# Isolation, identification and characterization of β-carotene producing endophytic fungi- Penicillium citrinum from bark of Taxus baccata

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# Abstract

C40 carotenoids with the chain of conjugated double bonds are known antioxidants with specific biological activity. Besides the known microbes used for β-carotene, we isolated a new fungus from *Taxus baccata* tree and identified its carotenoid synthesizing property. Isolated unknown fungi with carotenoids biosynthetic property were identified at the species level by analysing its internal transcribed spacer (ITS) and 18S rDNA region. Confirmation of β-carotene synthesis was done using UV-vis spectroscopy where the three peak spectra (434, 456 and 482 nm) of β-carotene confirmed its presence. Also, the infra-red spectrum gave absorption at a certain frequency which is indicative of the abundance of carotenoids with the major functional group as C-H, CH3 and C = C bonding. MS data of analytes was observed using the electrospray ionization method (ESI) with triple quadrupole (TQD) mass spectrometer, which resulted in the elimination of functional group and production of precursors ions of different carotenoids. Mass spectra showed a high degree of fragmentation with characteristics peak of carotenoids at 536.9 (β-carotene), 538.9 (Zeacarotene), 544.4 (Phytoene), 554.0 (β-cryptoxanthin), 569.18 (Zeaxanthin) and standard characteristics peak 536.6 of β-carotene in standard as well as sample confirmed presence.


# Introduction

Natural pigments like carotenoids are considered as an important group of pigments due to their applications in food as feed supplement, antioxidant, natural colorants, pharmaceuticals and biotechnological uses. From natural sources about 700 carotenoids have been isolated so far but among them only 50 carotenoids have shown provitamin A activity in humans (Rodriquez-Amaya 2003). Their important physiological functions include the reduction of cellular or tissue damage due to their anti-cancerous effects (Mayne 1996) and are found to be useful in the prevention of many deadly diseases like cancer, heart disease, stroke, diabetes, and arthritis (Smith 1998). It is used mainly as provitamin A in animal nutrition among all food producing animals, where β-carotene is metabolised fully in the enterocytes but humans absorb a relatively high quantity of β-carotene unchanged. In the small intestine enterocytes, Vitamin A as retinyl ester (20–75%) can be formed after cleavage of β-carotene molecule (Patrick 2000). The pigment which remains intact i.e., not converted is absorbed as intact β-carotene or β-carotene cleavage non-vitamin A products by the lymphatics with incorporation into chylomicrons (Johnson and Rusell 1992). However, besides its availability as a variety of natural and synthetic carotenoids, only β-carotene and astaxanthin are produced commercially by natural sources especially from microbes. β-carotene which is an important food colorant is known to be the precursor of Vitamin A, which plays a crucial role in a number of functions like embryonic development, immunity, cell differentiation, vision and reproduction (Tanoury et al. 2013). However, this vitamin is easily susceptible to oxidation due to light, heat and oxygen. The therapeutic potential of β-carotene has been known for many years and it has the key place as an antioxidant by reducing tissue and cellular damage and also as natural colorants for food products like soft drinks, ice creams, and many baked food products (Kim et al. 1997). Other than that, they also exhibit a number of physiological properties like safeguarding from

cardiovascular diseases, osteoporosis and improved strengthened immunity (Mascio 1991). For industrial use β-carotene is produced mainly by yeasts, fungi, algae, some bacteria, and lichens. The most common carotenogenic microorganisms used commercially for β-carotenoid synthesis are *Blakeslea trispora* (Mantzouridou et al. 2008), *Rhodotorula glutinis* (Malisorn and Suntornsuk 2008) *Sphingomonas* sp. (Silva et al. 2004), and *Phycomyces blakesleeanus* (Mehta 1997). For commercial production currently fungi *Blakeslea trispora* of class zygomycetes is the most utilized mucorales which is known to be fast growing saprophyte by feeding on dead organic matter present in soil. Currently carotenoids biotechnological production methods are used more than the chemical synthesis process as consumers are more aware and attracted to natural products. This is the reason that extraction of naturally synthesized carotenoids is becoming an important option mainly from microbes due to its cost effectiveness and less time consumption to synthesis it to a greater level. Other than these microbes a new fungi *Penicillium citrinum*, belonging to the family –Trichocomaceae was isolated from natural source of Ladakh, India. Most of the endophytic fungi are known plant growth promoter, producing a number of metabolites helping in plant growth as well as act as a defence mechanism from pathogens. The discovered fungi on isolation form bark of *Taxus baccata* and on growth in Potato dextrose agar media produced natural orange- red carotenoid β-carotene. It has been reported in many literature that *Penicllium citrinum* produce various important metabolites like growth promoting giberellins, extrolites like tanzowaic acid A, asteric acid, compactin and mycotoxin like citrinin (Khan et al., 2008). Some researchers also isolated several bioactive compounds like anthraquinone derivatives, benzopyrans, stemphol sulfates from marine derived (*P.citrinum* ) in the south china sea (He et al., 2017), but no work on carotenoids isolation form this fungus has been done so far. This current article is focused on identification of these carotenoids producing property of microbe *P. citrinum* isolated from colder region of Ladakh, India.

# Materials And Methods

### Materials and chemicals

Microbial media were procured from Hi Media Laboratories (Mumbai, India). Analytical grade chemicals were procured from Merck (Mumbai, India). Chromatography grade solvents were procured from SD fine chem (Mumbai, India). β-carotene (Cas no. 7235-40-7) was procured from Sigma Aldrich (Bangalore, India).

### Isolation of fungal culture

For endophyte isolation, whole *Taxus baccata* bark was used by passing serially through ethanol, hydrogen peroxide, calcium hypochlorite and sodium hypochlorite (Jain and Hoggman 2007). Processed bark was transferred to the conical flasks containing sterilized water and kept overnight. After that, bark was sliced with the help of sharp scalpel into small pieces, placed over the potato dextrose agar (PDA) medium under aseptic conditions in petri dishes at pH 7.0 and 28 ± 0.5 $^0$C for 5 days for sporulation. The pH of the media was adjusted to 6.0 before autoclaving then allowed to ferment at 28± 2$^0$C. Purity of

strain were analyzed by sub-culturing in PDA medium. Seed culture (3 x $10^6$ cells per ml) prepared is added to fermentation medium at different level for β-carotene production. Highest carotenoid producing strain were selected by culturing the fungal cell in liquid PDB (Potato Dextrose broth) media (An et al. 1989) for 3-4 days at 28$^oC$ with agitation speed of 180 rpm (An and Johnson 1990).

