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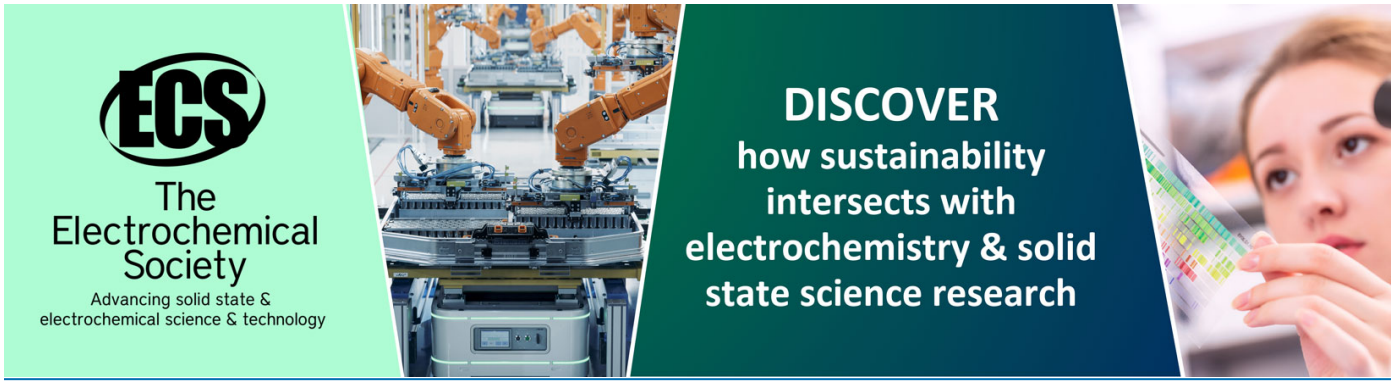


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# Identification of Penicillium Species of Fruits Using Morphology and Spectroscopic Methods

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**Abstract.** This study aimed to investigate and identify the isolated five species of Penicillium from different rot fruits. The *P.implicatum, P.aurantiogriseum, P.purpurogenum, P.citrinum, and P.notatum* Penicillium species were found. Penicillium species are important in the field of medicine, industry, and produces secondary metabolite, some penicillium species are contaminant for bread, grains, fruits, and processed food, so the identification and characterization of these species are very important. By using the microscopic technique and spectroscopic methods for identification and characterization that species isolated from infected fruits. The spectroscopic methods are Raman spectroscopy and UV-Visible spectrophotometry, they are fast analysis techniques, sensitive, use a small quantity of sample, and depend on biochemical components for the discriminate of Penicillium spp. Raman vibrational spectra and UV-Visible absorption spectra showed differences for five Penicillium species. Our results proved that spectroscopic techniques showed spectral differences for five Penicillium species, and this agreed with the study of morphological characteristics for that species. The conclusions of this study demonstrate that spectroscopic methods in combination with morphological techniques could be accurate and reliable approach for discrimination between different Penicillium species.

## 1. Introduction

Penicillium is a genus of one of the most common fungi found in different environments and suitable environment (temperature, humidity, pH). Penicillium species (spp.) are the main pathogens of different fruits. Penicillium spp. produced extracellular enzymes that play an important role in the microbiological decomposition of organic materials [1]. Many Penicillium species produced different chemical types of secondary metabolites, while some of them are important in the field of medicine, others are used for the production of mycotoxins, important drugs, and some of the Penicillium species are used in industry, especially penicillin production. On the other hand, there are penicillium species can cause contamination of bread, grains and processed food [2], for this reason, it is important to identify and classify penicillium at the species level and the ability to predict its structure information and biochemical composition in order to distinguish types of penicillium precisely. Asexual Penicillium species such as *Penicillium notatum* has been used in penicillin synthesis [3]. *Penicillium purpurogenum* has positive characteristics to be used in biotechnology, it has an active cellulase for

