JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

ISOLATION AND CHARACTERIZATION OF t-RESVERATROL AND α-VINIFERIN, A BIOACTIVE SECONDARY METABOLITE OF AN ENDOPHYTIC FUNGUS *ASPERGILLUS STELLIFER* AB4, FROM VITIS VINIFERA

Chetana Roat and Meenu Saraf

Address(es): Department of Microbiology & Biotechnology, University School of Sciences, Gujarat University, Ahmedabad-380 009, India.

*Corresponding author: chetana.roat@gmail.com

doi: 10.15414/jmbfs.2020.9.4.708-713

ARTICLE INFO

Received 24. 4. 2019
Revised 16. 10. 2019
Accepted 23. 10. 2019
Published 3. 2. 2020

Regular article

OPEN ACCESS

ABSTRACT

The secondary metabolite t-resveratrol and α-viniferin is widely recognized bioactive molecules that is known for preventing and slowing the occurrence of many diseases, have attracted great research interest. The aim of this work is to isolate the endophytic microorganisms that possess t-resveratrol and α-viniferin producing capability from the plants of *Vitaceae* family: *Vitis vinifera , Vitis quinquangularis Rend and Cayratia trifolia*. Twenty isolates were obtained from the different tissues of all the three plants and all were able to produced t-resveratrol and α-viniferin in different concentrations in their consecutives second subcultures. *Aspergillus stellifer* AB4 isolated from leaf of *Vitis vinifera* had shown stable production capability of t-resveratrol 288 µg/L and α-viniferin 301 µg/L in liquid culture, while 19 isolates producing capability diminished after third subculture. Optimization of the conditions for t-resveratrol and α-viniferin production by *A. stellifer* AB4 were studied, an inoculum size of 10% v/v ($2x10^4$) spore number/ml, a rotation speed of 100 rpm, and a temperature of 28°C and pH 7 was optimum for the production of t-resveratrol and α-viniferin. Growth of *A. stellifer* AB4 increased during cultivation, reached high level of biomass 1.98± 1.7 on 7^{th} day and the highest production of t-resveratrol 300 µg/L and α-viniferin 324 µg/L was reached on 9 day of fermentation. This work indicates that endophytic fungi *A. stellifer* AB4 is expected to be potential source of bioactive molecules t-resveratrol and α-viniferin.

Keywords: t-resveratrol, α-viniferin, Endophytes , *Aspergillus*, HPLC

INTRODUCTION

Endophytes reside within the specific chemical environment of host plant tissue and adapt the plant physiology in order to produce the bioactive molecules like secondary metabolites same as the plant produced. They colonize inside the living plant tissue without causing any harmful effect to the host plant (**Albert *et al*., 1990**). The endophytes establish either mutualisms or antagonistic relationship with the host plants. Host plant restrict the growth of endophytes utilize many mechanism to adapt its living environment. The studies on endophytes, especially in the field of disease management of humans and plants, are currently emerging to forefront. The microorganisms like fungi and bacteria have been reported as endophytes for the production of bioactive secondary metabolites. The most commonly known endophytes are fungi for production of bioactive secondary metabolites production (**Aly *et al*., 2011**).

The plant derivative metabolites such as t-resveratrol (3,5,4'-trihydroxystilbene) and α-viniferin are commonly used as medicinal ingredient and nutritional supplements. They are a small class of polyphenol stilbenes and are derived from the phenylpropanoid acid including cinnamic acid and p-coumaric acid that derived from aromatic amino acid phenyalanine, called as phenylpropanoid pathway by **Roat and Ramawat (2009)**. These two molecules are gaining much scientific attention due to its pharmacological, biological, neutriceuticals values and in treatment of metabolic syndromes (**Cherniack, 2011; Xianfeng-Huang *et al*., 2011; Zhu *et al*., 2011**). Epidemiological studies proved that moderate consumption of red wine containing reseveratrol reduces the risk of coronary heart disease, cancer, platelets thrombus formation, Alzheimer's disease refereed as "French Paradox" (**Chen *et al*., 2013**).

First time the resveratrol and its derivatives were isolated from the white hellebore, *Veratrum grandiflorum O. Loes* in 1939 (**Takaoka M, 1939**). Later it has also been reported in root extract of the Japanese Knotweed Polygonum *cuspidatum syn. Fallopia japonica*, which is known as a Chinese herbal medicine, well-known commercial source of resveratrol (**Nonomura *et al*., 1963**). Subsequently, the presence of stilbenes molecules like t-resveratrol and α-viniferin also reported their presence in the plants of *Vitaceae* family (**Baur *et al*., 2006**). Grapes from the plant *Vitis vinifera* is the best and the most important dietary sources of these stilbenes (**Goldberd *et al*., 1996**).

This is the first report of endophytic fungi *A.stellifer* AB4, being isolated from the plant *Vitis vinifera*, and used for the production and optimization of t-resveratrol and α-viniferin. Biosynthesis of t-resveratrol and α-viniferin by using endophytic microorganisms is a novel approach, in microbial biotechnology as it can be used to produce round the year within shorter turnover period of production time. The shortage of plant material from where t-resveratrol and α-viniferin is being extracted and the time it takes to grow the plants make this new microbial method more viable and advantageous, subsequently, less information is available for production of stilbenes from endophytes.

MATERIAL AND METHOD

Chemicals

Standards Compounds: *trans*-t-resveratrol and α-viniferin were 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

Plant Materials

Microbial endophytes where isolated from three grape plants from Vitaceae family, (i) *Vitis Vinifera* L. cv. Merlot (cultivar Merlot) (ii) *Vitis quinquangularis* Rehd and (iii) *Cayratia trifolia* (wild Vitis) were collected from three States of India (Gujarat, Rajasthan and Maharastra) during June to October. Different parts of plant like stems, leaves, and fruits were gathered for further studies as these plants were known for the secretion of t-resveratrol and α-viniferin.