### Optical Microscopy for phenotypic identification

For phenotypic identification the strains isolated was further cultured on potato dextrose agar (PDA), petri dishes at pH 7.0 and 28 ± 0.5 $^oC$ for 5 days for sporulation. The pH of the media was adjusted to 6.0 before autoclaving then allowed to ferment at 28± $2^0C$. The colonies grown on the plate were studied for fungal colony characteristics and lactophenol cotton blue staining (Raper and Fennell 1965; Sappa and Rai et al. 1954). The morphological characteristics were observed under light microscope.

### Molecular identification

Species level identification was performed through the sequencing of the housekeeping ITS region 5.8s rRNA and 18 S rRNA gene. In this study primer sequence for ITS1, ITS4 and 18 S rDNA are used for amplification. PCRs contained total volume of 50 µl in a 0.2 ml thin-walled PCR tube with its components volume nuclease free water 33 µl, Colony from slant 1 pick, Forward Primer (10µM) 2.0, µl Reverse Primer (10µM) 2.0 µl, 10X Reaction Buffer 10 µl, dNTP Mix (10 mM) 2 µl, Taq DNA polymerase (2.5U/µl) 1µl, for a final volume of 50 µl. The amplification was carried out in a Master cycler® Thermocycler (Eppendorf) with the steps: Initial denaturation carried out at 94$^oC$ for 10 min followed again for denaturation at 94$^oC$ for 1 min, annealing at 56$^oC$ for 1 min, extension at 72$^oC$ for 1 min and final extension at 72$^oC$ for 10 min. From denaturation to extension steps are repeated for 35 cycles.

### Gel extraction

The PCR products from ITS gene PCR reactions were purified to remove unincorporated dNTPS and primers before sequencing using PCR purification kit (GENEASY GEL ELUTION KIT).

**Sequencing:** Both strands of the ITS and rDNA region amplified by PCR were sequenced by automated DNA sequence -3037xl Genetic analyzer from Applied Biosystems using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data were aligned and dendrograms were generated using Sequence analysis software version 5.2 from Applied Biosystems. The sequences obtained for plus and minus strands were aligned using appropriate software prior to phylogenetic analysis.

### Phylogenetic analysis

Sequences were compared to the non-redundant NCBI database using BLASTN, with the default settings used to find sequences closest to each other. The Expected value and e values were noted for the most

similar sequences. Ten similar neighbours were aligned using CLUSTAL W. The multiple-alignment file thus obtained was then used to create a Phylogram using the MEGA 7 software.

### Estimation of β-carotene using UV-Vis spectroscopy

Growth and pigment production by the endophytic fungus was carried out in 250 mL Erlenmeyer flask containing 20 ml of potato dextrose broth inoculated with 1 loop full of fungal culture. Flasks were incubated on rotary shaker at 180 rpm for 72 hours. From submerged fermentation liquid media fungal cells were harvested. Cell pellets were isolated by filtration and centrifuged at 1008 x g for 15 min in refrigerated centrifuge as described by Buzzini and Martini (1999). The remaining cells left after filtration and centrifugation were again washed with distilled water and two ml of pre-warmed DMSO (Dimethyl sulfoxide, heated at 55°C for 30 min) was added in 0.5 g of fungal biomass and vigorous shaking was carried out in agitator for 1 min and left for 30 min at rest. With this 6 ml of acetone was added and solution was first homogenized and centrifuged at 1745 x g for 10 min and supernatant was collected (Persike et al. 2002). Again, the same procedure was repeated for the residue. Supernatants were pooled for determination of β-Carotene quantification (Sedmak et al. 1990). The volume was made up by acetone and absorption spectra was analysed in the range of 400–520 nm (Carvalho et al. 2012) using double beam UV-visible spectrometer (Shimadzu, Japan).

### β-carotene analysis by high performance liquid chromatography

Extracted carotenoid extract from fungal cells were also analysed to confirm for β-Carotene by high performance liquid chromatography (Shimadzu, Japan) with Photodiode-Array Detection (PDA) at multiple wave length equipped with C18 column (5uM particle size) and 20 uL loop injector. Mobile phase was mixture of solvent A: acetonitrile + water (90:10) and B: ethyl acetate with isocratic elution in ratio of 50:50 (flow rate 0.5ml/min). The runtime was kept at 20 min, detection was carried out in a wavelength of 400 nm to 500nm (Chi et al. 2015).

### Fourier transforms infrared analysis (FTIR)

FTIR analysis of the fungal extract was carried out by varian 7000 FTIR (Varian Inc. CA, USA) using potassium bromide disk technique. For this the mixture of powdered sample (dried cell pellets) and KBR were mixed into a uniform powder and compressed into disk at 10,000 psi. Pellet formed was then scanned at $4mm^{-1}$ over a wave number ranging from 400-4000 $cm^{-1}$ with resolution of 2 cm using Win-IR software (Meganathan et al. 2010) to detect the presence of functional groups in the extracted pigment. For pigment analysis, FTIR was considered as a complementary technique that could provide the molecular and structural information of organic and inorganic molecules present in the pigment.

### Preparative chromatography and Mass spectrometry

To identify the main carotenoids from mass spectra, extract from *Penicillium citrinum* were prepared with DMSO/Acetone separation method used for carotenoids (Michaen et al. 2012). The extract obtained

was concentrated using rota evaporator and final concentrate was separated on active and preparative silica gel plates (20 x 20 cm) (SD fine chem, Mumbai, India). Pre optimized mobile phase used for preparative TLC was Chloroform: Methanol: Acetone (10:22:53). With solvent system affinity compounds are moved along with the mobile, different TLC bands formed were scraped off and collected coloured band is mixed with methanol (LC-MS) grade. Mass spectroscopy of carotenoids was carried out by mass detector (SYNAPTIC MS-TOF, Waters, USA) interfaced with electro spray ionization (ESI) probe.

