) with a mobile phase of 1% (v/v) TFA in water: acetonitrile (30:70), at a flow rate of 1 ml/min. Artemisinin was monitored at 220 nm with a PDA detector (Shimadzu: SPD M20A). The chromatographic system was controlled by LC solutions (version 1.2) software. Filtered samples of standard artemisinin (Sigma-Aldrich) (1mg/ml), arteannuin B (0.5mg/ml), and the methanolic extract of the experimental sample were loaded on to the column. The retention time for artemisinin was 6.3 min and that of arteannuin B was 5.4 min. One unit (U) of enzyme activity was defined as the amount enzyme that catalyzed the conversion of arteannuin B to artemisinin per min at 30^{0}C.

Results

Purification of the enzyme

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) was performed according to Laemmeli (1970), using 10% polyacrylamide gels in amersham gel electrophoresis unit (Amersham Biosciences, Minnesota USA). The active fraction obtained from gel-filtration chromatography was subjected to SDS-PAGE analysis.The purified enzyme shows the molecular weight of 38.5 kDa.

Fig 3. SDS-PAGE analysis of purified JIC protein involved in bioconversion MALDI Characterization



The amino acid sequence obtained as per MALDI TOF analysis revealed that the enzyme under consideration is a dioxygenase with 46% sequence similarity to the putative dioxygenase subunit alpha yeaW protein of *Mycobacterium marinum* M. As the match percentage is less it is presumed to be a hitherto unknown protein

Fig 4. MALDI-TOF analysis. The peaks represents the fragments generated by Trypsin digestion of JIC protein

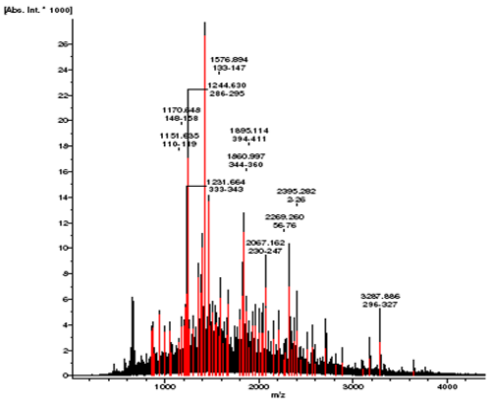
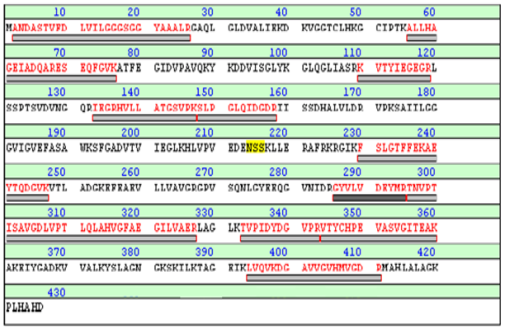


Fig 5. Trypsin finger print of JIC protein



Expression and purification of JIC enzyme encoding gene

The encoding gene and the pRSET A vector were transformed into competent *E. coli* BL21 (DE3) as described in materials and methods section. Based on previous publications about protein expression (Colleluori *et al*., 2005 and Xiong *et al*., 2006), preliminary expression experiments of JIC encoding gene were done in shake flask with or without IPTG induction at 37^{0}C. At this temperature, a band corresponding to JIC protein with the expected molecular weight (Fig.8) was shown for the expressing strain with IPTG induction. However, the majority of protein was accumulated in insoluble form, the minority in soluble form. Subsequently, by performing the expression experiments at 37, 30, 28, and 23^{0}C, the soluble fraction was remarkably increased (Fig.9), especially at 28^{0}C, the soluble JIC protein arrived at 88% of the total cellular protein from a 500 ml culture (Fig.10 & 11), which was determined by densitometric scanning of SDS-PAGE gels, and the inclusion bodies were correspondingly decreased. This suggest that a lower temperature is beneficial for expression of JIC protein in the form of soluble fraction in *E. coli.*

Fig.9 Fig.8

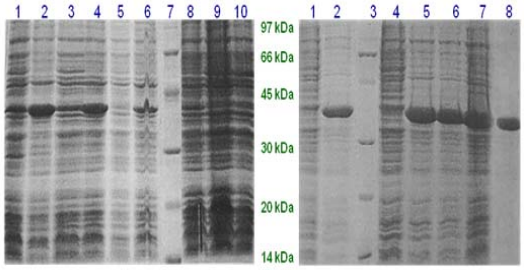


Fig.8 Lanes 1,3,5,10- uninduced cell extracts of E. coli cells from 4 colonies; 2,4,6,8,9- induced cell extracts of E. coli cells from 5 colonies; 7-MWT marker

Fig.9 Lanes 1- uninduced; 2- induced; 3- MWT marker; 4- Cell lysis supernatant; 5- Cell lysis pellet;6- 6M Urea solubilisation pellet; 7- 6M Urea solubilisation supernatant

Fig.11 Fig.10

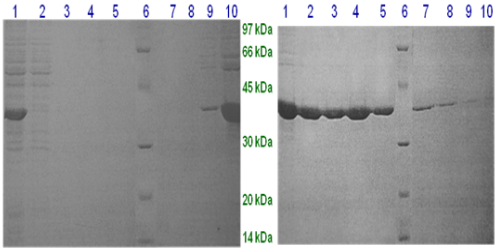


Fig.10 Lane 1- load; 2- flow through; 3 to 5- washing; 6- MWT marker; 7- elution fraction (50 mM Imidazole); 8,9- elution fractions (100 mM Imidazole); 10- elution fraction (125 mM Imidazole)

Fig.11 Lane 1 to 3- elution fractions (150 mM Imidazole); 4,5- elution fractions (175 mM Imidazole); 6- MWT marker; 7,8- elution fractions (200 mM Imidazole); 9,10- elution fractions (250 mM Imidazole)

To determine initial seed age with the highest yield of protein, the expression levels at 28^{0}C were compared between cultures at OD_{600} of 0.4, 0.5, 0.6,

0.7, 0.8, 0.9, 1.0 followed by IPTG induction, respectively. The results of SDS-PAGE analysis for whole cells indicated that the cell cultures grown at OD_{600} of about 0.6 results in the highest yield. The time of harvest had a less significant effect on the protein yield which was achieved after 3 h. Moreover, the yield of JIC protein was not significantly improved when increasing IPTG concentration from 0.5 to 1.0 mM, the highest yield of JIC protein was achieved at 0.5 mM/l.