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the production of several extracellular xylanase and used in cellulose pulp bio bleaching [4]. *Penicillium citrinum* plays an important role in the production of β-galactanase, xylanase, an endoglucanase, and enzymes used in biological and industrial fields. The enzymes such as β-glucosidases characterize *Penicillium citrinum* [5]. *Penicillium aurantiogriseum* can produce lipase, which is used in food industry, laundry detergents and in perfumery [6]. Fungi to species level have so far been named depending on genetic metabolic by using molecular characterization diversity and based on morphological identification [7]. Classification of fungi such as penicillium at the species level depends on morphological characteristics (colony features, hypha, the arrangement of spores), which cause fungal differentiation, so the morphological study is the most diagnostic and consistent method [8]. As fungi are morphologically complex microorganisms differ in their structural forms due to the surface or submerged form of growth, the nature of the growth, physical environment and some species are cryptic with very similar morphology, therefore, it is difficult to identify them by the morphological method only. The primary structure of fungi consists of tubular filaments, known as hypha that arise from a single reproductive spore. It repeats itself to form a mass of strands known as mycelium [9]. Most of the important morphological features are identified from mycelium. Penicillium spp. determined by microscopic identification technique based on macroscopic morphological features such as colony types, colony color, size, shape, and hyphae were observed by the naked eye and microscopic features (arrangement of spores and conidia) of different Penicillium spp. observed under a compound microscope. The morphological identifications of fungi by the microscopic examination method alone sometimes are not suitable for several reasons, including the prokaryotes, small cells, and some cells close to the limits of the accuracy of the optical microscope [10]. The identification of Penicillium to species level is not always possible based on the morphological method, so we implemented spectroscopic methods such as Raman spectroscopy and UV-vis spectrophotometry. By using spectroscopic methods to characterize fungi by describing the biochemical components and obtain more traits are used to discriminate between species with more accurate and clear results. Raman spectroscopy is simple technique, requires a small amount of sample, not affected by water, rapid, non-destructive, ease of use at low cost, high sensitivity and provides information about biochemical fingerprinting of the fungi mass, chemical composition, structure, and interactions of biomolecules inside the cells of microorganisms [11, 12]. Raman spectroscopy has been used for the rapid and accurate identification of fungi species in recent years, thus, appears to be a good and useful technique for studying and distinguishing fungi. It is an inelastic process that occurs when a sample is illuminated with a strong monochromatic light source (such as a laser beam) [13] in the visible range or near-infrared. Raman spectra of samples are obtained when collecting inelastic scattered photons from the studied sample. We proved that Raman spectroscopic technique is important for classification and identification of fungi and that is because of fingerprints based on Stoke scattering [14], each sample has a special fingerprint that distinguishes it from the other samples. Raman spectra can distinguish between fungi species through the difference in the intensity peaks, presence of some peaks in the spectra of samples and absence of some peaks from other spectra. UV/Vis spectrophotometry is also used to strengthen the result obtained from morphology and Raman spectra [15]. In this study, we are the first used UV / VIS Spectrophotometry to support our study to identify and differentiate Penicillium species.

## 2. Materials and methods

### *2.1. Fungi species and sample preparation*

The various strains of pencillium (*Implicatum, Aurantiogriseum, Purpurogenum, Citrinum and Notatum*) used in this study were isolated from infected fruits. The isolates were inoculated as one point in 9-cm petri dishes containing potato dextrose agar (PDA) at 28±2 °C in the darkness for a one week. When fungal growth and colony diameters were observed on PDA media, the plates for fungi were examined by using lactophenol cotton blue (LPCB) for identification penicillium spp. by using microscopic technique (labomed vision 2000). Penicillium colonies were suspended in 250 ml conical

flask containing potato dextrose (PDB) and cultured again for a period of 1-3 weeks in darkness at 28±2 °C and mycelium and spores of sample were filtered using Whatman No.1 filter paper, then, washed three times with sterile distilled water. Samples were dried and used in powder form for further study and characterization using spectroscopic methods.

### *2.2. Micromorphological and macromorphology identification of fungal isolates*

After completion of one week of fungi culture, morphological characteristics were performed by preparing the slides using lacto phenol blue cotton and observed with optical microscope. Under the microscope we observed the growth rate, diameter, color, smooth and texture of colonies and microscopic features (stipes, the number of tips, branching of individual hyphae, conidiophores, ornamentation of conidia, conidium shape, length) [16-18] and hence performed.