### Statistical analysis

Data are presented as mean ± SD of three replicates. The results were statistically analysed by ANOVA and means compared using Duncan's multiple range tests.

# Results

### Morphological and Microscopic examinations

Isolated endophytic spores were grown by culturing six Petri plates on PDA media. Culturing fungus in PDA media showed rapid growth and attained its maximum final mycelial structure within 5 days at 28± $2^0$C. This strain of fungal cells with reddish-orange color and wrinkled growth pointing in the centre (2-3 mm in diameter) and plane dense surface on the sides with hyphae was developed having entire margin and smooth consistency form (Fig. 1(a), (b). For microscopic examination fungal cells was observed in 40X magnification under light microscope. On closer examination vegetative reproductive spores which are termed as conidia is observed along with specialized stalks (conidiophores characteristic feature of Ascomycota). (Fig. 1c).

### Molecular identification of the Fungi

DNA was isolated from the fungal culture (AS_F). Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed (Fig. 2a). Fragment of ITS region was amplified by PCR. A single discrete PCR amplicon band of ~700 bp was observed when resolved on agarose gel (Fig. 2b). Similarly, the 18S rDNA region was amplified by PCR. A single discrete PCR amplicon band of ~1300 bp was observed when resolved on agarose gel (Fig. 2c). Ladder specification was presented in Fig. 2d. The sequencing results and Bioinformatics analysis of ITS region and 18S rDNA region are given in supplementary Figure 1 and 2 respectively. For sequence analysis for AS_F, the blast results and phylogenetic tree analysis of these sequences are compared with existing sequences in the NCBI database using the BlastN program (Figure 3 a, b). Sequences producing significant alignments with score and expect values are presented in Table 1 (a) (for ITS) and Table 1(b) (for 18S rDNA).

Table 1

Sequences producing significant alignments with score and expect values for ITS Region (a) and for 18S rDNA (b).

(a)

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|
| *Penicillium citrinum* isolate RSO_OC17_74 | 917 | 917 | 100% | 0 | 99.61% | 518 | MN634643.1 |
| *Penicillium citrinum* strain PeciHA21B02 | 917 | 917 | 100% | 0 | 99.61% | 530 | OM373020.1 |
| *Penicillium citrinum* strain NSF4 | 917 | 917 | 100% | 0 | 99.61% | 526 | JQ712839.1 |
| *Penicillium citrinum* strain | 917 | 917 | 100% | 0 | 99.61% | 529 | MW014916.1 |
| *Penicillium* sp. isolate A21 | 916 | 916 | 100% | 0 | 99.42% | 577 | KX611014.1 |
| *Penicillium citrinum* strain ZSF5 | 916 | 916 | 100% | 0 | 99.42% | 579 | KT844552.1 |
| *Penicillium* sp. clone SR-5 | 915 | 915 | 100% | 0 | 99.23% | 562 | MK246010.1 |
| *Penicillium citrinum* strain GKF39 | 915 | 915 | 99% | 0 | 99.61% | 513 | MK713439.1 |
| Fungal sp. isolate PS3782 | 915 | 915 | 99% | 0 | 99.61% | 1049 | KY776337.1 |
| *Penicillium citrinum* isolate AASF-2 | 915 | 915 | 100% | 0 | 99.23% | 584 | MT141138.1 |

(b)

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|
| *Penicillium citrinum* strain GPF021 | 1890 | 1890 | 98% | 0 | 99.24% | 1293 | MK615877.1 |
| *Penicillium citrinum* isolate PA-33 | 1890 | 1890 | 98% | 0 | 99.24% | 1316 | MG456717.1 |
| *Penicillium citrinum* strain: PSU3 | 1890 | 1890 | 98% | 0 | 99.24% | 1593 | LC127087.1 |
| *Penicillium* sp. yz11-22N2 | 1890 | 1890 | 98% | 0 | 99.24% | 1319 | KU182953.1 |
| *Penicillium* sp. Y28 18S | 1890 | 1890 | 98% | 0 | 99.24% | 1683 | KP872503.1 |
| *Penicillium citrinum* strain MF410 | 1890 | 1890 | 98% | 0 | 99.24% | 1544 | KM096252.1 |
| *Penicillium citrinum* strain MF408 | 1890 | 1890 | 98% | 0 | 99.24% | 1539 | KM096251.1 |
| *Penicillium citrinum* strain MF399 | 1890 | 1890 | 98% | 0 | 99.24% | 1542 | KM096250.1 |
| *Penicillium citrinum* strain MF396 | 1890 | 1890 | 98% | 0 | 99.24% | 1543 | KM096249.1 |
| *Penicillium citrinum* isolate F3P1Lb | 1890 | 1890 | 98% | 0 | 99.24% | 1364 | ON026131.1 |

### UV-Vis spectroscopy and High performance liquid chromatography of fungal Pigment

Identification of the extracted pigment was done by using UV-Vis spectrophotometric wavelength scan in the region 400 to 550 nm. Extract from the sample was run to find the lambda max (λ max) in this region. As shown in supplementary Fig. 3 and supplementary table 2, the absorption spectra wavelength of maximum transmittance of sample is at 450 nm.

### High performance liquid chromatography of fungal extracts

β-carotene extract prepared above was also standardized with mobile phase, Solvent A - methanol: acetonitrile (1:9) and Solvent B –Ethyl acetate in ratio of 50:50 for purification of this pigment using HPLC. Using reversed phase chromatography better resolution could be achieved for confirmation, as interfering peaks could be removed with similar resolution times in the chromatogram. With the mobile phase, solvent A: acetonitrile + water (90:10) and solvent B: ethyl acetate, isocratic elution in ratio of 50:50 (flow rate 0.5ml/min), we were able to resolve β-carotene HPL chromatogram and UV spectra of standard as well as two samples under normal conditions (Fig. 4 a, b & c).

### Fourier transform infrared spectroscopy of β-carotene

The infrared spectra of sample and standard was recorded between 450 to 3900 $cm^{-1}$. In the FTIR spectrum certain group of chemical bonding give rise to bands with certain frequency. As seen in the Fig. 5 (a,b), standard β-carotene showed peaks at 2929.87 $cm^{-1}$, 2860.43 $cm^{-1}$, 1433.11 $cm^{-1}$ ,1373.32 $cm^{-1}$ while sample peaks were at 2933.73 $cm^{-1}$, 2895.15 $cm^{-1}$ ,1625.99 $cm^{-1}$,1438.90 $cm^{-1}$ ,1375 cm-1.