Fusion tags play a key role in the expression and purification of recombinant proteins. Fusion tags permit specific detection and purification strategies for proteins and enable measurements of recombinant protein solubilities and expressed protein quantities. The His-tag is most commonly used for affinity purification, has a small size and strong binding affinity to immobilized metal ion affinity chromatography matrices under both native and denaturing conditions. In this study, the nickel-affinity chromatography was performed with the supernatant containing protein with His-tag to purify. About 8 mg of highly purified protein was obtained from one liter of cell culture as calculated by determination of total protein concentration under this expression and purification scheme.

Identification of purified protein

To verify the identity and bioconversion activity of the purified recombinant protein, the enzyme was assayed for activity and products determined by TLC and HPLC. The conversion results were shown in Fig. 12, 13, 14, and 15. A clear conversion of arteannuin b to artemisinin was observed by using enzyme, which confirms that the purified protein is the indeed.

Fig.12 TLC analysis of Standard samples of AB, AN and methanolic extract of test sample obtained by incubating crude extract with AB and co-factors

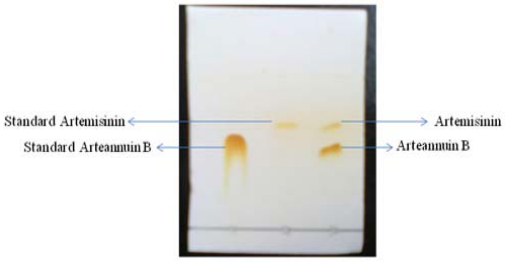
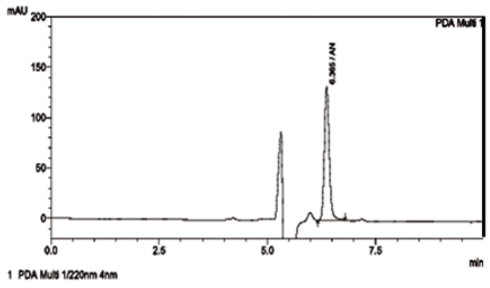


Fig.13 HPLC elution profile of standard Artemisinin (1.0 mg/ml)



HPLC elution profile of Standard Artemisinin (1.0 mg/ml)

Fig.14 HPLC elution profile of Standard Arteannuin B (0.5 mg/ml)

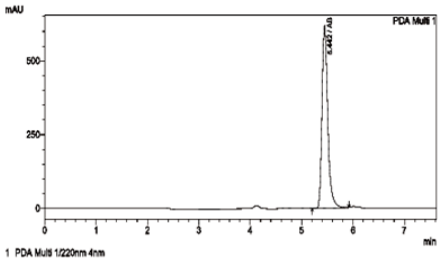
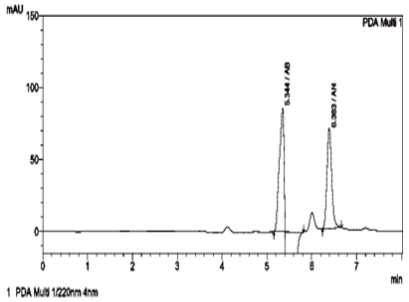


Fig.15 HPLC elution profile of test sample incubated with the enzyme



Discussion

Considering the fact that very low amounts of the enzyme are obtained in the native organism i.e. *Streptomyces pactum,* it is very important to develop a strategy for large-scale cost–effective production of such an important protein with potent bioconversion activity.

In this study, the successful expression and purification of an enzyme involved in bioconversion of arteannuin B to artemisinin has been achieved. The enzyme was produced using pRSET A, an expression vector system in *E. coli*. Initially, the expression was tested at 37^{0}C that is optimal temperature for *E. coli* and the protein was accumulated primarily in the form of inclusion bodies in *E. coli.* Using 8 M urea for denaturation followed by dialysis and refolding, the protein was extracted from the inclusion bodies. Unfortunately, this did not lead to a significant recovery of soluble enzyme, although some other researchers have reported that under such a temperature (37^{0}C) sufficient amounts of soluble proteins could be successfully purified and refolded from the inclusion bodies (Colleluori *et al*., 2005). Subsequently, we decreased the post-induction temperature from 37^{0}C to 28^{0}C in shake flask culture, the production yield of soluble protein in *E. coli* was increased from 35 to 80%. This suggested that a lower temperature (28^{0}C) is beneficial for improving the expression level of soluble protein. Furthermore, to determine the time point when the highest amount of protein can be obtained, the expression levels at 28^{0}C were compared between the cultures with different optical density values. The experimental data showed that the time of harvest had

a less significant effect on the protein production, although the yield of soluble protein was the highest after 4 h of expression at 28^0C. In addition, proper optimization of induction conditions was also performed. The optimized conditions for protein expression in *E. coli* are a temperature of28^0C, a seed concentration of 0.6OD, IPTG concentration of 0.5mMol and an induction time of 4hrs.

Acknowledgements

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