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STUDY OF ENZYME ACTIVITY FOR RESVERATROL PRODUCTION BY ENDOPHYTIC FUNGUS ASPERGILLUS NIGER VVE1

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ABSTRACT *Trass*-resveratrol is a polyphenolic compound belonging to the group of stilbenes. The compound has gained increasing interest over the last few years. Resveratrol synthesized via phenypropanoid pathway.The aim of present study was to establish an enzymatic reaction system and studied the participation of key enzymes involvement in resveratrol production. Liquid enzyme extract were prepared from *Aspergillus niger* VVE1, an endophytic fungus from *Cayratia trifolia* plant. The observed concentration of different enzymes phenylalanine ammonia lyase (PAL), Cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) 102.34±25, 61.78±1.23 and 298.54±1.77 U/mg protein. The production of resveratrol in the presence of these enzymes was confirmed by thin layer chromatography (TLC) and High performance liquid chromatography (HPLC).

KEYWORDS : *Aspergillus niger.,* endophytic fungus., enzymatic activity., resveratrol, TLC, HPLC.

INTRODUCTION:

Resveratrol (tras-3,5,4'-trihydroxystilbene) is a phenolic compound that belongs to that stilbene class and was first isolated from white hellebore in 1040[1] . It is widely found in different plants like Japanese knotweed, grapes, peanuts, blueberries, cranberries and ferns[2,3,4]. Reveratrol, a plant-derived stilbenes, has been found to have many potential medicinal use, including in extending lifespan; as an anticancer, anti-angiogenic, anti-inflammatory, immunomodulatory, antidiabete, and cardioprotective drug; in lowering body weight; in reversing social deficit induced by pubertal stress; and as an antioxidant[5,6,7].

It is a phytoalexin in plants and is synthesized by phenylpropanoid pathway in response to stress conditions. It begin with the synthesis of phenylpropanoic acid from aromatic amino acid phenylalanine (Phe) or tyrosine (Tyr) via the shikimate pathway[4]. In the following steps, there are two branches. TAL/PAL catalyzes the desamination reaction of Tyr to form p-coumaric acid. Next, 4 CL COMBINES p-coumaric acid and coenzyme A (CoA) to produce 4-coumaroyl-CoA. In the separate branch, cinnamic aid is formed as the product of Phe catalysis by PAL/PTAL[8,9,10].

Enzymes contributing to the biosynthesis of resveratrol are found both in plants and microorganisms[10]. However, some endophytic fungi have been found to produce resveratrol during cultivation in vitro[11].

The objective of this study was to evaluate the presence of key enzyme in the biosynthetic pathway of resveratrol and their efficiency to produce resveratrol. The goal of our experiment was to investigate the efficiency of enzyme for the production of resveratrol.

MATERIAL AND METHODS:

1.Chemicals and Microorganisms

Standards Compounds: *trans*-t-resveratrol was purchased from Sigma.

All chemicals used for extraction and purification were of AR grade Merck.

Thin-layer chromatography (TLC) was performed using percolated silica gel 60 GF254 plates. The endophytic microorganisms was isolated from *Cayratia trifolia* plant was taken from the Microbiology lab, Gujarat University.

2.Preparation of enzyme extracts: Enzyme extracts were prepared from *Aspergillus niger* VVE1 cells (3.0 g) using sodium phosphate buffer (8.0 ml, pH 9.0) containing 0.1 g/l $MgSO_4$, 0.1 g/l $CaSO_4$ and 0.6 mmol/l DTT[11,12]. After centrifugation at 10,000 × g for 10 min at 4° C, the supernatant was obtained and then precipitated using ammonium sulphate at saturated of 75% at 4°C. The protein sediment was collected by 10 min centrifugation at 8000 × g, and then dissolved in 5 ml of the above mentioned sodium phosphate buffer. The obtained enzyme solution was dialyzed against the same buffer using a MD25 dialysis tube to remove SO_4^{-2} and stored at 4°C.

3.Measurement of Enzyme Activities:

The main enzymes participating in resveratrol biosynthesis (7), phenylalanine ammonia lyase (PAL), Cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) in the prepared enzyme extract, were tested according to the method reported by Zhang et al. One unit (U) of the enzyme was defined as an increase of 0.01 of OD value per hour, and the enzyme activity was expressed as the unit of enzyme per milligram protein (U/mg). Protein concentration in the enzyme extract was determined using a visible spectrophotometer, UV mini -1240 (Shimadzu). Bovine serum albumin was taken as standard. Determination of the enzyme activity was conducted in triplicates and the mean value were reported with their standard deviations.

4.Extraction and determination of resveratrol

Dried *Aspergillus niger* VVE1 cells 50 mg were extracted in acetone-water 3:2 (*v/v*) for 12 hours (room temperature) on a test tube rotator, centrifuged at 2000 rpm for 15 minutes and then the supernatant was concentrated under vacuum at 40°C till the complete removal of acetone, the aqueous extract was partitioned twice with equal amount of ethyl acetate; finally the ethyl acetate phase was concentrated under vacuum till dryness.