### *2.3. Raman spectroscopy*

Raman spectra were recorded using Raman microspectrometer equipped with optical and digital microscope and high-resolution digital camera from Olympus. Green laser light (520–570 nm) was used to induce the fungi samples and Raman scattered light was collected at the region 3800-200 $cm^{-1}$.

### *2.4. UV–Vis spectrophotometry*

UV spectra were recorded in the range of 190–1100 nm by a UV–visible spectrophotometer (ELCO duple beam 1100 with a resolution 0.72 nm, accuracy ± 0.5 nm and 1.8 nm bandwidth). Samples were prepared by adding 5 mg of the penicillium sample powder to a solution consisting of 40 ml distilled water and 10 ml ethanol and stirred using magnetic stirrer to obtain a homogeneous solution.

## 3. Results and discussion

### *3.1. Morphological characterization of fungi:*

***Penicillium citrinum*** exhibits moderate sporulation growth on potato dextrose agar (PDA) at 28±2 °C.P. citrinum can be recognized by its penicilli, which consists of 3 -5 divergent structures and vesiculate metulae [16].The color of the colonies is grey green, mycelium inconspicuous, soluble pigments yellow, Figure 1A, conidial colour grey green to dark green and conidia were spherical smooth to finely roughened surface and were bearing long well defined columns [17]. ***Penicillium notatum*** grew on (PDA) at 28±2 °C. P. notatum has penicilli, which consists of 2-5 divergent and smooth walls of biverticillate structure. The color of the colonies is brown in age, surrounding colonies is yellow in color and have loose-textured, soluble pigments absent light yellow. Conidia (spores) celandine are green, globose, subglobose and smooth-walled [19] Figure 1A. ***Penicillium aurantiogriseum*** is moderately rapid growing on (PDA) at 28±2 °C.P. aurantiogriseum has penicilli mostly terverticillate, sometimes biverticillate with branches strongly diverges. The color of the colonies is dark green as in Figure 1A, obversed mycelium white to yellow, soluble pigment very faint, conidial color greyish green and conidiophores were mononematous ,subglobose to ellipsoidal and smooth walled [20,21]. ***Penicillium purpurogenum*** was spreading and moderately growing on (PDA) at 28±2 °C.P. purpurogenum has narrow penicilli. As shown in Figure1 velvety colonies has dark green color, long and smooth hyphae, mycelium is observed yellow in color, pigmented in red and conidial color is very dark green ,ellipsoidal, rough and heavy walled conidia [17,22-23]. ***Pencillium implicatum*** is moderately growing on (PDA) at 28±2 °C.P. implicatum has strictly monoverticillate penicilli. Intense colonies with bluish grey color, velvety and extent floccose in the center, Figure1 B. It is observed that mycelium has white color, yellow soluble pigment and conidial color is heavy grey-blue, ellipsoidal and smooth walled conidia [24-25]. By observing the morphological characteristics of penicillium species, we found that they differed through the color, internal structure, characteristics of colonies, and other properties studied by microscopy.

