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Optimization and purification of recombinant Isopentenyl diphosphate isomerase (IDI) from *Cassia angustifolia* Vahl.

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ABSTRACT

Isoprenoids are a diverse group of compounds found in all living organisms. The biosynthesis of isoprenoids in plants involves mevalonate and non-mevalonate pathway, which generate the C5 building block IPP and DMAPP. Cassia angustifolia Vahl. is a plant with high global demand as a laxative due to the presence of sennosides, which are anthraquinones synthesized through the MEP pathway. Isopentenyl diphosphate isomerase (IDI) catalyses the conversion of IPP to DMAPP and plays a crucial role in sennoside biosynthesis. To obtain a better understanding of IDI properties from senna (caIDI), it is necessary to establish a standardized protocol for its overexpression and purification, as the current information on this protein is uncharacterized. The presented study attempted to overexpress and purify recombinant caIDI in E. coli in a native form and standardized the inducible overexpression of the enzyme. Total RNA was isolated from senna, and the IDI gene was cloned into pET28a(+) with a 6X His tag at the N-terminal. Two E. coli strains, Rosetta (DE3) and BL21 (DE3), were tested to determine the most effective expression for caIDI. Different concentrations of IPTG, time, and temperature were examined to obtain the native form of the protein. Results were analysed by SDS PAGE and GelAnalyzer software v.19.1. Rosetta (DE3) is a more suitable strain for caIDI overexpression, induced by 0.5mM IPTG for 4 hrs at 37°C. The study provides in-vitro insights into this novel protein and creates golden possibilities of a genetically engineered pathway.

Key words: *IDI, IPPS, Indian senna, sennoside, Isoprenoids, Overexpression.*

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Abbreviation
IPP, Isopentenyl pyrophosphate; IPPS, Isopentenyl pyrophosphate synthase; DMAPP, dimethylallyl pyrophosphate; IPTG, Isopropyl-1-thio-b-D-galactopyranoside; IMAC immobilized metal affinity chromatography; Ni-NTA, nickel-nitrilotriacetic acid; SDS-PAGE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

INTRODUCTION

Isoprenoids are a highly diverse group of compounds, with over 65,000 different structures known to date [1]. They are found in all living organisms and have diverse functions including cholesterol and vitamin A synthesis in animals, respiratory electron transport, bacterial cell wall biosynthesis, defense against environmental stresses, and secondary metabolite production [2]. In plants, isoprenoids play critical roles in many physiological processes. They are essential components of the photosynthetic machinery, serving as light-harvesting pigments (chlorophylls and carotenoids) and electron carriers (quinones and plastoquinone) [3]. Some isoprenoids, such as gibberellins and abscisic acid, act as hormones that regulate plant growth and development [4]. They are also attractants for pollinators or repellents for herbivores and contribute to the characteristic flavors and aromas of many fruits and vegetables [5].

The biosynthesis of isoprenoids in plants involves a complex network of enzymatic reactions that occur in different subcellular compartments. In general, the mevalonate pathway operates in the cytosol and generates the C5 building block IPP (Isopentenyl pyrophosphate), which is then transported into the plastids where it is used to synthesize larger isoprenoid precursors. Alternatively, the non-mevalonate pathway (also known as the MEP pathway) operates in the plastids and generates IPP directly [6]. Isopentenyl diphosphate isomerase (IDI; EC: 5.3.3.2) also called Isopentenyl pyrophosphate synthase (IPPS) catalyzes the conversion of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP), which are the building blocks for longer isoprenoids. There are two types of IDIs: type I and type II. Type II is a flavoprotein found in bacteria and archaea which requires Mg^{+2} and flavin mononucleotide in reduced form [7]. On the other hand, type I is present in various organisms including *Escherichia coli* [8] humans [9], *Rhodobacter capsulatus* [10] *Saccharomyces cerevisiae* [11] and plants. Identified as a key enzyme for isoprenoid biosynthesis, proteomic study of various plant IDIs has been attempted in the last two decades. Mutation in IDI disrupts concentration of DMAPP and IPP pool leading to dwarfism, decreased pigmentation and male sterility in *Arabidopsis thaliana* [12]. Silencing of *IDI* gene in *Nicotiana benthamiana* resulted in disruption in thylakoid membranes and 80% reduction in pigments [13]. Several functional analysis of plant IDIs have also been attempted to understand the protein in detail and broaden its applicability [4,14,15].

