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Biotransformation of Artemisinin by using a Novel Soil Isolated Microorganism

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***Abstract*—**Artemisinin is a potential antimalarial drug effective against the multidrug resistant forms of Malarial Parasites. The current production of artemisinin is insufficient to meet the global demand. In the present study microbial biotransformation of arteannuin B, a biogenetic precursor of artemisinin to the later has been investigated. Screening studies carried out on several soil borne microorganisms have yielded one novel species with the bioconversion ability. Crude cell free extract of 72h old culture of the isolate had shown the bioconversion activity. On incubation with the substrate arteannuin B, crude cell free extract of the isolate had shown a bioconversion of 18.54% to artemisinin on molar basis with a specific activity of 0.18 units/mg.

***Keywords*—**Arteannuin-B, *Artemisia annua*, Artemisinin, Bioconversion

I. Introduction

MALARIA is one of the most devastating infectious diseases of global importance with an estimated number of 225 million clinical episodes with 781,000 deaths in 2009 [1]. This pernicious infectious disease of humans is caused by four species of a protozoan parasite belonging to the genus *Plasmodium*: *Plasmodium falciparum Plasmodium malariae Plasmodium ovale* and *Plasmodium vivax*. *Plasmodium falciparum* is the most virulent parasite, and causes a large majority of the clinical cases and mortalities [2]. The alarming spread of drug resistance has made combination therapy preferably an artemisinin-based combination therapy (ACT), a strategic and viable option to outweigh the disease [3]. Artemisinin is a potential antimalarial drug produced from aerial parts of *Artemisia annua* L (quinghao) plant belonging to Asteraceae, an aromatic annual herb, native of China [4]. Artemisinin and its derivatives are among the internationally accepted drugs of choice in combination therapy as they are fast-acting, highly potent and complementary to other classes of treatment.

The structure of artemisinin was determined by X-ray analysis in 1979 [5]. Artemisinin is an oxidized sesquiterpene lactone with a unique endoperoxide bridge. Its unique activity was found to be associated with the alkylation of malarial-specific proteins [6]. *Artemisia annua*.,L, the only natural source of artemisinin produces very low quantities of artemisinin as compared to it's biogenetic precursors artemisinic acid and Arteannuin-B [7], [8].

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The concentration of artemisinin in *Artemisia annua* is very low, in the range of 0.01 – 0.8% [9]. This short-fall in the production of artemisinin can be overcome by alternate approaches like biotransformation [10]. Earlier biotransformation of arteannuin-B, to artemisinin was carried out with crude leaf homogenates of *Artemisia annua* [11]. The present study is oriented towards achieving a microbial source for bioconversion of arteannuin-B (Fig .2), a sesquiterpene with an unusual α-methylene-γ-lactone [12], [13] to artemisinin. This article mainly focuses on the isolation and identification of the role of a novel soil borne *Streptomyces* sp in the bioconversion of arteannuin-b to artemisinin.

II. Methodology

A. Isolation of Microorganism

Soil samples have been collected from the lawns of the campus of National Institute of Technology, Warangal, Andhra Pradesh, India.

Isolation of microorganisms from the soil samples were carried out separately on selective media like nutrient agar medium (pH 7.2), potato dextrose agar medium (pH 4.5) and glycerol yeast extract medium (pH 6.8) for bacterial, fungal and actinomycete cultures respectively by pour plate method. Pure cultures of the isolates were maintained in glycerol stocks.

Shake flask cultures of the isolates were tested for their ability to convert arteannuin-B to artemisinin, by the protocol that is described in detail in the later sections. Out of several isolates studied, a novel species has exhibited the bioconversion activity. The isolate has been named as DBT-5 in our laboratory. Morphological features and biochemical characteristics of the culture have been studied.

B. Culturing Of DBT-5

The isolate DBT-5 was cultured in malt-yeast extract medium (yeast extract-4 g, malt extract-10 g, and glucose-4 g distilled water-1000 ml pH 7.2) at 30^{o}C in an orbital shaker at 120 rpm for 72h.

C. Growth Versus Bioconversion Activity

The isolate DBT-5 cultured in malt-yeast extract medium (pH-7.2) at 30^{o}C in an orbital shaker at 120 rpm was harvested at 8 h interval and the crude cell free extract was assayed for bioconversion activity.

D. Preparation Of Crude Cell Free Extract

The process of cell free extract preparation was carried out at a temperature of 4^{o}C. A 72 h old culture was harvested by

centrifugation at 12000rpm for 10 min. The pellet was homogenized with extraction buffer (50mM Tris (P^H 7.2), 10mM β-mercaptoethanol, 2mM EDTA, 5% Glycerol and 10µM PMSF) in a mortar and pestle with acid washed-sterile sea sand as abrasive. The homogenate was centrifuged at 12000 rpm for 30 min at 4°C. The supernatant obtained on centrifugation was used as total protein extract for carrying out bioconversion reaction. The concentration of protein in the crude extract was estimated by Lowry method [14] with bovine serum albumin (BSA) as the standard.

E. Evaluation Of Enzyme Activity

1. Incubation System

Assay of the bioconversion ability of the crude cell free extract was performed by incubating a mixture of 2ml of crude protein solution, 100µg of arteannuin-B (precursor), and the co-factors 0.1mM ATP,1.0mM Mg^{+2} & 1.0mM Mn^{+2} . The total volume of the reaction mixture was made up to 3ml with Tris buffer (pH 7.2) and was incubated for a period of 180 min at 30°C. A negative control was run parallel to this with all the other components except the substrate i.e. the precursor arteannuin-B [11].