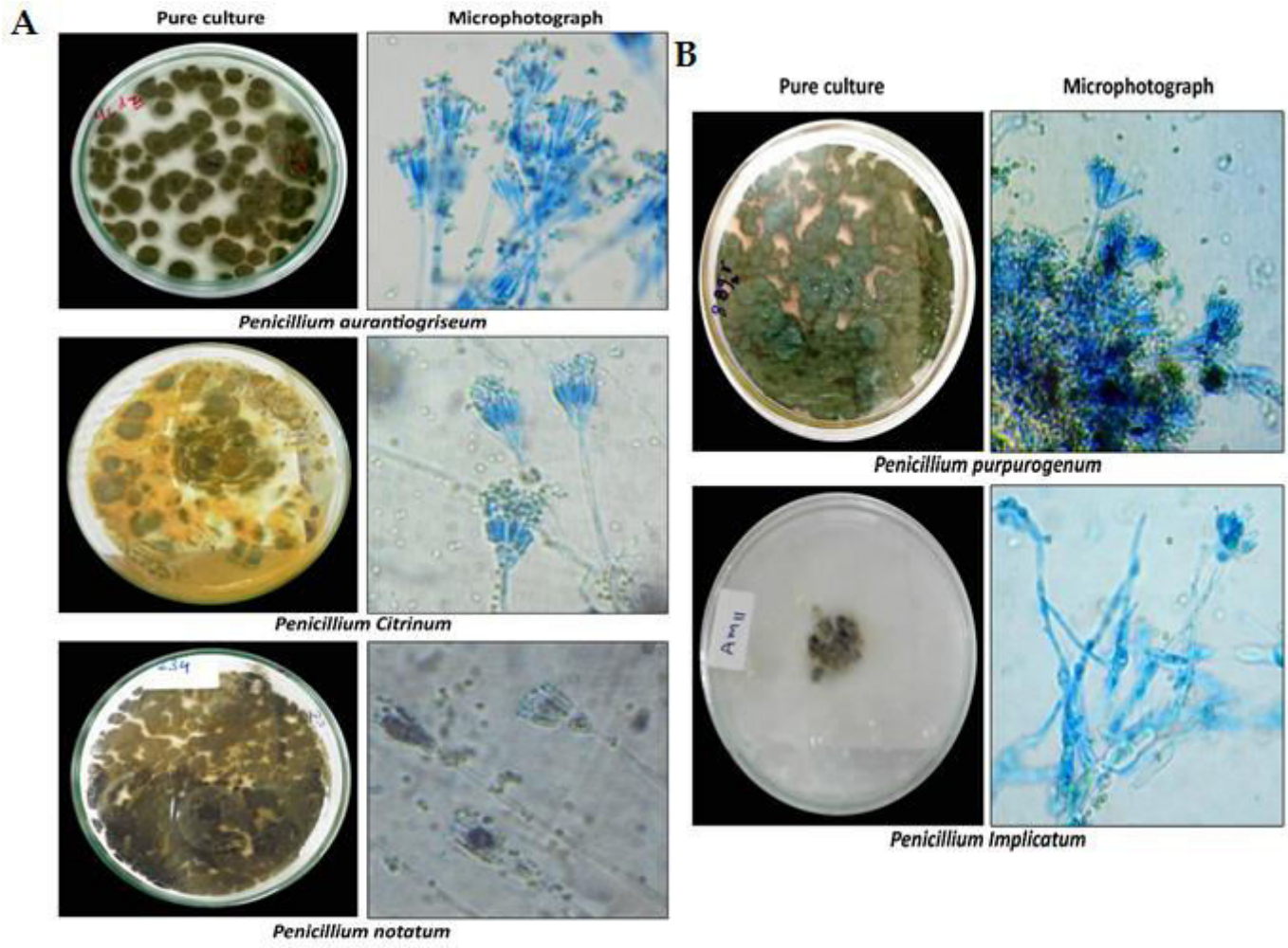
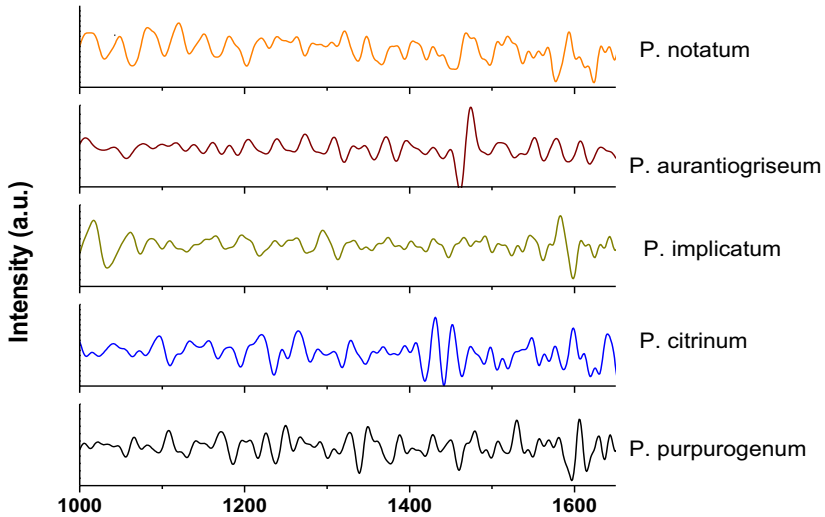



**Figure 1.** Observation of pencillium species colonies and microscopic morphologies: (A) three species (Aurantiogriseum, Citrinum and Notatum) and (B) two species (Purpurogenum and Implicatum), Colonies after incubation for 7 days at 28±2 °C on PDA.