Cassia angustifolia Vahl. Also known as Indian senna has high global demand as laxative, which is due to anthraquinones called sennosides [16]. Research has suggested that MEP pathway plays a crucial role in sennoside biosynthesis. It is suggested that IDI is an important enzyme producing DMAPP which is ultimately converted into anthraquinones [17]. Despite playing a substantial role in several vital biocomponents including sennosides, IDI from Indian senna has not been studied and characterized. The presented study has attempted to overexpress and purify recombinant senna IDI (caIDI) in *E. coli* in a native form. Standardization of inducible overexpression and thermal stability of enzyme have been also performed. This study not only gives *in-vitro* insights to this novel protein but also creates golden possibilities of genetically engineered pathway.

MATERIAL AND METHODS

RNA isolation and cloning of caIDI

Total RNA isolation was carried out from young leaves of Indian senna by TRIzol method [18]. Quality and quantity check of the isolated RNA was performed by the absorbance microplate reader Epoch machine (BioTek, Vermont, United States). A good quality RNA was procced further for first-strand cDNA synthesis by oligo dT primers using the Revert Aid First-strand cDNA synthesis kit (ThermoFisher, Massachusetts, United States) following the manufacturer instructions. cDNA was stored at -80˚C util use. Cloning primers were designed with incorporated NheI and EcoRI recognition sites to perform directional cloning in pET28a(+). The clone was designed to have 6X His tag at the N-terminal of the gene. Primer designing was performed by SnapGene software (https://www.snapgene.com/), and the parameters assessment was carried out by OligoCalC server (http://biotools.nubic.northwestern.edu/OligoCalc.html) [19]. The synthesized cDNA was amplified using cloning primers (Forward: 5' ATT GCT AGC ATG GGA GAC GTT CCT 3′; Reverse: 5' GCC GAA TTC TTA AGT CAA CTT GTG 3') with a stop codon at reverse primer. The PCR (Polymerase chain reaction) run was designed to have 5 min initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58° C for 30 sec, extension at 72° C for 1 min and a final extension at 72° C for 10 min on Veriti™ 96-Well Fast Thermal Cycler system (form ThermoFisher, U.S.). The amplified product was run on 1.5% agarose gel followed by gel extraction using NucleoSpin gel and PCR clean-up kit (NucleoSpin, Takara, Shiga, Japan). The gene was cloned into the expression vector pET28(+) by digestion ligation protocol to generate pET_IDI. pET_IDI was transformed into *E. coli* DH5α and positive clones were sequenced using T7 promotor and T7 terminator primers.

Optimization of IDI overexpression

Research has suggested that IDI is a water-soluble cytoplasmic enzyme [20]. Due to lack of structural information about caIDI, we have focused to purify the protein in native form. To achieve the same, we have examined two *E. coli* strains; Rosetta (DE3) and BL21 (DE3). To optimize the most effective induction in the suitable strain, different concentration of Isopropyl-1-thio-b-D-galactopyranoside (IPTG), time and temperature variation have been investigated to obtain most of the protein in native form (excluding inclusion bodies). All the samples were analysed on 12% SDS-PAGE and the band intensity was evaluated by GelAnalyzer software v.19.1 (http://www.gelanalyzer.com/).

Selection of suitable strain

pET_IDI was transformed separately in chemically competent cells of *E. coli Rosetta (DE3)* and *BL21(DE3).* The transformants were cultivated overnight (16 hrs) at 37˚C in 10 mL LB (Luria-Bertani) media with

50µg/mL kanamycin. In case of Rosetta, 34 µg/mL chloramphenicol was also added in the LB broth. Inoculum (1%) from the overnight grown culture was added to fresh LB media (25mL) with appropriate antibiotics as described before and kept at 37˚C with shaking at 120 rpm. Cells were grown until OD600 reaches 0.6 and expression was induced by 1mM IPTG at 37˚C for 4hrs at vigorous shaking (150rpm). Cells were harvested by centrifuging at 7000 rpm for 10 min followed by two washes of 50mM phosphate buffer pH 8.0. The pallet was stored in -20˚C until use. Cell lysis was carried out by mixing the cells with 50mM phosphate buffer and 8mg lysozyme and kept at 37˚C for 20 min. The cells were centrifuged at 12,000 rpm for 15 min at 4˚C, supernatant was collected, and pallet was mixed with SDS loading buffer. The collected samples were run on 12% SDS-PAGE and protein band was analysed after staining by coomassie brilliant blue R-250.