Media

Four different types of media were used for isolation and screening of endophytes : Potato dextrose agar (PDA: potato (peeled and diced) 300 g, dextrose 20 g, agar 20 g, and water 1 L) Beef-protein medium (BP: beef extract 3 g, peptone 10 g, NaCl 5 g, agar 20 g, and distilled water 1 L; pH 7.4–7.6), Gause medium G-1(GA1: soluble starch 20 g, KNO3 1 g, K2HPO4 0.5 g,MgSO4·7H2O 0.5 g,

NaCl 0.5 g, FeSO4·7H2O 0.01 g, agar 20 g, and distilled water 1 L; pH 7.4–7.6) and Nutrient agar. The liquid form of these media were used to quantify the t-resveratrol and α-viniferin production by the isolates. Further specified media Czapek yeast extract agar medium (sucrose 30 g, yeast extract 5 g, NaNO33 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $K_2HPO_4$1 g, agar 20 g, and water 1 L) was used for the further strains identification of the genera *Alternaraia*, *Aspergillus* and *Penicillium* (**Pereyra *et al*., 2011**).

Endophytes Isolation

Endophytes were isolated from the stems, leaves, fruits of *Vitis vinifera, Vitis quinquangularis* and *Cayratia trifolia*. All samples were surface sterilization by 7% of sodium hypochlorite for 5-7 minutes under aseptic conditions and inoculated on BP, GA1, PDA and NA medium **(Larran *et al*., 2002; Naik *et al*., 2009) Xing and Guo (2011)**, the inoculated plates were kept at 28±1 °C under dark conditions. The colonies were subculture on fresh medium from which the isolates were obtained for further purification. All experiments for each test were conducted in triplicate.

Screening of endophytes for t-resveratrol and α-viniferin production

Morphological study was done for all the endophytes isolated, containing bacteria and fungi. All the isolates obtained were inoculated in 100 mL of the respective liquid medium having 1% inoculums size and kept under dark conditions at 28±1 °C on shaker with 100 rpm for 6-7 days, centrifuged at 3000 × g for 15 min was done for separation of cells from the liquid. The liquid nitrogen was used to crush the cells and then extracted with acetone: water (60:40) to obtain the t-resveratrol and α-viniferin from inside the cells. The extract was concentrated under vacuum below 40°C till the complete removal of acetone. The aqueous phase was, then partitioned three times with 20 mL ethyl acetate; finally the ethyl acetate phase was concentrated under vacuum till dryness. This extract was used for the next steps of separation.

TLC and HPLC measurement

The extract of bacterial and fungal culture was dissolved in methanol and used for TLC profile. 10µl of authentic samples of t-resveratrol, α-viniferin 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 : ethyl: actetate (85:15:3.v/v). The plates were air dried and observed under visible and UV Light.

Separation of t-resveratrol and α-viniferin was done by HPLC with the following method: Solvent A- 0.0025% trifluoro acetic 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 monitored at λ exc 300 nm and λ em 390 nm using fluorescence detector .The spent medium was extracted with 100 ml ethyl acetate and analysed by HPLC for t-resveratrol and α-viniferin released in the medium (Roat *et al* 2009).

The Standard compounds were dissolved in methanol to yield a final concentration of 1.0 mg ml^{-1} and standard curve were prepared for both the molecules with concentration ranging from 25 to 1200 ng ml^{-1}. The amount of the compound were calculated on the basis of their standard curve.

Identification of t-resveratrol and α-viniferin producing endophytic fungus

The morphological identification and microscopy study and molecular identification of the isolated strain was done (**Yuan *et al*., 2011; Dey *et al* 2011; Suetsuna *et al*., 1990**). Fungi Identification PCR Kit (TaKaRa, Kyoto, Japan) and primers, ITS5: GGAAGTAAAAGTCGTAASAAKG and ITS4: TCCTCCGCTTATTKATDTGC′, were used for Genomic DNA study.

Profiles of cell culture growth of isolated species for t-resveratrol and α-viniferin production

t-resveratrol and α-viniferin production was carried out *in vitro* in 100 mL liquid Czapek yeast extract medium in a 250-mL flask at 28 °C with a rotation speed of 100 rpm and the cell growth profile of isolated strain was studied. The distribution of t-resveratrol and α-viniferin both intracellular and extracellular was done at days 5 and 7 within the cultures and their study was done by similar extraction method. Dry mass of cells were measured at every day after drying the mycelia at 50 °C for 48 h, and the production of t-resveratrol and α-viniferin measured using the above mentioned methods from days 3 to 20 of cultivation. The resulting t-resveratrol and α-viniferin content in the liquid culture (micrograms / litre) and within the cells (micrograms / gram) was determined and reported as the average of three parallel samples.

Optimization of physicochemical parameters for production of t-resveratrol and α-viniferin

Optimization of time course study, pH, and incubation period and inoculums size was studied. The isolated strain AB4 was inoculated into Czapek yeast extract (CYE) agar medium and grown at range of temperatures varying from 15°C to 48 °C. *Aspergillus* strain AB4 growth and synthesis of t-resveratrol, α-viniferin at each temperature was determined (**Thompson *et al*., 1997**). Different pH values ranging from 3 to 11 were used after adjusting pH of the CYE medium. The effect of incubation period was determine from 3 to 20 days for the growth and active metabolite production by the isolated strain. To determine inoculums size, the culture flasks were also inoculated separately with inoculums volume 5%, 10%,15%,20% spores ranging from $1x10^4$, $2x10^4$, $3x10^4$, $4x10^4$ (spores/ml), for growth and production of t-resveratrol and α-viniferin by strain AB4. Each experiment was carried out in triplicate.

Statistical Analysis

All results were averaged over three separate analyses from three flasks for the estimation of t-resveratrol and α-viniferin and three consecutive experiments with six replicate flasks in each treatment for growth value determination. The results were expressed as g/L of cell growth.