### Preparative chromatography and Mass spectroscopy for β-carotene estimation.

For chromatographic separation, individual pigments present in the fungal extract was separated on a preparative chromatographic silica plate and separated into three bands –upper brown band, middle yellow band and lower pink band. These separated bands were dissolved in different LCMS grade methanol, filtered and observed for mass spectroscopy. For mass ion transitions and MS parameters of analytes, electrospray ionization method (ESI) with triple quadrupole (TQD) mass spectrometer was done performed in positive ion mode which resulted in elimination of functional group and production of precursors ions of different carotenoids (Figure 6). To distinguish between these protonated molecules, carotenoids ions transitions were monitored with m/z value of ions reported in literature and observed value m/z value of protonated molecules as precursor ions. Mass spectrum observed showed high degree of fragmentation as shown in Fig. 6(a), with characteristics peak of carotenoids at 536.9 (β-carotene), 538.9 (Zeacarotene), 544.4 (Phytoene), 554.0 (β-cryptoxanthin), 569.18 (Zeaxanthin) and standard characteristics peak 536.6 (Fig. 6.b) of β-carotene in standard as well as sample confirmed presence of this carotenoids.

## Discussion

Microscopic examinations of fungal strain shows that the reproductive spores or conidia commonly contain one nucleus which is usually dispersed by wind or water helps in asexual mode of reproduction. Use of PDA media accelerate the growth of fungal cells and conidia formation by providing required nutrients for development. For molecular identification of the fungi, the DNA sequence of AS_F was found to be best match with *Penicillium citrinum* based on the nucleotide homology analysis for ITS and 18S rDNA. A high score and low expect value indicate greatest homology. The sequence of the ITS and 18S rDNA gene from AS_F and that of matching sequences from 10 nucleotide sequences were aligned by using the Maximum Likelihood method based on the Kimura 2-parameter (K2P) model. The tree with the highest log likelihood is shown Fig. 3 (a, b). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein J, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1434 positions in the final dataset for ITS sequence

analysis and 1040 positions in the final data sheet for 18S rDNA gene. Evolutionary analyses were conducted in MEGA7. For evolutionary divergence between sequences analysis, the number of base substitutions per site from between sequences are shown in supplementary Table 1 (a, b) for ITS and 18S rDNA gene.

For presence of β-carotene in fungal extract, UV-Vis spectroscopy was done in the fungal liquid extract which showed the typical three peak spectra (434.00; 456.80 and 482.80 nm) of β-carotene in UV spectrum. As reported from literature (Supplementary Fig. 4) structure of β-carotene have larger pi system (11) and it absorbs in the visible region as β-carotene energy levels are greater and requires longer wavelength or less energy (Clark,2020). Here this wavelength corresponds to the ΔE required for electronic transition where wavelength will be strongly absorbed (Supplementary Fig. 4). In terms of high-performance liquid chromatogram, a linear regression curve ($y = 2963.74x + 20636.4$, $r^2 = 0.9905$) was obtained with peak areas for different concentrations of standard β-carotene ranging from 20 to 100 ppm (Supplementary Fig. 5). The standard curve was plotted based on the values of peak area and concentration of standard and concentration of sample was calculated based on this equation. Single peak within retention time (3.944min) for standard A and sample B (3.932 min) and sample C (3.721 min) at 450 nm was observed. Comparing the retention time presence of β-carotene was confirmed and calculated in our fungal strain (0.976 mg/g).

In FTIR graph, range from 2850–3300 $cm^{-1}$ indicates about the C-H stretching mode in saturated system (alkanes) depending on the hybridization. Strong peak at about 1438.90 $cm^{-1}$, 1375.2 for standard and 1433.11 $cm^{-1}$, 1373.32 for sample indicates the presence of $CH_2$ and $CH_3$ bending in medium range, 1480 – 1440 indicates medium $CH_2$ bending (Bacher 2002), 1465–14440, 1390 – 1365 gives medium $CH_3$ bending (Fig. 5). Also, absorption at 1625.99 $cm^{-1}$ is due to weak conjugated C = C alkene ring stretching. Based on these vibrational modes within a molecule due to its dipole momentum different wavenumbers with difference in hybridization was observed. These absorption at certain frequency are indicative of the abundance of carotenoids with major functional group as C-H, $CH_3$ and C = C bonding. From MS graph (Fig. 6) we compared spectra of sample with standard β-carotene which also confirmed presence of fungal pigment in the extract. With the observed m/z value of precursor ion and comparing with product ion reported in literature (Rivera et al. 2014). We compared biosynthetic pathway for formation of carotenoids in other microbes and plotted best possible biosynthetic pathway (Fig. 7) based on m/z value of different carotenoids (Andrewes et al. 1976; Scalcinati et al. 2012; An et al. 1999). Here carotenoid biosynthetic pathway starts with geranylgeranyl diphosphates with formation of phytoene. Further desaturation steps (dehydrogenation) gave lycopene which is cyclized at each end to produce α-carotene or β-carotene using  or β-cyclase. With hydroxylation at C3 or C3’ of carotenes zeaxanthin and lutein are produced (Fig. 7). While incubating this microbe in PDA Media we allowed microbe to grow for 8–9 days then the standard incubation time of 5–6 days which allowed this hydroxylation step and formation of these xanthophylls from β-carotene.

Microbial production of carotenoids like β-carotene is increasing due to its better industrial potential. From the results it is concluded that the common bio pigment β-carotene was identified and extracted in a new fungal strain *Penicillium citrinum*, belonging to family –Trichocomaceae. Unknown fungal strain was confirmed by phenotypic molecular biomarkers. We observed highly specific results for conformation of presence of this pigment in the extract of endophytic fungus from bark of *Taxus baccata*. We saw presence of number of important carotenoids like β-carotene, Zeacarotene, Phytoene, β-cryptoxanthin, and Zeaxanthin in MS spectra. But we focused only on β-carotene in our present research work for confirmation in our fungal extract. Plotting biosynthetic pathway based on MS spectra it offers a basis in future for further making particular carotenoids using different stimulants. Results from UV-visible with characteristics triband peak and similar retention time for standard as well as sample and abundance of carotenoids with major functional group in FTIR graph showed that viable synthesis of this carotenoids β-carotene from this microbe under normal growth conditions. Therefore, looking at results we could use this fungal strain as source for carotenoids extraction and need further observation about the new metabolites.