Optimization of induction temperature

The effect of various temperature at different induction periods were examined by inoculating (1%) pre-cultured *E. coli Rosetta* (DE3) with pET_IDI in three different flasks of LB media (10mL each) until OD600 reaches 0.6. The cells were induced by 1mM IPTG at two different induction temperature: 37˚C and 30˚C for 4 hrs. The induced cells were harvested analysed on SDS-PAGE as mentioned before.

Optimization of IPTG concentration

Single colony of *E. coli Rosetta* (DE3) with pET_IDI was grown overnight under conditions described above. The culture was inoculated (1%) in four different flasks with 25mL fresh LB media with 50µg/mL ampicillin and 34µg/mL chloramphenicol at 37˚C with shaking (120 rpm). The cells were induced at an OD600 of 0.6 by 0.25, 0.50, 0.75 and 1mM IPTG. Induced culture was kept at 37˚C for 4 hrs with high shaking. The cells were harvested, lysed and analysed as described before.

Optimization of induction time

A fresh LB medium (25mL) with 50µg/mL ampicillin and 34µg/mL chloramphenicol was inoculated by 250µL of overnight grown culture of *E. coli Rosetta* (DE3) with pET_IDI until cells achieve the OD600 0.6 at 37˚C. The culture was induced by 0.50mM IPTG and kept at 37˚C with vigorous shaking. The induced culture was collected (3 mL) every hour from 1 to 6 hrs. The collected cells were harvested, lysed, and analysed on SDS-PAGE.

Large scale production and purification of recombinant caIDI

Large scale production of IDI protein was carried out under optimized conditions. Pre-inoculated culture of *E. coli Rosetta* (DE3) with pET_IDI was added to fresh LB media (150mL) and kept at optimum temperature. One the OD600 reaches 0.6, the cells were induced with 0.50mM IPTG and kept at 37˚C for 4 hrs. The cells were palette down by centrifuging at 10,000rpm for 15 min at 4˚C followed by two washes of resuspension buffer (25mM NaH_2PO_4 and 250mM NaCl). The pallet was mixed in 8mL of resuspension buffer with 8mg/mL lysozyme and kept at 37˚C for 20 min. The lysed cells were centrifuged at 12,000 rpm for 15 min at 4˚C and the supernatant was proceeded further with purification. From this prepared sample, 50µL of it was kept aside for SDS-PAGE analysis.

IDI was purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA agarose beads of Genetix (New Delhi, India). The purification procedure was carried out at constant 4˚C to avoid probable protein denaturation due to temperature. At everystep of purification, sample was collected for SDS-PAGE analysis. Ni-NTA column was made manually by adding 4mL of Ni-NTA resins in a 5mL sterile luer lock syringe followed by centrifugation at 2000rpm for 1 min. The resin was washed three times with 8-10mL of resuspended buffer to equilibrate the column. The cell lysate sample (8mL) was added to the prepared column, mixed carefully and kept at 4˚C for an hour to have strong binding of caIDI N-terminal 6X His tag with Ni-NTA resin. The column was centrifuged at 2000rpm for 2 min and supernatant was removed. The resin was washed four times with washing buffer (resuspension buffer + 25mM imidazole) and supernatants were removed at every step after centrifugation. The protein was eluted by two repetitive steps of addition of 4mL of elusion buffer (resuspension buffer + 500mM imidazole). All the collected samples including elute were kept at -20˚C until analysis.

The elutes were quantify by Bradford estimation considering 0.01µg/mL BSA as standard. Various concentration of standard (2, 4, 6 and 8 ug/mL) was added in a microtiter plate and volume was made up to 150µL by 10mM phosphate buffer (pH 8.0). To this mixer, 150µL of Bradford reagent was added and kept room temperature for 10-15 min following by spectrophotometric analysis at OD410.