2. Extraction Of Artemisinin

The reaction was stopped by adding ethanol and chloroform in 1:1 proportion on completion of the incubation time. From the reaction mixture, artemisinin was extracted twice with hexane in a separating funnel. The hexane fraction was collected in a separate collection beaker and was allowed to evaporate. The residue dissolved in methanol was further tested for the presence of artemisinin.

3. Detection Of Artemisinin

Artemisinin was detected by performing thin layer chromatography (TLC) on silica gel plates with a mobile phase of ethyl acetate and hexane in 2:8 ratios. Artemisinin and arteannuin-B were detected by spraying iodine vapors.

4. Quantification Of Artemisinin

Quantitative analysis of artemisinin produced by bioconversion of arteannuin B was carried out by HPLC [15]. HPLC was performed on Shimadzu – LC-10AT VP Series using a Phoenomenex column (250 X 4.6 mm, C18, ODS with particle size of 5 µm) with a mobile phase of 1% TFA in water: acetonitrile (30: 70), at a flow rate of 1 ml/min. Artemisinin was monitored at 220 nm with an UV-VIS detector (Shimadzu UV-Visible SPD-LC 10A VP Series). The chromatographic system was controlled by Spinchrom CFR (version 2.2) software. Filtered samples of standard artemisinin (Sigma- Aldrich) (1mg/ml), standard arteannuin B (1mg/ml) and the methanolic extract of the experimental sample were loaded on to the column. The retention time for artemisinin was between 6.9 and 7.1 min and that of arteannuin B was between 5.7 and 5.9 min.

F. Effect of pH And Temperature On the Enzyme Activity

The pH optimum of the bioconversion reaction was studied by incubating the reaction mixture at different pH values within the range of 5-10 in Citrate buffer, Phosphate buffer, Tris buffer and Carbonate buffer systems. The optimum temperature for the bioconversion was analyzed by incubating the incubation mixture at different temperatures in the range of 0°C -60°C. In both the above cases the bioconversion activity was assayed by the standard protocol mentioned earlier and the artemisinin produced in the reaction was quantified by HPLC.

III. Results and Discussion

Artemisinin is a potential antimalarial but its effective use is hindered by the insufficient production. Microbial bioconversion of arteannuin-B, the biogenetic precursor of artemisinin to the later will form one of the potential alternatives in enhancing artemisinin production.

Among an array of soil isolates studied, species named as DBT-5 has shown the bioconversion activity

TABLE I
MOPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATE DBT-5

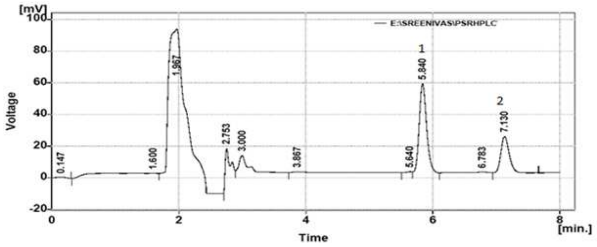
Test	Result
Gram's staining	Grram Positive
Spore chain morphology	Rectiflexibles
Utilization of sugars	
Arabinose	+
Fructose	-
Galactose	-
Glucose	+
Mannitol	+
Sucrose	-
Xylose	+
Utilization of	
Valine	+
Proline	+
Arginine	+
Guanine	+
Pottasium nitrate	+
Phenylalanine	+
Hypoxanthine	+
Threonine	+
Histidine	+
Hydrolysis of	
Casein	-
Starch	-
Tween 80	-
Gelatin	-

+ = Positive, - = Negative

Crude cell free extract of the harvested culture was incubated with the precursor arteannuin-B and co-factors at a temperature of 30°C for 3 h in Tris buffer system (pH-7.2). The end products extracted from the reaction mixture were qualitatively detected by thin layer chromatography. Rf values of authentic arteannuin B and artemisinin are 0.4 and 0.5 respectively, allowing for an easily noticeable resolution on the TLC plate.

Artemisinin produced by the crude enzyme in test sample was further quantified by HPLC. The HPLC analysis of the standard artemisinin (1mg/ml) and arteannuin B (1mg/ml) and the methanolic extract of the test sample were carried out

Under the experimental conditions specified earlier, the retention times of artemisinin and arteannuin B were 6.9-7.1min and 5.7–5.9 min respectively. HPLC elution profile of the test sample has shown peaks complementary to the standard arteannuin B and artemisinin. The HPLC chromatogram of the experimental sample with the crude cell free extract of DBT-5 has shown the elution of arteannuin B and artemisinin at the retention times of 5.840min and 7.13min respectively (Fig.1).



Peak 1 corresponds to Artemisinin and peak 2 to that of arteannuin B

Fig. 1 HPLC elution profile of methanolic extract of the test sample incubated with crude enzyme

Crude cell free extract of DBT-5 *has* shown a 18.54% to artemisinin on molar basis with a specific activity of 0.18 units/mg.

IV. Conclusion

Biotransformations with microbial enzymes can from a potential alternative for producing artemisinin from its precursors. A 18.54% conversion of arteannuin –B to artemisinin showed by crude cell free extracts of DBT-5. Dhingra et al have reported a % bioconversion of 21.75 from crude extract of *Artemisia annua* leaves. Whereas in the present study 18.54% bioconversion has been reported from the crude cell free extract of DBT-5 culture. With regards to the amount of plant material that is required for extraction of enzyme, time required for the growth and harvest of enzyme from *Artemisia annua* the 18.54% bioconversion reported from DBT-5 is a viable option to enhance the production of artemisinin.

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