### *3.2. Raman study*

The Raman spectra for the chemical components present in the five strains of pencillium are shown in Figure 2 and Figure 3 in two regions, 1000-1700cm$^{-1}$ and 400-1000 cm$^{-1}$. Raman bands in spectra have been assigned to biomolecules such as protein, lipids, saccharides carbohydrates and nucleic acids. From figure 2 and 3, it is observed that all species of penicillium contains these biochemical components. Peak intensity helps us to identify the species because it indicates the quantity of the chemical components and it is observed different in different species. The different vibrational modes in Raman spectra for penicillium species gives us information about the genetic and phenotypic signatures of those species which is important to classify fungi. The bands of Amide I and Amide II were observed in the range 1643-1630 cm$^{-1}$ and 1584-1530 cm$^{-1}$ which indicates the presence of protein in all penicillium spp. Each region represents C-N stretching, C-C and C-N stretching and N-H deformation groups [26-27] of Amide I and Amide II, respectively. All peaks in the spectrum indicate the presence of protein in all samples, but their peak intensity and wavenumber values are different for different species. This denotes that each sample contains a specific quantity of protein. The bands associated with the $CH_2$ deformation vibrations were observed in the range 1479-1404 cm$^{-1}$, and 1396-

1332 $cm^{-1}$, which assigned to lipid contribution [27-28] and glycogen (polysaccharide of glucose) [27-29] contribution, respectively. Raman bands at 1322, 1327, 1328 $cm^{-1}$ indicates that contribution of amide III [27-30] for proteins in *P.purpurogenum, P.citrinum and P. notatum*, respectively. All pencilium species showed bands in the regions of 1295-1220 $cm^{-1}$ assigned to amide III of protein [27-31]. The intensity of bands in the region 1171-1120 $cm^{-1}$ corresponding to C-C and C-O stretching groups for polysaccharides [29] with high intensity in case of *p.notatum* and low intensity in caseof *p.aurantiogriseum*. The bands for nucleic acids corresponding to O–P–O (DNA) and Adenine, Cytosine, thymine, phosphodiester band in DNA and RNA [27-28, 32-33] have been observed for all species in regions 1097-1061 $cm^{-1}$, 677-625 $cm^{-1}$ and 839-707 $cm^{-1}$, respectively. But the peak intensities are different for different species. The peaks in the low wavenumber region 897-842$cm^{-1}$ and 565-472 $cm^{-1}$ (Figure 3) indicate to contribution of carbohydrates assigned to COC stretching and C–C stretching and deformation (COC) glycosidic ring, deformation (CCC) and chitin, respectively [27-29, 31, 33]. All species show high intense and complex peaks in this region, this means penicillium species contain more quantity of carbohydrates compared to other biochemical components. The Raman peak for the polysaccharide beta (1,3)-D-glucan in the cell wall, is observed, in the range of 427 - 406 $cm^{-1}$[28], Figure 3. The strong and high intense peaks are observed for *P.purpurogenum, P.aurantiogriseum, P.notatum* and *P.citrinum* whereas these are weak and low intense for *P.implicatum*. This illustrates that cell wall in four species of penicillium contains a more polysaccharides. Aromatic amino acids present in proteins that are an essential component of fungi. Earlier [34], we observed the same results using FTIR-ATR spectroscopic method for the *P.purpurogenum, P.aurantiogriseum, P.notatum, P.citrinum* and *P.implicatum*.