Thermal stability test of purified protein

The purified caIDI was dialyzed three time in dialysis buffer (10mM NaH_2PO_4, pH 8.0) at 4˚C for 6 hours of each dialysis to remove the imidazole. The dialyzed sample was concentrated using protein concentrators by Pierce™ Protein Concentrators PES (ThermoFisher, U.S.). Qualitative analysis of the concentrated sample was performed by SDS-PAGE and protein quantification was performed by Bradford method as

described before. The thermal stability was checked by keeping the purified caIDI at -20˚C, 0˚C and 4˚C overnight.

RESULTS AND DISCUSSION

Cloning of caIDI

The cloning strategy for IDI in an expression vector pET28a(+) was via double digestion (EcoRI and NheI). Amplification of plasmid via gene specific primers at the desire molecular weight was the initial screening of positive clones **(Fig 1)**. The clones were then sequenced for concrete proof.

Optimization of Induction protocol

Host selection

pET_IP was expressed in two strains of *E. coli*: *Rosetta* (DE3) and *BL21* (DE3). Both the strains were induced at same conditions by 1mM IPTG at 37˚C for 4 hrs. After cells lysis, supernatant and pallet was loaded on SDS PAGE. As expression in pallet refers to inclusion body formation of the recombinant protein, only the band intensity of supernatant was taken under consideration. The results showed that *Rosetta* (DE3) had a significantly higher expression of caIDI compared to *BL21* (DE3), with over a 161% increase **(Fig 2)**. A reason behind the intense increment could be the nature of *E. coli Rosetta* (DE3) for a eukaryotic gene. As the strain contains the rare codons, expression of eukaryotic gene in *E. coli* is highly facilitated [21]. Keeping these results into consideration, further analysis was carried out in *Rosetta* (DE3) only.

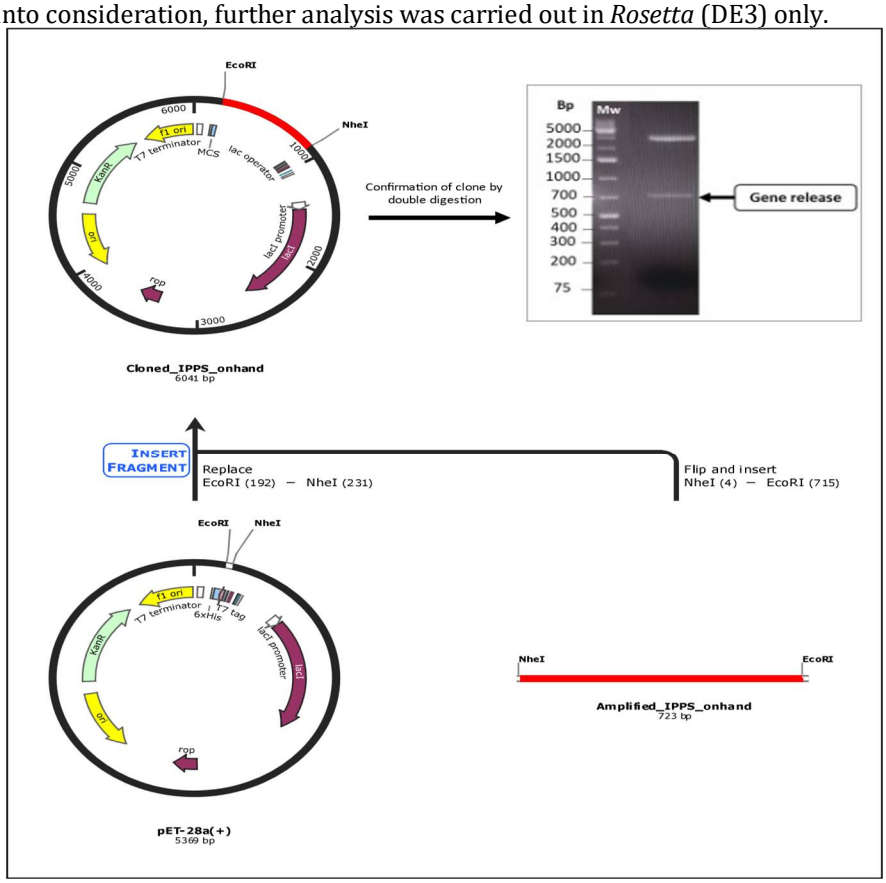

Figure 1 Graphical representation of the cloning strategy and confirmation of clone by double digestion.