# Declarations


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### COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

### CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

### PATIENT CONSENT STATEMENT

No human subjects were used in the study.

### Data Sharing & DATA AVAILABILITY STATEMENT

All the Data were provided as supplementary file in table and in figure form.

### Authors contribution statement

First author, Shweta Nagal (ORCID ID: 0000-0002-7362-4565): Contributions to the conception of the work, analysis and writing of manuscript.

Corresponding author, Bibhu Prasad Panda (ORCID ID: 0000-0001-5110-2945): Concept of research work, result analysis & drafting the work.

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# Figures

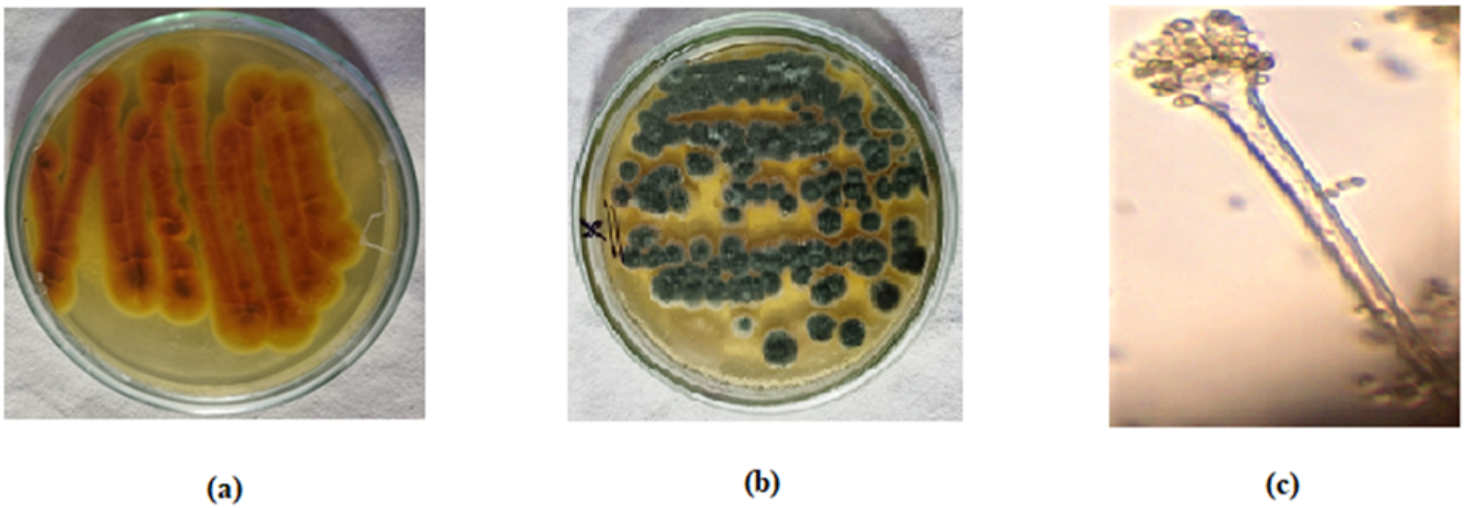
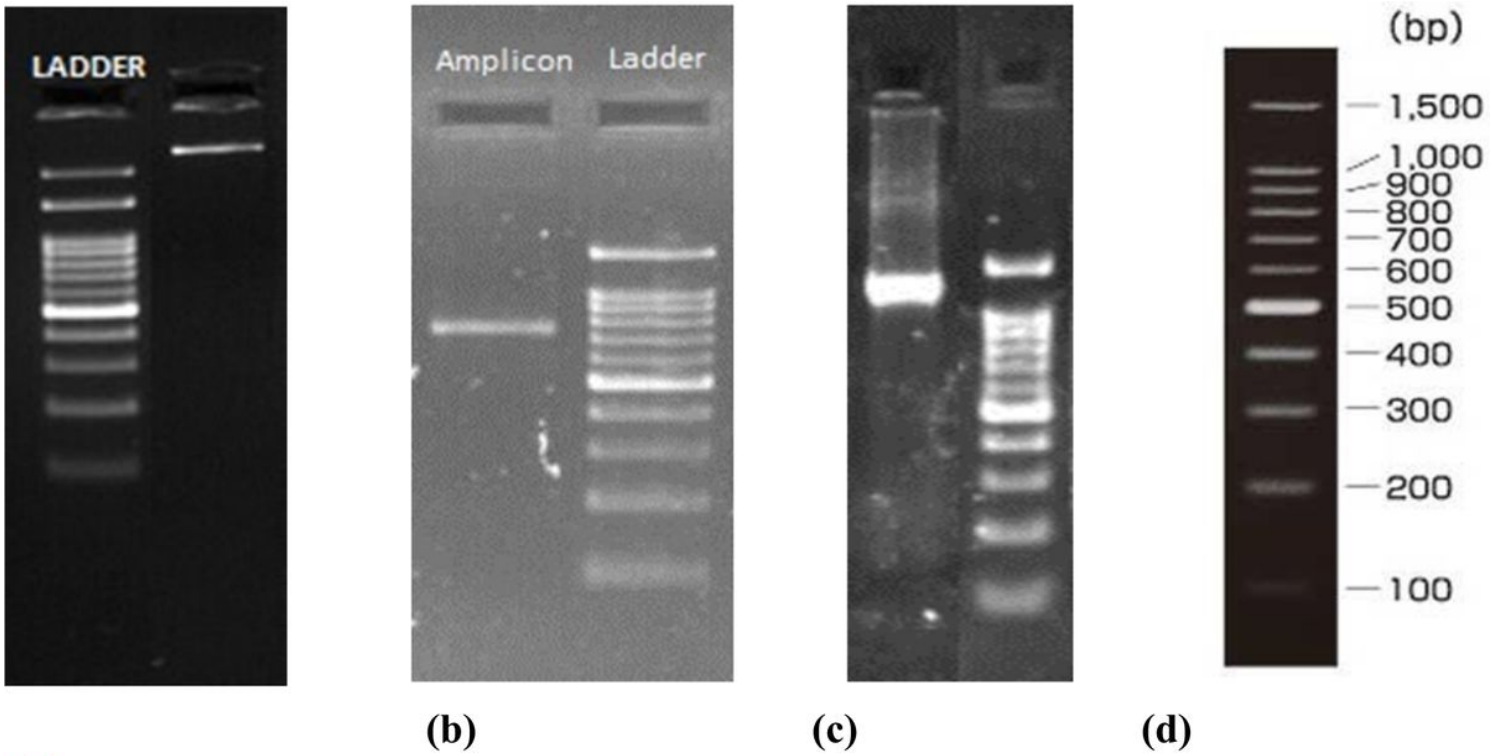