RESULT AND DISCUSSION

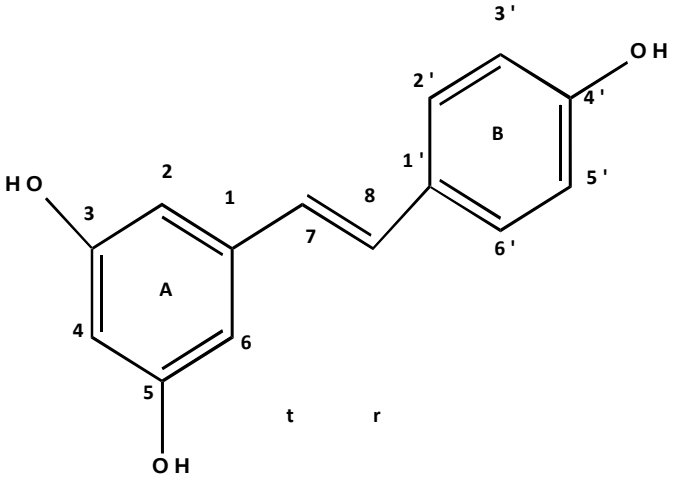
Identification and Production of t-resveratrol and α-viniferin by isolated endophytes

Vitis vinifera, Cayratia trifolia and Vitis quinquangular were studied, total of twenty isolates were obtained from these three plants, ten from *Vitis vinifera*, five from *Cayratia trifolia*, and five from *Vitis quinquangularis* (Table I). Fifteen isolates were identified as fungi and remaining five were bacteria. Among all the isolates, the only one fungal isolate which was isolated from the leaf of *Vitis vinifera* produced maximum t-resveratrol (300µg/L) and α-viniferein (324µg/L). The chemical structure of t-resveratrol and α-viniferin (Figure I). The production of t-resveratrol and α-viniferin gradually decreased for few isolates after second and third subculture. The only one fungal strain AB4, isolated from leaf of *Vitis vinifera* plant was stable and retained the production capability on CYE medium.

Table 1 Endophytes isolated from *Vitis vinifera, Vitis quinquangularis, Cayratia trifolia*

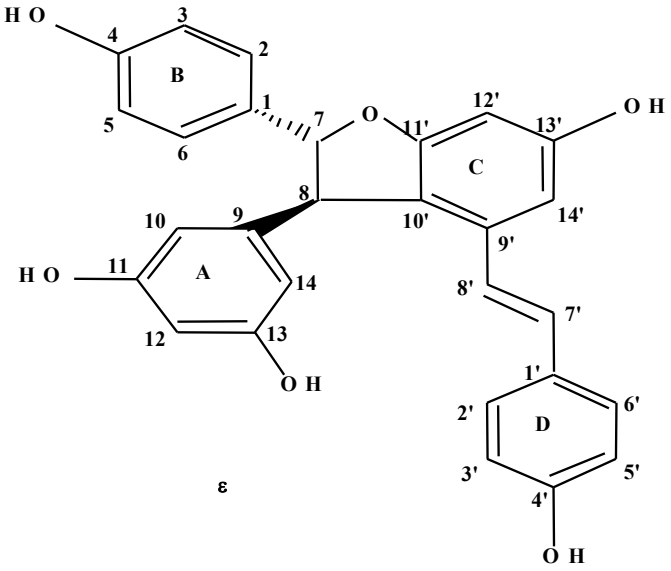
Plant	Strain	Types	Tissue	Medium	Number of isolates
Vitis vinifera (AB)	AB1	Bacterium	Skin of fruit	BP	1
	AB2	Bacterium	Stem	GA	1
	AB3	Bacterium	Leaf	NA	1
	AB4	Fungus	Leaf	PDA	**1**
	AB5	Fungus	Stem	PDA	1
	AB6	Fungus	Skin of fruit	GA	1
	AB7	Fungus	Tendril	PDA	1
	AB8	Fungus	Seed of friut	PDA	1
	AB9	Fungus	Cob	PDA	1
	AB10	Fungus	Root	PDA	1
Cayratia trifolia	CD1	Bacterium	Leaf	BP	1
	CD2	Bacterium	Stem	NA	1
	CD3	Fungus	Cob	GA	1
	CD4	Fungus	Fruit	GA	1
	CD5	Fungus	Root	PDA	1
Vitis quinquangularis	EF1	Bacterium	Fruit	BP	1
	EF2	Fungus	Stem	GA	1
	EF3	Fungus	Cob	GA1	1
	EF4	Fungus	Root	PDA	1
	EF6	Fungus	Leaf	PDA	1

(A)



t r

(B)



ε

Figure 1 Chemical structure of (A) t-resveratrol **($C_{14}H_{12}O_3$)** (B) α-viniferin (**$C_{28}H_{23}O_6$**)

Alternaria (Five strains), *Penicillium* (Six strain strains), *Cephalosporium* (Three strains), *Aspergillus* (Six strains) selected for t-resveratrol and α-viniferin production (Table II). Maximum production of t-resveratrol and α-viniferin was studied by *Aspergillus* strain AB4, which showed highest production of both the compounds. Strain AB4 isolated from leaf of *Vitis vinifera* on CYE agar medium, reaching 16-18 mm in 7 days, and 25-35 mm in diameter in 14 days at 25 ºC. Initially light brown, later becoming dark brown, centrally rising, close textured, velvety, with regular margins. The colony growth showed a concentric appearance with the mycelium showed subhylaine branches. Conidiophores were 100-250 × 4.5-6 µm, vesicle: hemispherical, 10-15 µm, merging into supporting conidiophores, biseriate sterigmata, primaries crowed, parallel 6-7 × 2-3 µm, second arise closely packed 5-7 × 1-2 µm. These morphological characteristics were resembles with genus *Aspergillus* (Figure II).