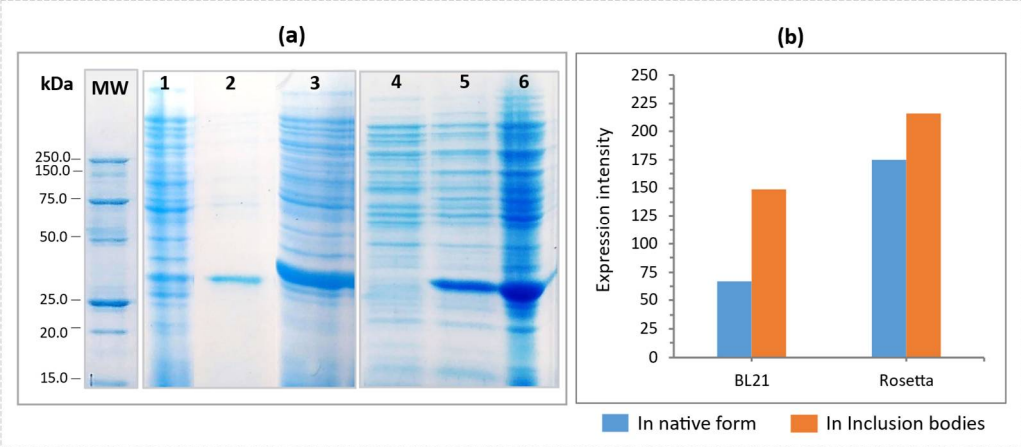

Figure 2 Selection of suitable strain for caIDI overexpression in native form. **(a)** SDS-PAGE of overexpressed caIDI in *E. coli Rosetta* (1-3) and *BL21* (4-6). **(1 & 4)** non-induced culture, **(2 & 5)** caIDI expression in native form, **(3 & 6)** caIDI expression in inclusion bodies. **(b)** Graphical representation of expression intensity in each sample fraction calculated by GelAnalyzer 19.1.

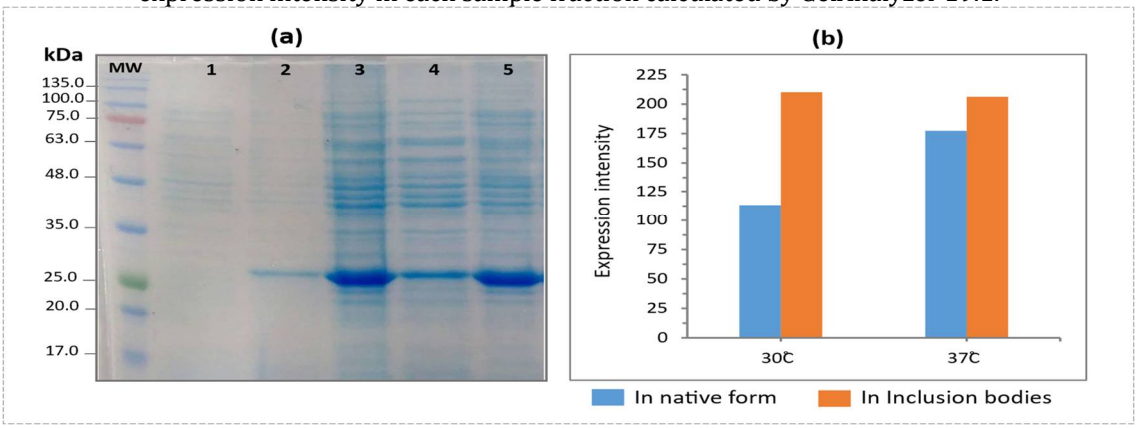

Figure 3 Optimization of induction temperature for caIDI overexpression in native form. **(a)** SDS-PAGE of overexpressed caIDI in *E. coli Rosetta* 30˚C (2 & 3) and 37˚C (4 & 5) for 4hrs. **(1)** non-induced culture, **(2 & 4)** caIDI expression in native form, **(3 & 5)** caIDI expression in inclusion bodies. **(b)** Graphical representation of expression intensity in each sample fraction calculated by GelAnalyzer 19.1.