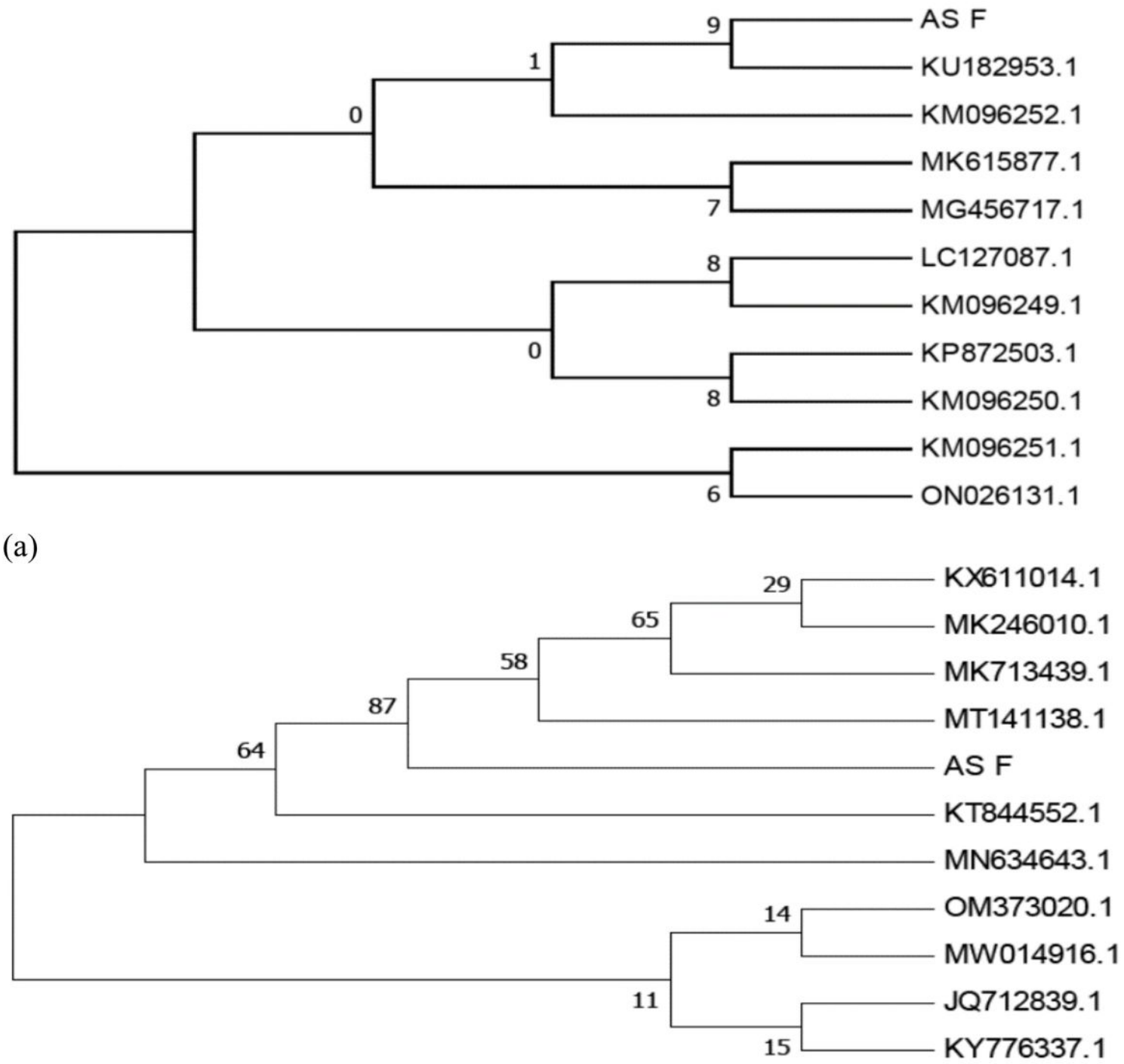

**Figure 1**

(a): Dorsal view of petri plate showing fungal culture in potato dextrose agar media for 4-5 days at 28± $2^0$C (b) Frontal view of petri plate showing fungal culture in potato dextrose agar media for 5 days at 28±$2^0$C (c) Microscopic examination of fungal cells at 40X magnification under light microscope.