Table 2 t-resveratrol and α-viniferin producing fungi

Plant	Tissue	Strain	Genus	t-resveratrol production (µg/L)			α-viniferin production (µg/L)		
				No. of Subcultures					
				I	II	III	I	II	III
Vitis vinifera	Fruit	AB1	*Penicillium*	122±2	129±4	10±2	112±3	122±2	122±6
	Stem	AB2	*Alternaria*	125±3	135±4	112±2	113±3	111±3	10±2
	Leaf	AB3	*Aspergillus*	145±1	155±5	160±3	115±2	132±4	140±2
	Leaf	AB4	*Aspergillus*	115±5	165±3	288±3	120±3	155±5	301±4
	Stem	AB5	*Alternaria*	116±3	143±2	118±7	15±3	11±4	0±2
	Skin of fruit	AB6	*Cephalosporium*	117±4	134±3	116±7	14±3	28±4	0±1
	Tendril	AB7	*Penicillium*	117±2	120±4	123±4	20±4	45±3	20±4
	Friut	AB8	*Alternaria*	13±3	14±4	12±3	0±0	0±0	0±0
	Cob	AB9	*Aspergillus*	111±2	123±4	131±4	18±4	16±3	21±2
	Root	AB10	*Penicillium*	12±2	12±0	16±5	0±0	0±0	0±0
Catratia trifolia	Leaf	CD1	*Aspergillus*	12±4	121±4	140±2	22±3	167±2	78±3
	Stem	CD2	*Alternaria*	114±4	121±3	126±3	130±3	127±5	86±5
	Cob	CD3	*Cephalosporium*	10±2	0±1	0±2	18±2	46±4	43±4
	Fruit	CD4	*Penicillium*	132±3	132±3	154±3	32±2	51±3	40±7
	Root	CD5	*Penicillium*	112±3	121±3	152±5	12±3	13±4	38±5
Vitis quinquangularis	Fruit	EF1	*Aspergillus*	112±2	124±4	136±5	42±3	18±4	11±3
	Stem	EF2	*Cephalosporium*	0±3	0±1	0±5	32±2	16±3	15±2
	Cob	EF3	*Alternaria*	132±4	138±3	143±2	0±1	0±2	3±3
	Root	EF4	*Aspergillus*	143±2	145±4	140±7	0±1	26±3	14±3
	Leaf	EF5	*Penicillium*	0±2	0±4	0±3	12±3	16±2	18±3

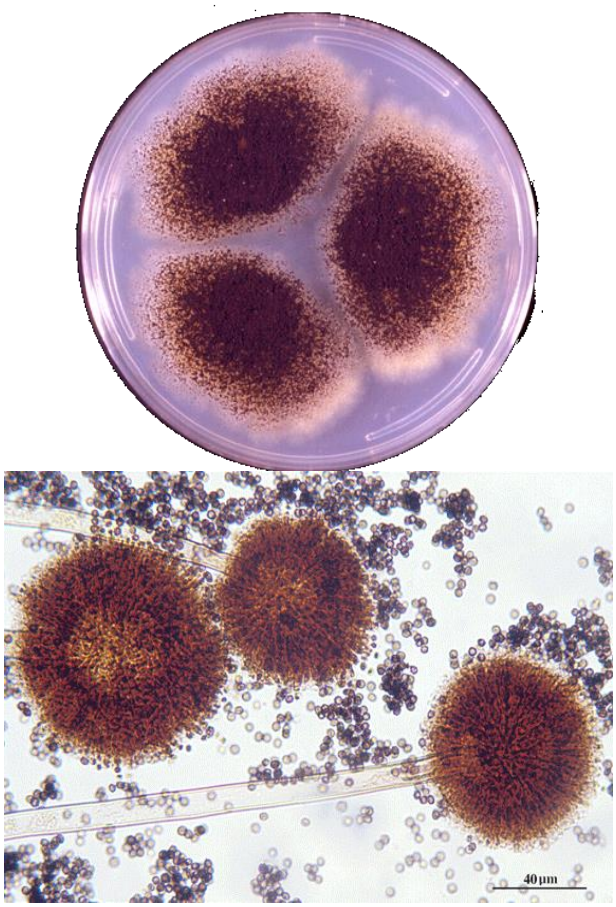
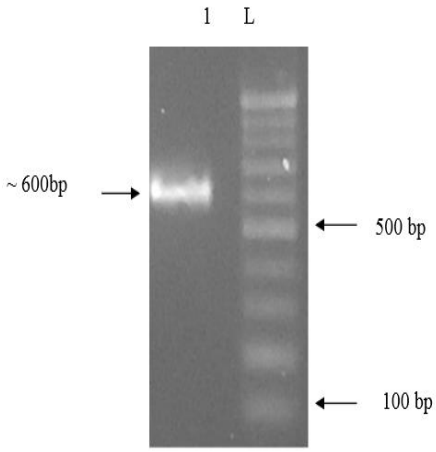


Figure 2 Morphology of *Aspergillus stellifer* AB4

The DNA sequence of the ITS regions of fungal strain AB4, together with the 18S rRNA, was 573 bp long and archived in the Gene Bank database under the accession number KT258010. Closest related species available at Gene Bank with the ITS sequence data for strain AB4, which was identified as *Aspergillus stellifer* (Figure III) and were used for the construction of a phylogenetic tree **Saitou and Nei, (1987) (Tamura et al., 2007; Huang *et al*., 2001).**

(A)



PCR amplification of ITS region from fungal sample. The size of PCR amplified product is ~ 600bp

(B)