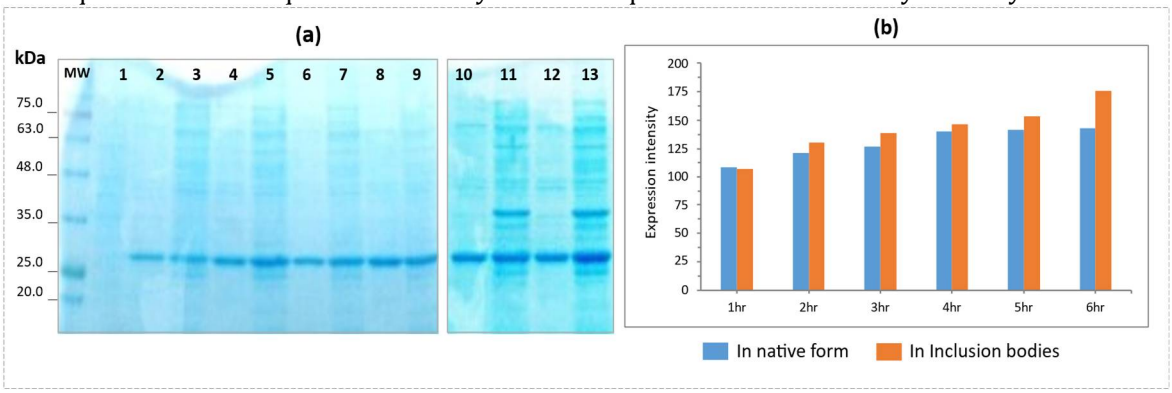

Figure 4 Optimization of IPTG concentration for caIDI overexpression in native form. **(a)** SDS-PAGE of overexpressed caIDI in *E. coli Rosetta* 37˚C for 4hrs using 0.25-1mM IPTG. **(1)** non-induced culture, **(2 & 3)** expression by 0.25mM IPTG in native form and inclusion bodies respectively, **(3 & 4)** expression by 0.50mM IPTG in native form and inclusion bodies respectively, **(5 & 6)** expression by 0.75mM IPTG in native form and inclusion bodies respectively, **(7 & 8)** expression by 1.0mM IPTG in native form and inclusion bodies respectively, **(b)** Graphical representation of expression intensity in each sample fraction calculated by GelAnalyzer 19.1.