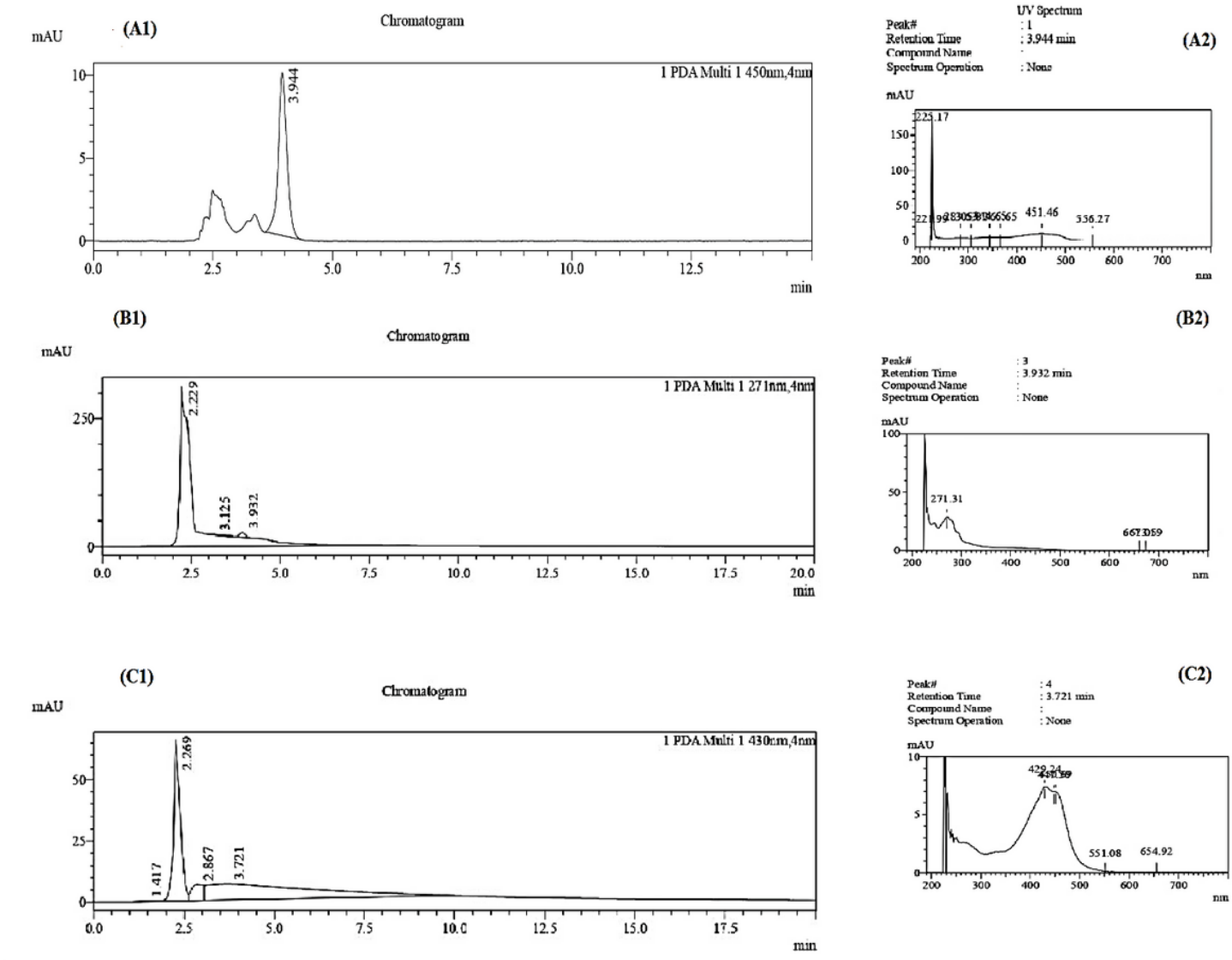

## Figure 2

(a) Genomic DNA of fungal culture (AS_F); (b) PCR amplicon of ITS region; (c) PCR amplicon of 18S rDNA region; (d) ladder specification.



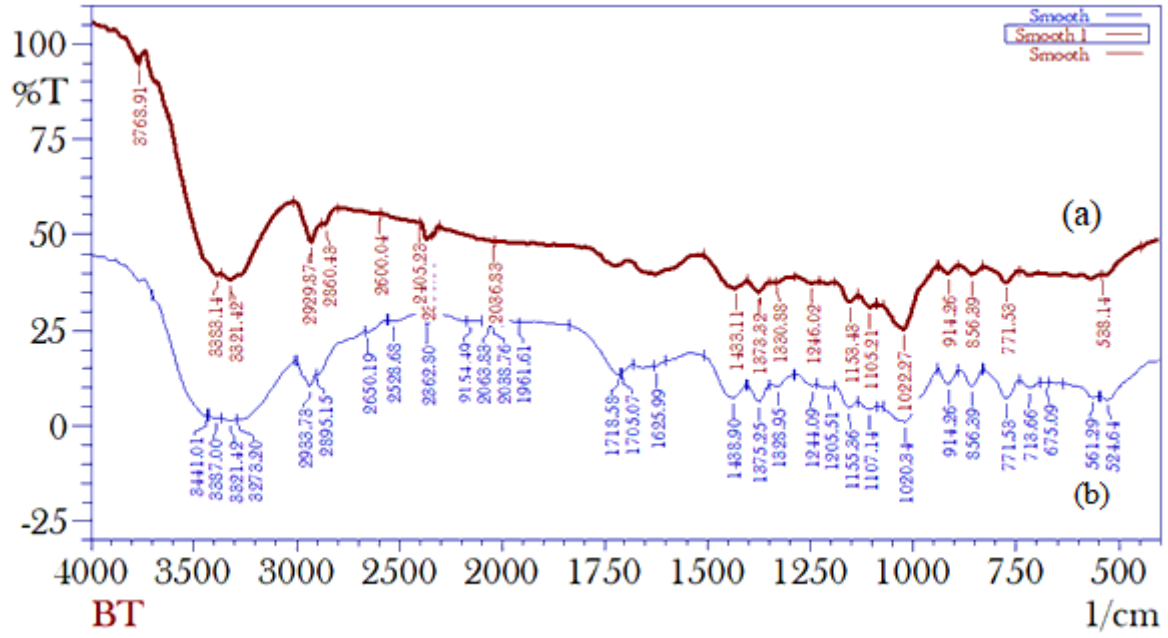

## Figure 3

Molecular Phylogenetic analysis by Maximum Likelihood method for ITS region (a) for 18S rDNA (b).