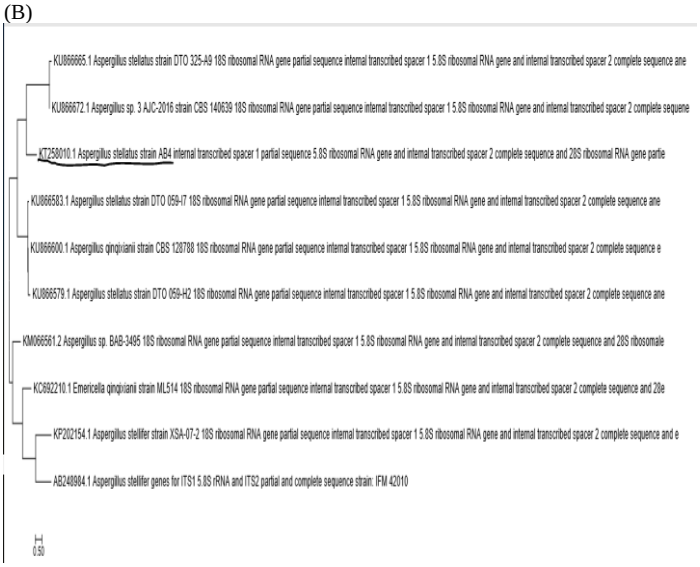


Figure 3 (A) PCR amplified 18S rDNA ITS region (B) Phylogenetic tree of the *Aspergillus* sp. AB4 and related organisms

Profiles of cell culture growth of *Aspergillus stellifer* AB4 for t-resveratrol and α-viniferin production

The highest cell mass was recorded at early of the stationary phase of 9th day while the maximum growth of cells were observed at late log phase of 7th day with 1.98± 1.7µg/g dry cell weight and production of the t-resveratrol 172± 1.6 µg/g and α-viniferin 180± 1.3 µg/g while early of stationary phase 9th day the production of t-resveratrol and α-viniferin was recorded 300± 2.3µg/g and 324± 2.6µg/g. Middle of the stationary phase 11th day cell growth and mass was noticeably decreases, 1.74± 1.8µg/g dry cell weight with less production of the t-resveratrol 100 µg/L and α-viniferin 143µg/g. While synthesis of t-resveratrol and α-viniferin started from the lag phase of cultivation and the maximum value reached on early of the stationary phase of cultivation both intracellular and extracellular. In log phase, more growth was observed and in early stationary phase of cell cycle, more accumulation of t-resveratrol and α-viniferin was observed on day 9, both growth and production of the compounds was declined in late stationary phase on day 13 (Table III). Simultaneously t-resveratrol and α-viniferin amount inside and outside the cell was studied and no compound was detected in spent medium proved that fungal mycelium secreted both the compounds. Therefore, t-resveratrol and α-viniferin may be a constitutive product that accumulates within cells of *Aspergillus stellifer* AB4 (Figure IV).

Table 3 Cell culture growth pattern of *Aspergillus stellifer* AB4 for resveratrol and viniferin production

Sample collection (days)	Cell dry weight (µg/g)	t-resveratrol (µg/L)	α-viniferin (µg/L)
5	**1.91±2.36**	**161±2.31**	**168±3.21**
7	**1.98±2.22**	**172±1.61**	**180±1.30**
9	**1.80±2.1**	**300±2.31**	**324±2.6**
11	**1.74±3.2**	**100±1.64**	**143±2.11**
13	**1.00±2.1**	**23±2.22**	**41±2.00**

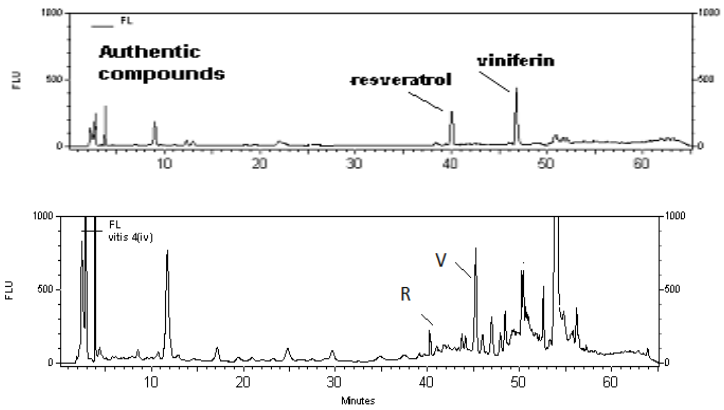


Figure 4 HPLC Profile of t-resveratrol and α-viniferin in *Aspergillus stellifer* AB4 culture

Optimization of physicochemical parameters for production of t-resveratrol and α-viniferin

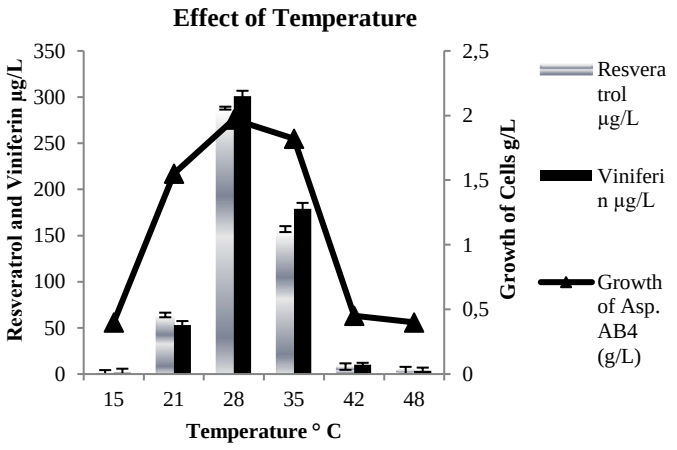
The optimum temperature was recorded at 28 °C, with highest t-resveratrol production (299 µg/L) and (322 µg/L) of α-viniferin with maximum growth (1.97 mg/ml) by isolated strain *Aspergillus stellifer* AB4. Whereas at 35 °C the growth of cells was recorded less 1.30 mg/ml and less production of t-resveratrol (157.46 µg/L) and α-viniferin (179.0 µg/L) (Figure V a). Likewise, very less growth and less bioactive metabolite production observed at low temperature 15 °C and at high temperature 48 °C. The time course study were studied from temperature 15 °C to 48 °C, and it was noted that growth of *A. stellifer* AB4 cells ceased at 42 °C. The design experiments proved that low temperature may stop the enzymes activity which involved in the metabolism of the fungus and high temperature may degraded the fungus. Similarly, (**Shi *et al*., 2012**) also reported the isolation of antifungal and antitumor agent from endophytic fungi at 25 °C and 7-9 days of incubation period