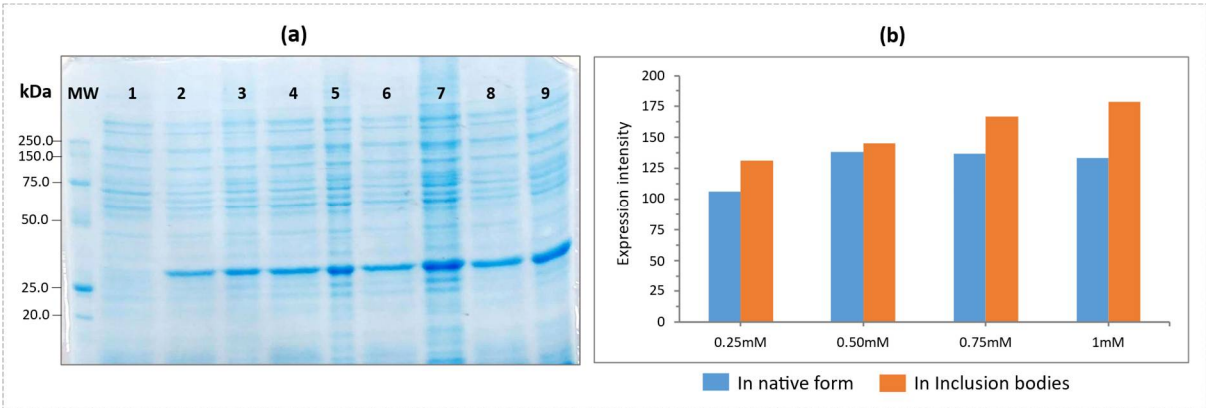

Figure 5 Optimization of induction time for caIDI overexpression in native form. **(a)** SDS-PAGE of overexpressed caIDI in *E. coli Rosetta* 37˚C by 0.50mM IPTG for 1-6hrs. **(1)** non-induced culture, **(2 & 3)** expression in 1hr in native form and inclusion bodies respectively, **(4 & 5)** expression in 2hr in native form and inclusion bodies respectively, **(6 & 7)** expression in 3hr in native form and inclusion bodies respectively, **(8 & 9)** expression in 4hr in native form and inclusion bodies respectively, **(10 & 11)** expression in 5hr in native form and inclusion bodies respectively, **(12 & 13)** expression in 6hr in native form and inclusion bodies respectively, **(b)** Graphical representation of expression intensity in each sample fraction calculated by GelAnalyzer 19.1.

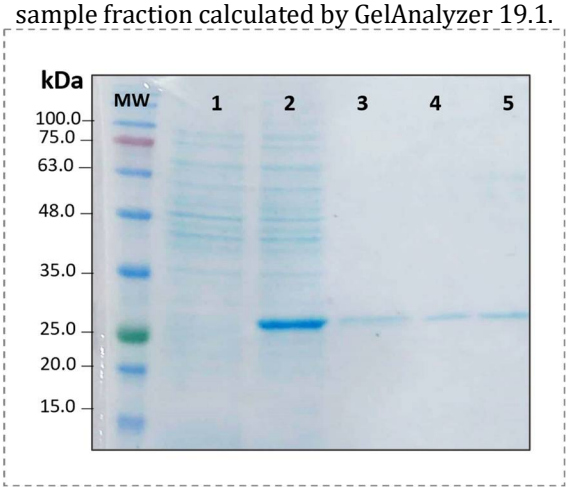

Figure 6 SDS_PAGE of caIDI purification in native form. **(1)** non-induce culture, **(2)** caIDI overexpression in native form **(3)** purified caIDI by Ni-NTA agarose **(4)** purified protein after dialysis by 10mM phosphate buffer **(5)** concentrated protein by protein concentrator

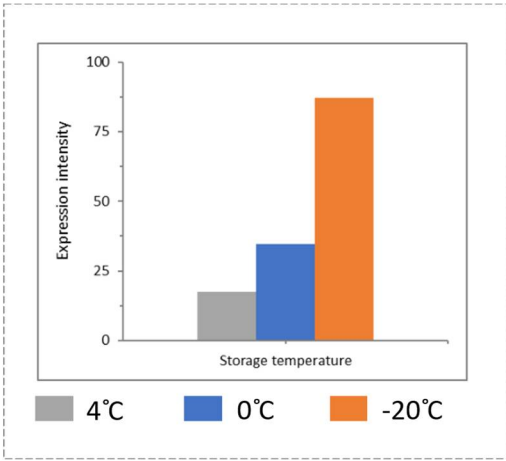

Figure 7 Optimization of storage temperature for caIDI. Graphical representation of expression intensity in each sample fraction calculated by GelAnalyzer 19.1.

Optimization of induction temperature

To optimize the post induction temperature, *Rosetta* (DE3) with pET_IP was induced by 1mM IPTG for 4hrs at two different temperatures: 30˚C and 37˚C. After lyses of the induced cells and analyzing them on SDS-PAGE, it was observed that the protein was expressed at a higher level at 37˚C compared to 30˚C. The expression of caIDI in its native form showed a 55.86% increase at 37˚C **(Fig 3)**. Only 35.14% caIDI was expressed in native form at 30˚C keeping more than 60% expression as inclusion bodies which were undesirable. On the other hand, at 37˚C 46.22% protein was expressed in native form. Though the optimal temperature for protein expression may differ for different proteins, it is believed that higher temperatures increase metabolic activity and protein synthesis rates in bacterial cells [22].