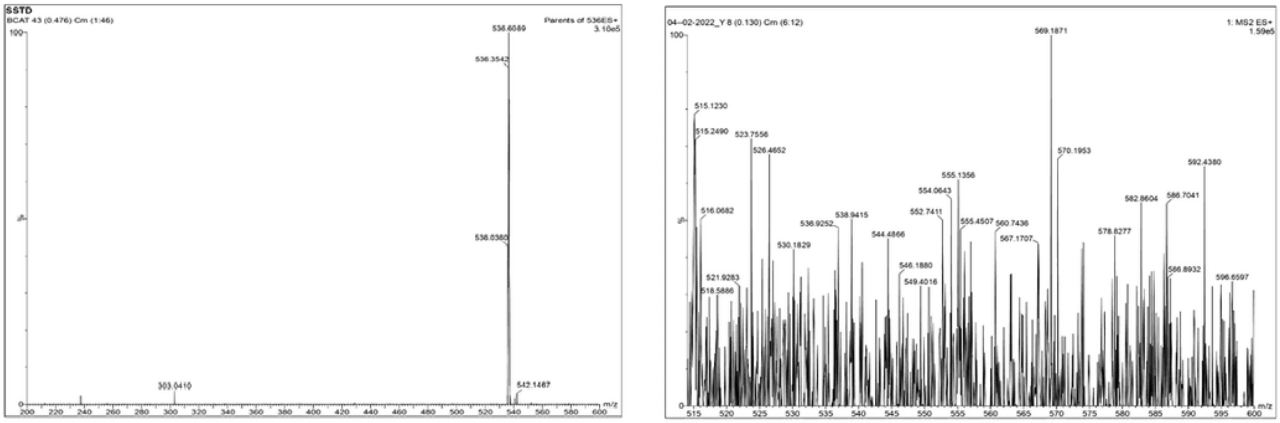

## Figure 4

HPL chromatogram of the (A1) standard β-carotene and (B1), (C1) extract of *P. citrinum* showing retention time (minutes) and absorption spectra. Peaks were identified by matching retention time and absorption spectra with standard β-carotene.



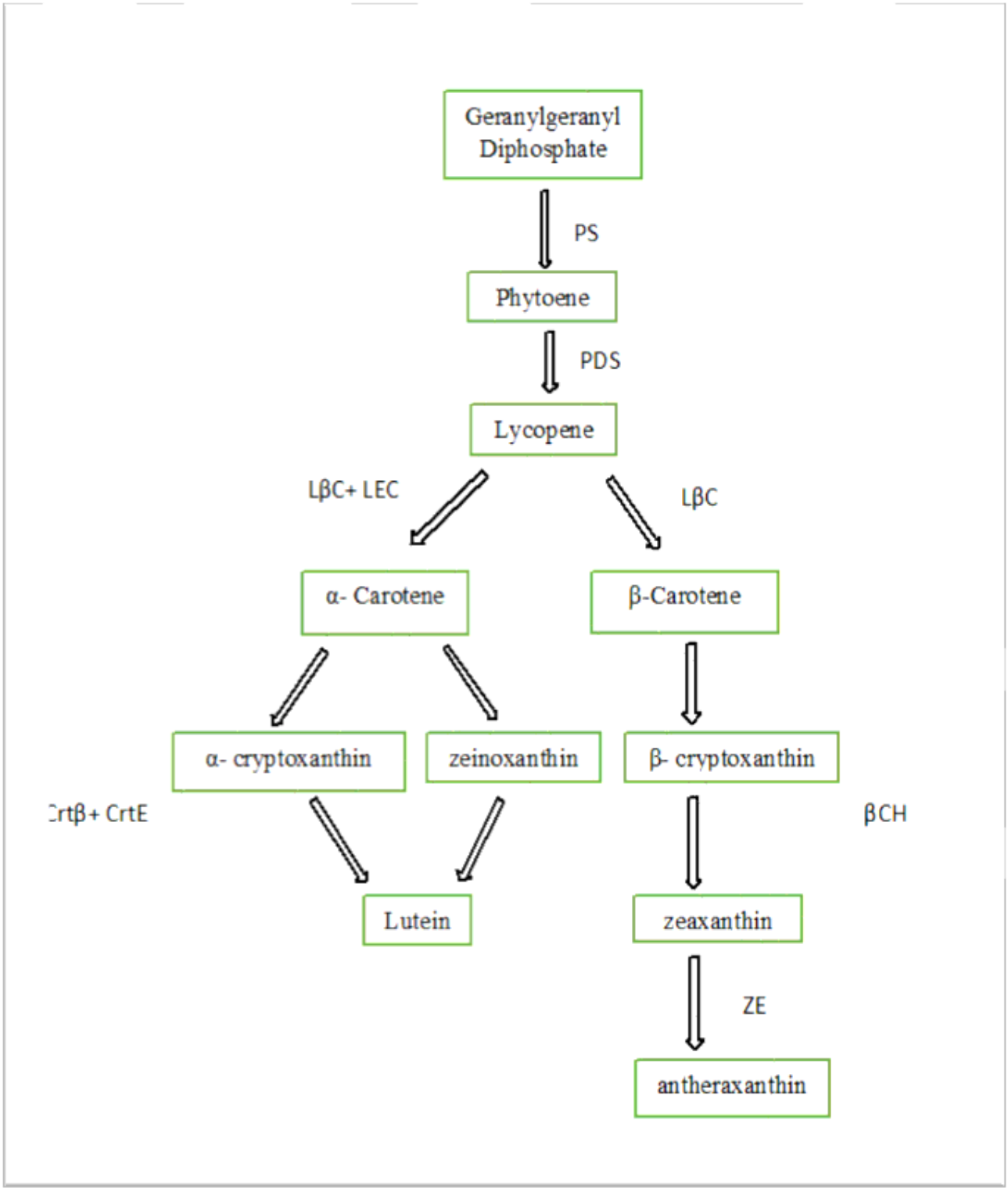

## Figure 5

FTIR spectra of standard β-Carotene (a) and fungal pigment extract of *Penicillium citrinum* (b).




## Figure 6

(a) Positive ion MS peak chromatogram of the standard β-Carotene (b) ESI-MS positive ion spectra for carotenoid of the fungal extract of *Penicillium citrinum* in positive ion mode.




## Figure 7

Postulated biosynthetic pathway of β-Carotene in *Penicillium citrinum*. PS-phytoene synthase, PDS-phytoene desaturase, LβC- lycopene β cyclase, L C- lycopene cyclase, βCH- β-Carotene hydroxylase, ZE-zeaxanthin epoxidase, Crtβ- β-ring hydroxylase, Crt - ring hydroxylase.

# Supplementary Files

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