The neutral pH-7 was recorded best for growth (1.90 mg/ml) and production of the molecules, 300 µg/L t-resveratrol and 324 µg/L α-viniferin by *A. stellifer* AB4 (Figure V b), the cell growth was increased from pH-5 to 7 and then slight decline in growth at pH-9, this study suggested that growth and bioactive metabolite production was also there to the more and less of pH-7. But highly acidic pH 3 and highly alkaline pH 11 were not supported the growth and production of the compounds. **Shin J *et al*., 2012**, also, reported that highest production of t-resveratrol by *Alternaria* sp. MG1 was at pH 7. pH acts significant role for the metabolism and or the biosynthesis of secondary metabolites because it is connected with cell wall and permeability properties of the cell membrane for ion uptake or loss to the nutrient medium. **Rubini MR *et al*., 2005** reported the growth of endophytic fungal community and its antibacterial compounds production at neutral pH.

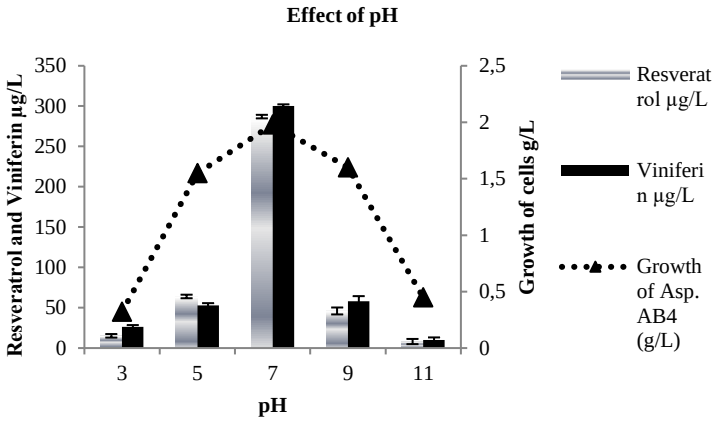
The growth of cells, in terms of incubation time started from the initial stage of log phase, and the maximum growth was observed at late log and early stationary phase. In the present study maximum growth 1.98µg/L was observed at 7 day and maximum production was recorded at 9 day, 300 µg/L t-resveratrol and 324µg/L α-viniferin by *A. stellifer* AB4 (Figure V c). Growth of cells is declined at early of stationary phase, while production enhanced at its maximum value, and at the end of the stationary phase both growth and production of compounds were significantly decreased. Stinson et al (**Thakur *et al*., 2009**) also reported similar results in the case of endophyte *Gliocladium* sp. Effect of incubation period on the production of bioactive compound (Fumonisin B1) by *Fusarium moniliforme* was investigated by (**Albert *et al*., 1990**). They observed that the production of metabolite commenced after 12 days.

The inoculums size has significant role in growth and production of metabolites. More the inoculums size, more is the growth of cell but not necessary more the production of the compound because at early stationary phase growth was decline and production of metabolites was enhanced. **Thakur *et al*., 2009** also reported the importance of inoculum density in increasing mycelia growth and metabolite production by *Streptomyces species.* In the present study, it was observed that the optimum inoculum size of 10% ($2x10^4$ spores /ml) was optimum for maximum growth and yield of metabolite by the *Aspergillus stellifer* AB4 (Figure V d).

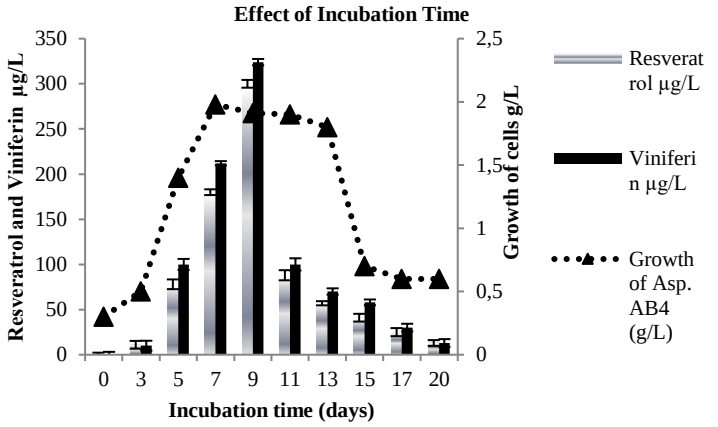
(A)



(B)



(C)



(D)

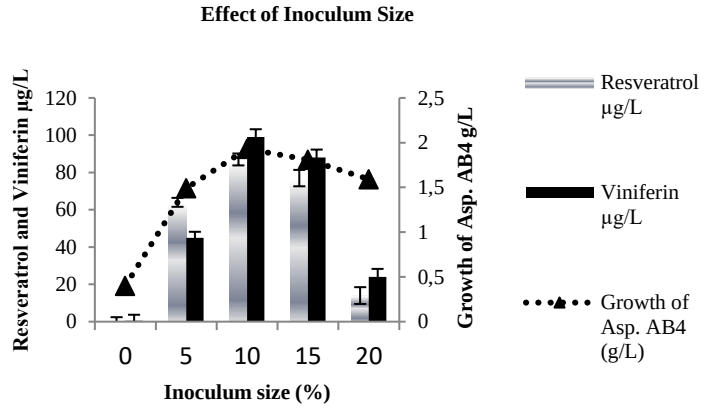


Figure 5 Optimization of physicochemical factors for production of t-resveratrol and α-viniferin by *Aspergillus*sp. AB4 (A) Incubation temperature (B) Ph (C) Incubation time (D) Inoculum size