IPTG concentration and induction time optimization

In order to achieve optimal expression of a desired recombinant protein, it is essential to optimize the expression conditions, such as the induction concentration, post-induction time, and temperature (Gutiérrez-González et al., 2019). By investigating different induction concentrations ranging from 0.25-1mM IPTG, it was observed that the maximum expression of the protein in its native form was achieved with 0.50mM IPTG **(Fig 4)**. Induction with higher than 0.50mM IPTG resulted in a higher proportion of the overexpressed protein being in the form of inclusion bodies, rather than in its native form.

To optimize the final factor: post-induction time, all other conditions were kept constant. The *Rosetta* (DE3) transformed by pET_IP was induced using 0.50mM IPTG at 37˚C and induced cells were collected from 1-6 hrs. The cells were lysed, and the protein band intensity was analyzed on SDS PAGE. Based on the data, it was observed that the increase in the native expression of the protein was 22.70% between 1 and 4 hours of induction, while only a 1.71% increase in expression was observed after 4 hours **(Fig 5)**. There was 38.8% increase of caIDI as inclusion body from 1 to 6 hours suggesting the expression of protein shifts towards inclusion bodies. One of the reasons for these results could be that the expression level of caIDI is very high, and the host cell is unable to fold it correctly, leading to the accumulation of the protein in the form of inclusion bodies. Additionally, the cellular stress caused by prolonged overexpression might trigger the formation of inclusion bodies.

Purification and thermal stability of caIDI

Utilizing all the optimized data together, 150mL of LB media inoculated with pre-cultured *Rosetta* (DE3) pET_IP was induced by 0.50mM IPTG at 37˚C for 4hrs. The induced cells were lysed by lysozyme and native protein was purified by IMAC using Ni-NTA agarose beads. **Fig 6** represents the data of caIDI purification in native form showing 64.529% yield of purified protein. The represented data shows efficient purification of caIDI protein in its native form, which is essential for downstream applications such as structural and functional studies. To avoid interference of downstream processes like structural analysis and functional assays, it was necessary to remove imidazole and high salt concentration. This was achieved by dialyzing the purified sample in 10mM phosphate buffer at 4°C and then concentrating the protein using a protein concentrator to increase the yield. To determine the quantity of protein, Bradford estimation was performed using BSA as a standard. The regression formula from the standard curve ($y = 0.0369x + 0.0116$) was utilized to calculate the protein content in the purified and concentrated caIDI sample. After quantification, it was determined that the sample contained 625.8 µg/mL of protein. Additionally, the SDS-PAGE analysis of the sample revealed a single band with strong intensity, indicating a high level of both quantity and quality of the purified caIDI.

The purified sample was kept at three different temperatures: 0˚C, 4˚C and -20˚C overnight (16hrs) to optimize the storage temperature for caIDI. The samples were analyzed on SDS PAGE as represented in **Fig 7**. The data indicated that storing caIDI at 0˚C and 4˚C resulted in a significant loss of protein with 79.8% and 60.10% reduction, respectively. Conversely, when the sample was stored at -20˚C, it exhibited stability for up to 20 days. his study on caIDI stability at different temperatures is important to optimize the storage condition for this protein, which can help to maintain its stability and preserve its activity. Additionally, it can provide insights into the protein's structural properties and its sensitivity to environmental factors. The results of this study can be used as a guide for optimizing the storage condition for other temperature-sensitive proteins, which can have significant implications in various fields of research and biotechnology.

CONCLUSION

In conclusion, this study focused on the overexpression and purification of recombinant caIDI in *E. coli* in a native form, and the standardization of inducible overexpression and thermal stability of the enzyme. The research suggests that the MEP pathway plays a crucial role in sennoside biosynthesis, and IDI is an important enzyme in producing DMAPP, which is ultimately converted into anthraquinones. Despite playing a substantial role in several vital biocomponents including sennosides, IDI from Indian senna has not been studied and characterized before. The successful expression and purification of caIDI provides in vitro insights into this novel protein, and also creates golden possibilities of genetically engineered

pathways. The study will contribute to the understanding of the isoprenoid biosynthesis pathway, and potentially lead to the development of new therapeutic agents or the improvement of existing drugs based on the sennoside compounds.

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CONFLICT OF INTEREST
The authors have declared that no competing interest exists.

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