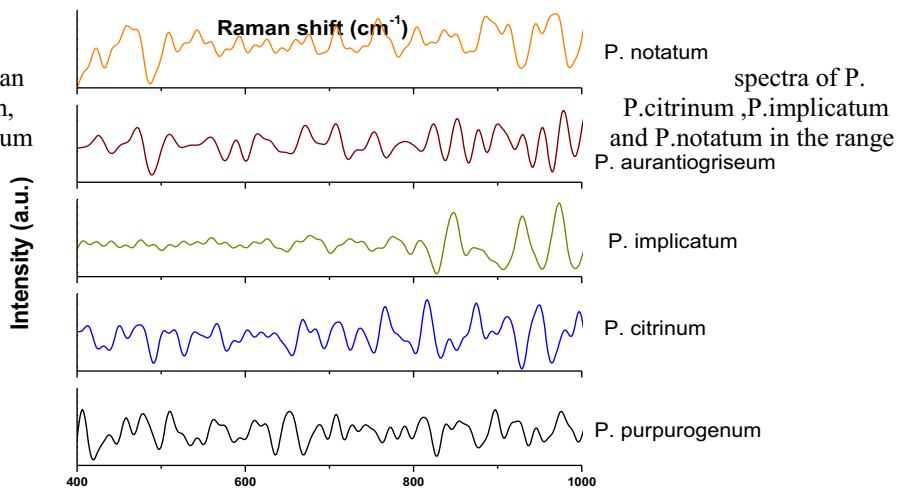




**Figure 2.** Raman spectra of P. purpurogenum, P.citrinum ,P.implicatum ,P.aurantiogriseum and P.notatum in the range 1000-1700 $cm^{-1}$

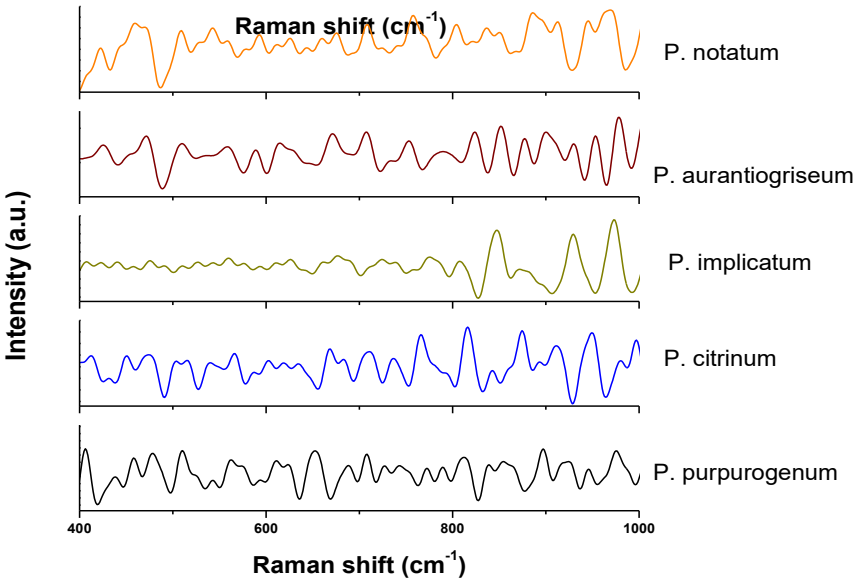



**Figure 3.** Raman spectra of P. purpurogenum, P.citrinum, P.implicatum ,P.aurantiogriseum and P.notatum in the range 400-1000 $cm^{-1}$