The extract of fungal culture was dissolved in methanol and used for TLC profile. 10µl of authentic samples of resveratrol and isolated extract were applied on pre-coated silica gel 60 F_{254} TLC plate (0.2 mm Merk5554). The plate were kept in the mobile phase of chloroform: MeOH:Water(85:15:3.v/v). The plates were air dried and observed under visible and UV Light. Characterization and separation of compounds was done by HPLC with the following method: Solvent A- 0.0025% trifluoroacetic acid in water; solvent B-80% acetonitrile (E. Merck, India) in solvent A. The mobile phase consisted of solvent (A) and (B). The step gradient programme of solvent A was as follows: 0-3 min: 86%-82%; 3-12 min: 82%-82%; 12-25 min: 82%-78%; 25-**30 min: 78%-78%; 30-38 min: 78%-60%; 38-43 min: 60%-60%; 43-46 min: 60%-40%; 46-48** min: 40%-30%; 48-50 min: 30%-30%; 50-52min:30%-20%;52-54 min: 20%-20%;54-56 min: 20%-15%; 56-58 min: 15%- 0%;58-60 :0-0%; 60-62 min: 0%-86%; 62-65 min: 86%-86%. Flow rate of 1.0 ml min^{-1} and chromatographic peaks were monitored at λexc 300 nm and λem 390 nm using fluorescence detector .The spent medium was extracted with 100 ml ethyl acetate and analyzed by HPLC for resveratrol released in the medium[13].

5.Statistical analysis

All results were averaged over three separate analyses from three flasks for the estimation of t-resveratrol and three consecutive experiments was done. The results were expressed as U/mg protein.

RESULT

Enzyme related activity for resveratrol biosynthesis

The result of experiment showed the protein content was 0.432 ± 0.016 mg/ml in the enzyme extract. The activity of PAL, C4H, 4CL in the enzyme extract was 102.34±25, 61.78±1.23 and 298.54±1.77 U/mg protein, respectively, which indicated the required enzymes for the resveratrol biosynthesis were found in the enzyme extract.

Confirmation of resveratrol production in the enzymatic reaction system:

The verification of resveratrol production was confirmed by TLC and HPLC. In the TLC, the refractive index (Rf) of standard and the resveratrol produced by enzyme extract was same (0.14). The compound having the same retention time (40 min) as standard resveratrol (40 min) at λexc 300 nm and λ em 390 nm using fluorescence detector in the sample with enzyme extracts, but not in the enzyme extract without substrates. Production of resveratrol in the presence of this enzyme was done by TLC (fig 1) and HPLC (fig.2).

DISCUSSION:

The low resveratrol production of the enzymatic reaction system might be because the biosynthesis of resveratrol uses a series of enzymes that have different operating pHs, Which could not be achieved in an uniform reaction system. Loss of enzyme activity and essential coenzyme factors caused by the disruption of cells might be another important factor that decreased the overall efficiency. In order to improve resveratrol production and up regulate the expression of key enzymes, it is crucial to construct a resveratrol biosynthesis pathway via introduction of the entire pathway with the initial addition of substrate like phenylalanine. Wu et al.[13] established an *E.coli* system by adding the whole pathway genes to obtain resveratrol at a level of 35.02 mg/L. Addition of malonyl-CoA, ATP, CoA, p-coumaric acid and phenyalaninme was separately carried out in an attempt to improve resveratrol production because they are key compounds in the resveratrol biosynthetic pathway[10].

CONCLUSION

The key enzymes PAL, C4H, 4CL showed efficient resveratrol production in the enzyme extract. Although, many approaches have been developed for improving resveratrol production by over-expressing key enzymes using an in vitro platform. To introduction of non-pathway genes, increasing the proximity of enzymes, and promoting fungal interspecific interaction are additional strategies to enhance resveratrol production by improving enzyme activity and regulating metabolic flux.

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FIGURE LEGENDS:

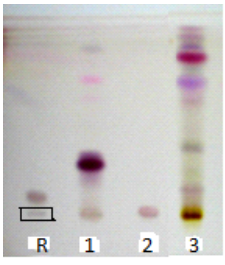
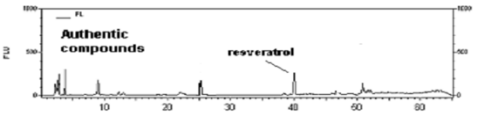
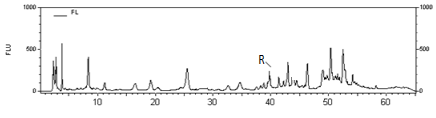


Fig 1: TLC profile R is authentic resveratrol , 1 is PAL, 2 : C4H, 3: 4CL

(R)



(1)



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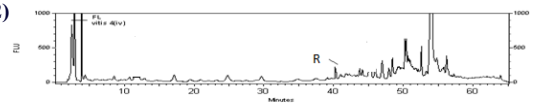

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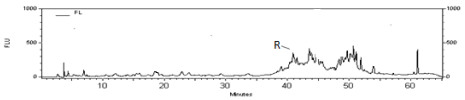

Fig 2: HPLC profile R is authentic resveratrol , 1 is PAL, 2 : C4H, 3: 4CL

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