Plants are very well known for secondary metabolites production in pharmaceutical and neutracuticals science. But plant both *in vivo* and *in vitro* take time to grow and produce the bioactive compounds. The *in-vitro* culture of *Cayratia trifolia* in MS medium containing 0.25 mg l-1 of NAA and 0.2 mg l-1 Kinetin after 15-day cultures produced t-resveratrol, 35µg/l and α-viniferin, 182µg/l (Roat et al., 2009). The endophytic microorganisms resides inside the plant tissue adapt the plant machinery and can able to produce the secondary metabolite in very short period of time, which the plant produced (**Jasim et al 2013**). Production of these bioactive compounds from endophytes is new and acceptable challenge in pharmaceutical world. Among the endophytes, fungus is very well known for the production of many novel secondary metabolites like polyphenols (**Aly et al., 2011**).

The medium and the explants selection plays important role for the production of the bioactive compounds, only appropriate medium along with appropriate plant part helps to isolated the endophytes and then, production of the bioactive molecules. Among all endophytic microorganisms, fungi have huge diversity in different plant in different or same geographical area. (**Goldberd *et al*., 1996; Wang *et al*., 2010**). In the present study, the fungal stain AB4 isolated from leaf of the *Vitis vinifera* capable to produced t-resveratrol and α-viniferin from first to

third and then subsequent subculture, while other isolated strain also produced these compounds but in less quantity and some isolates stop producing the compound after second subculture, **Wang *et al.*, 2010**, also reported the similar result in endophytic fungus *Tubercularia* sp. TF5. The reason might be due to the enzymes or genes involvement in the biosynthesis of the compounds. Increased biomass and growth of *Aspergillus* cell supported the statement that t-resveratrol and α-viniferin is synthesized by *Aspergillus* strain AB4 .The findings revealed that t-resveratrol and α-viniferin producing microorganisms are present in the nature. But simultaneously *Aspergillus* produced toxin and also act as a pathogen and non-pathogen for plants by **Gonzalez and Tello, (2011).** In this study the t-resveratrol and α-viniferin producing capability by *Aspergillus sp*. indicated the possibility of new genes and enzymes mechanism for the biosynthesis of bioactive molecules in endophytic microorganisms.

Physicochemical parameters like inoculum size, temperature, pH, size of flasks were studied and optimized for the maximum production of t-resveratrol and α-viniferin by *Aspergillus stellifer* AB4. The effect of temperature and pH for the production of t-resveratrol and α-viniferin is depends on adaptability of the strain. The optimum temperature for t-resveratrol and α-viniferin production was the same as that for the cell growth of *Alternaria* sp. MG1 at 28 °C and pH 7. The inoculums size and time period is also important for synthesis of these bioactive compounds because low inoculums sizes resulted in less of cell growth and high inoculums sizes revealed more cell degradation (***Rubini et al., 2005***). In the current study, we first time report the *Aspergillus stellifer* AB4 as the novel source for production of bioactive secondary metabolites resveratrol and α-viniferin from *Vitis vinifera* plant in CYE medium. The presented data show new potential for improving the t-resveratrol and α-viniferin production capability may be achievable given appropriate physicochemical conditions and for the enhancement for the production polyphenols: t-resveratrol and α-viniferin.

CONCLUSION

In the current study, we report the *Aspergillus stellifer* AB4 from the plant *Vitis vinifera* as the source for production of secondary metabolites t-resveratrol and α-viniferin on Czapek yeast extract medium. t-resveratrol and α-viniferin have a significant roles in pharmaceutical and nutraceutical science. The presented data showed that the endophytic fungus *Aspergillus stellifer* AB4, has a potential for improving the t-resveratrol and α-viniferin production in short period of time. The optimization studies of physicochemical conditions could help in large-scale production for both the molecules. We have studied an effective production of t-resverarol is 300 µg/L and α-viniferin is 324 µg/L from *Aspergillus stellifer* AB4, The high growth rate and short generation time suggests that *Aspergillus stellifer* AB4 could be promising source of t-resveratrol and α-viniferin production. However, for the commercial development for the production of t-resveratrol and α-viniferin, requires a combination of biotechnological approaches such as genetic manipulation, strain improvement and fermentation.

Acknowledgments: This work was supported by the University grant Commission, Government (UGC), New Delhi, India for providing Dr. D.S Kothari Post Doctoral Fellowship with Ref. No.F.4-2/3006(BSR)/BL/16-17/0021&01-09-2063.

REFERENCES

Alberts, J.F., Gelderblom, W. C., Gthiel, P.G., Marasas, W.F., Schalkwyk, D. J. V., Behrend, D. J. V. (1990). Effects of temperature and incubation period on production of Fumonisin B1 by *Fusarium moniliforme*. *App Environ Microbiol*, 56:1729-1733.

Aly, A. H., Debbab, A., Proksch, P. (2011). Fungal endophytes: unique plant inhabitants with great promises. *Appl Microbiol Biotechnol*, 90:1829–1845. https://dx. doi.org/ 10.1007/s00253-011-3270-y

Baur, J.A., Sinclair, D.A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* 5:493-506. . https://dx. doi.org/ 10.1038/nrd2060

Cherniack, E. P. (2011). Polyphenols: planting the seeds of treatment for the metabolic syndrome.*Nutrition*, 27: 617–623. https://dx. doi.org/ 10.1016/j.nut.2010.10.013

Chen, Y. J., Chen, Y.Y., Lin, Y.F., Hu, H. Y., Liao, H F. (2013). T-resveratrol inhibits alpha-melanocyte-stimulating hormone signaling, viability, and invasiveness in melanoma cells. *Evid Based Complement Alternat Med*. 632121. https://dx. doi.org/10.1155/2013/632121