*3.3. UV-VIS study*

The aromatic amino acids such as tryptophan, tyrosine, and phenylalanine used as spectral absorption investigations for characterization of pencillium species using UV-Vis spectroscopy technique. Some aromatic amino acids absorb ultraviolet radiation in the 350-250 nm range. The spectral UV absorption spectroscopy is dependent on π–π∗ electronic transition of the amino acids generally available in protein such as tryptophan and tyrosine [35-38]. The UV absorption spectra were measured for five penicillium species, which included three wavelength intervals: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (100–280 nm) and also visible absorption region (400-700 nm) [39]. The UV–vis spectra, recorded for five samples of pencillium suspended in homogeneous liquid, consists of ethanol and water, showed absorption band in the range 256-520 nm (Figure 4). This is first attempt to distinguish and characterize fungi such as penicillium species using UV–vis spectroscopy. The UV-Visible spectrophotometry has been used to detect fungal pigments found in the different species of penicillium in the range of 400 nm to 700 nm for visible light [40, 41]. From Figure 4, UV-C radiation absorption peak are observed for *p.implicatum* (256 nm), *p.aurantiogriseum* (257nm), *p.notatum* (257 nm), *p.citrinum* (259 nm) and *p.purpurogenum* (259 nm). All penicillium species shows intense and strong absorption peaks between 256 to 259 nm, all these peaks confirmed the presence of tyrosine and tryptophan of the proteins [42]. The UV-B radiation absorption peaks are observed (in the range 282-287 nm) for *p.implicatum* (282 nm), *p.citrinum* (282 nm), *p.notatum* (284 nm), *p.purpurogenum* (284 nm) and *p.aurantiogriseum* (287nm). Absorption peaks in the region 282-287 nm attributed to tyrosine and tryptophan of the proteins [42]. From Figure 4, it is also observed that *p.citrinum* has weak absorption peaks in the visible range 400-700 nm at 520 nm, this absorption peaks represents the presence of pigments including melanin produced by *p.citrinum* [43]. Generally, the UV-vis absorption peaks are different for each species in terms of intensity and wavelength.

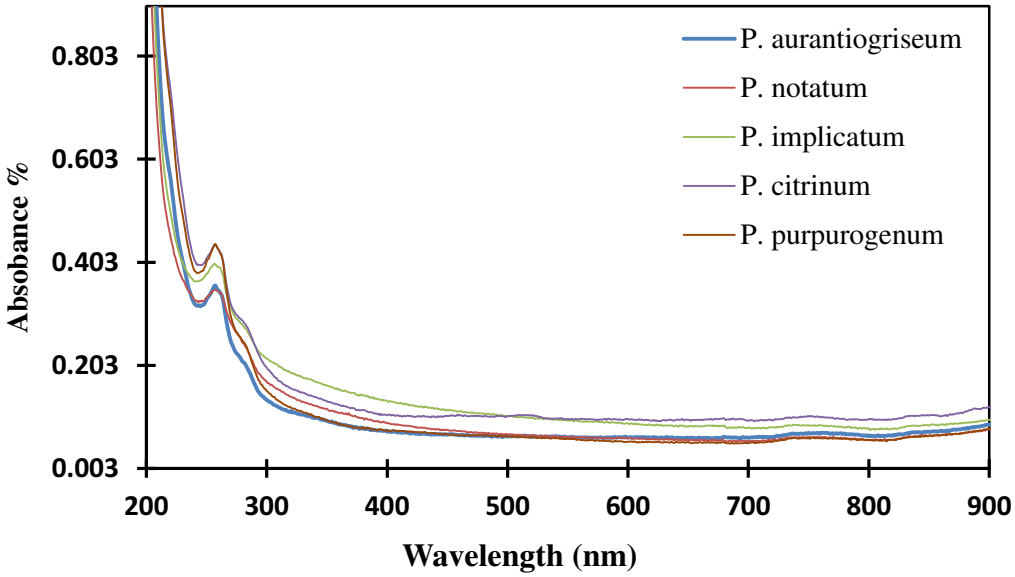



**Figure 4.** UV–vis absorption spectra recorded from the pencillium: Aurantiogriseum, Notatum, Implicatum, Citrinum and Purpurogenum at room temperature.

## 4. Conclusions

Pencillium species of rot fruit fungi have been characterized and identified using Raman spectroscopy, UV–Vis spectroscopy, and microscopy. From this study, it is found that each species has a special features which reflected in their Raman and UV-Vis spectra. Results showed that spectroscopic methods are coincide with morphological characteristics for identification and characterization of studied penicillium species. It is found that all five species have different Raman and UV-Vis spectra. All the methods used in this study are complementary in the presentation of biochemical components information about the types of penicillium and their classification. These methods are reliable methods for rapid and accurate identification and discrimination between different species. Some of the species contain a bioactive components can be captured using spectroscopic methods and used in the pharmaceutical industry.

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