Dey, P., Banerjee, J.D., Maiti, M.K. (2011). Comparative lipid profiling of two endophytic fungal isolates *Colletotrichum* sp. and *Alternaria sp*. having potential utilities as biodiesel feed stock. Bioresoure Technol 102:5815–5823. https://dx. doi.org/10.1016/j.biortech.2011.02.064

Goldberd, D., Tsang, E., Karumanchiri, A., Diamandis, E., Soleas, G., NG, E. (1996). Method to assay the concentrations of phenolic constituents of biological interest in wines. *Anal. Chem*. 68:1688–1694. https://doi.org/10.1021/ac951083i

Huang, Y., Wang, J., Li, G. Zheng, Z.S.W. (2001). Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxusmairei*, Cephalataxus fortune and Torreya grandis. FEMS. *Immunol Med Microbiol* 31:163–167. https://dx. doi.org/10.1111/j.1574-695X.2001.tb00513.x

Jasim, B., Joseph, A.A., John, C.J., Mathew, J., Radhakrishnan, E.K. (2014). Isolation and characterization of plant growth promoting endophytic bacteria from the rhizome of *Zinger officinal*. *3Biotech* 4:197-204. https://dx. doi.org/ 10.1007/s13205-013-0143-3

Larran, S., Perello, A., Simon, M.R., Moreno, V. (2002). Isolation and analysis of endophytic microorganisms in wheat (Triticumaestivum L.) leaves. *World Journal of Microbiology & Biotechnology*, 18: 683–686. . https://dx. doi.org/ 10.1023/A:1016857917950

Naik, B.S., Sashikala, J., Krishnamurthy, Y.L. (2009). Study on the diversity of endophytic communities from rice (*Oryzasativa* L) and their anatagonistic activities in vitro. *Microbiological Research*. 164(3):290–296. . https://dx. doi.org/ 10.1016/j.micres.2006.12.003

Nonomura, S., Kanagawa, H., Makimoto, A. (1963). Chemical constituents of polygonaceous plants. I. Studies on the components of Ko-J O-Kon. (Polygonum cuspidatum Sieb. Et Zucc.). *Yakugaku Zasshi* 83:988-990. https://doi.org/10.1248/yakushi1947.83.10_988

Pereyra, C.M., Cavaglieri, L.R., Chiacchiera, S.M., Dalcero, A.M. (2011). Mycobiota and mycotoxins contamination in raw materials and finished feed intended for fattening pig's production in eastern Argentina. *Vet Res Commun*. 35:367. https://doi.org/10.1007/s11259-011-9483-9

Roat, C., Ramawat, K. G. (2009). Elicitor induced accumulation of stilbenes in cell suspension cultures of *Cayratia trifolia* (L.) Domin. *Plant Biotechnol Rep.* 135–138. https://doi.org/10.1007/s11816-009-0082-y

Rubini, M.R., Rute, T.S.R, Pomella, A.W., Cristina, S.M., Arajo, L.W., Santos, D.R.D., Azevedo, J.L. (2005). Diversity of endophytic fungal community of cocao (Thebroma cacao L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom disease. *Int J Bio Sci*, 1:24-33. https://doi.org/10.7150/ijbs.1.24

Saitou, N., Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Shi, J., Zeng, Q., Liu, Y., Pan, Z. (2012). *Alternaria* sp. MG1, a t-resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for t-resveratrol production. *Appl Microbial Biotechnol.* 95: 369-379. https://doi.org/10.1007/s00253-012-4045-9

Stinson, M., Ezra, D., Hess, W.M., Sears, J., Strobel, G. A (20030. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. *Plant Sci.* 165: 913–922. https://doi.org/10.1016/S0168-9452(03)00299-1

Suetsuna, K., Osajima, Y. (1990). Cultural conditions for production of griseorhodin by a culture of Streptomyces californicus JCM6910. Suisan Diagakkakenkyn Hokoku. 38: 17-22.

Tamura, T., Dudley, J., Nei, M., Kumar, S. (2007). MEGA 4: molecular evolutionary genetic analysis (MEGA) software version 4.0. *Mol Biol Evol*, 24:1596–1599. https://doi.org/10.1093/molbev/msm092

Thakur, D., Bora, T. C., Bordoloi, G.N. Mazumdar S. (2009). Influence of nutrition and culture in conditions for optimum growth and antimicrobial metabolite production by Streptomyces sp.301. *J Med Mycol*. 19:161-167. https://doi.org/10.1016/j.mycmed.2009.04.001

Thompson, J., D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, 25:4876–4882. https://doi.org/10.1093/nar/25.24.4876

Wang, M.Z., Liu, S.S, Li,Y.Y., Xu, R.L.C., Shen, Y.M. (2010). Protoplast mutation and genome shuffling induce the endophytic fungus *Tubercularia* sp. TF5 to produce new compounds. *Curr Microbiol* 6:254–260. https://doi.org/10.1007/s00284-010-9604-7

Xianfeng-Huang, X.H., Zhu, H.L. (2011). t-resveratrol and its analogues: promising antitumor agents, Anticancer Agents. *Med Chem.*, 11: 479–490.

Xing, X.K., Guo, S.X. (2011). Fungal endophyte communities in four Rhizophoraceae mangrove species on the south coast of China. *Ecol Res* 26:403–409. https://doi.org/10.1007/s11284-010-0795-y

Yuan, Z.L., Rao, L.B., Chen, Y.C., Zhang, C.L., Wu, Y.G. (2011).From pattern to process: species and functional
diversityin fungal endophytes of *Abies beshanzuensis*. *Fungal* 115:197–213. https://doi.org/10.1016/j.funbio.2010.11.002

Zhu, X., Liu, Q., Wang, M., Liang, M., Yang, X., Xu, X. (2011). Activation of Sirt1 by resveratrol inhibits TNF-alpha induced inflammation in fibroblasts. *PLoS ONE* 6: 270-81. https://doi.org/10.1371/journal.pone.0027081