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APPLICATIONS OF RAMAN SPECTROSCOPY FOR CHEMICAL CHARACTERIZATION AND PROTEIN CONFORMATION OF AGARICUS BISPORUS (LANGE) IMBACH. (AGARICOMYCETIDAE) SPORES

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Abstract: A Raman spectra was obtained for a spore suspension of an edible mushroom *Agaricus bisporus* using iRaman 2013 with a variable laser power (max. upto 300mW), near infrared 785 nm diode laser and resolution time 3 cm^{-1}. The spore sample was exposed for 140s with excitation laser power 30mW to obtain optimum peaks. The spectra revealed Raman frequencies range from 242 to 2690 cm^{-1}. Raman spectroscopy shows a strong S-S stretching vibrational band in the region of 500-550 cm^{-1} suggesting that the g-g-g form is the most preferred conformation. The spectra also exhibited the presence of various compounds such as C=C aliphetic chain, S-S, C-S, C-O-C, Sulfonic Acid, C≡C, P-H, Sulfhydryl Group (S-H), phosphorus acid selenate, C-Br, C-F, Si-H and notably isocynate. In the biological and environmental research this kind of analytical capability is very useful for the chemical identification of fungi, such as, *Agaricus bisporus*.

Key Words *Agaricus bisporus*, organic and inorganic compounds, Raman spectroscopy, spore.

INTRODUCTION

Raman spectroscopy has attracted a great attention; firstly, with an aim to develop a rapid biological threat detection technique both for the military and homeland security and secondly, to analyze the cellular components of biological cells or characterizing molecular interactions in the biological cell.[1,2] Vibrational spectroscopic methods such as Raman and surface enhanced Raman scattering (SERS) provide rapid and detailed fingerprint information about the molecular composition of biomaterial in a non-destructive manner. Over past decades, tremendous advances have been made with respect to the technology, methodology and interpretation of biological Raman spectra. Raman spectroscopy is emerging as a rapid and information-rich method of investigating biological threats. Numerous studies have been performed utilizing Raman and Surface enhanced Raman spectroscopy for the detection and analysis of biological materials.[1]

Raman spectroscopy is nondestructive and does not require extrinsic contrast-enhancing agents. Similarly, it requires minimal sample preparation, and has acquisition times ranging from a few seconds to minutes.

Raman spectroscopy is based on vibrations between the chemical bonds of the atoms. In this technique, a beam of monochromatic visible laser light is focused on the sample. The radiation scattered by the sample contain weak lines, at frequencies both lower and higher than exciting radiations. This frequency difference is called Raman shift and is characteristics of the sample and independent of exciting frequency. The spectra, thus obtained is called Raman Spectra which arise from molecules, which scatters photons of the lower and higher frequency than that of exciting line. The molecules which scatter photons with lower frequency is called as strokes lines and that of with higher frequency is called Antistokes lines. Therefore, the chemical composition of a cell and resulting in a typical vibrational "fingerprint" for the identification and characterization of the sample.[3] Raman spectroscopy offers high sensitivity.[4] large informational content which have made it appealing for studies of various biological systems such as identification of bacterial and fungal cell (including mushrooms) and the differentiation between spores and vegetative growth states.[5]

Mushrooms are widely appreciated all over the world as a human food for their nutritional properties, texture, flavour, medicinal as well as tonic attributes.[6] They have also been considered a valuable source of many different nutraceuticals[7] which play an important role in the maintenance of equilibrium through the endogenous defense system.[8]

Out of more than 2000 species of mushrooms existing in the nature; less than 25 species are commercially used as foods. Amongst the various mushrooms, *A. bisporus* (Lange) Imbach (White Button Mushroom) is commercially cultivated and most commonly used. It totally accounts for 40% of the world edible mushrooms. The high nutritional value and good flavor has increased the demand of this product.[9]

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A. bisporus has a delicious taste and aroma, high nutritional value, high biological activity, and low toxicity.[10] It has many medicinal metabolites that have therapeutic activity against many ailments including cancer. Some of the important metabolites include polysaccharides, ergosterol, selenium, anti-hyper cholesterol agents, terpenoids, lectins etc.

Although, *A. bisporus* has several health benefits, yet few reports state it's hazardous effects on human health. It is reported that the button mushroom contains small amount of the carcinogenic mycotoxin agaritine, which is a health hazard.[10] Whereas, two cases of white mushroom industry workers suffering from asthma caused by hypersensitivity to basidiocarp and spores are reported.[11] It is well understood fact that presence of aerosols can cause respiratory diseases.[3,4] The present study was undertaken mainly for following reasons, a) This mushroom exhibit health benefits and there are several reports dealing with chemical composition of fruiting body and / or mycelial biomass, but no report was came across dealing with chemical composition of spores, b) There are a few cases reporting the negative allergic effect on the health of the mushroom workers due to spores. Hence, it becomes necessary to analyze chemical composition of the spores, c) Raman spectroscopy was effectively used largely in aerosol studies with few reports dealing with filamentous fungi. However, there was no report found on mushrooms.

MATERIALS AND METHOD

Sample collection

Fresh *A. bisporus* mushrooms (3.5 ± 0.4 cm cap diameter) packed (200 g lots) in 100 gauge perforated polyethylene packets were purchased from a local shop and immediately a spore print was obtained on cellophane paper following the method of Storey.[12]

Preparation of spore suspension

After obtaining a spore print, a loopful of spores was transferred aseptically in sterilized deionized water and mixed thoroughly. This suspension served as sample and was subjected to the analysis.

Raman spectra

Raman spectra of the spores was collected using a iRaman 2013 (IW and Tech. Comp., USA) equipped with a variable laser power (max. upto 300mW), near infrared 785 nm diode laser, with CCD detector and optical fiber attachment with cuvette holder and resolution time 3 cm^{-1}. The spore sample was exposed for 140 s with excitation laser power 30mW to obtain optimum peaks. The spectra with most prominent Raman peaks were obtained and interpreted using various literatures.[13 – 21]

RESULTS

The spectra revealed fifteen major Raman peaks in the range between 242 to 2690cm^{-1}. The Raman spectra obtained is represented in Figure 1 (A and B) and the groups assigned to respective Raman frequency is represented in Table 1. The Raman bands 242 – 395 cm^{-1} exhibited the presence of C=C aliphatic chain while peak at 415 cm^{-1} showed the presence of selenate. In the present study, the Raman frequency for S-S was observed in the range of 455 – 494 cm^{-1} which is near to 510cm^{-1}, suggesting the gauche-gauche-gauche (g-g-g) conformation of the proteins (http://epubs.surrey.ac.uk/184938/6/SeSpeciesInFoods HealthTableNov07.pdf). A peak was observed in the region of 568 – 658cm^{-1} which is characteristics for organobromine (C-Br). This observation is in agreement with Schrader[23] and Socrates.[24] Furthermore, the Raman bands 679 – 694 cm^{-1} were identified as C-S. Methionine and cystine residues have a C-S stretching vibration. A peak at 723 – 789cm^{-1} was identified for C-F. Whereas, the presence of glycosidic bond (C-O-C) was observed in the range of 867 – 940cm^{-1}. The Raman frequency 1025 cm^{-1} was assigned for sulfonic acid.

Table 1: Raman frequencies (cm^{-1}) and observed chemical species for spores of *Agaricus bisporus*.

Raman Frequencies (cm^{-1})	Chemical Species
242-395	C=C aliphetic chain
415	Selenate
455-494	S-S
568-658	C-Br
679-694	C-S
723-789	C-F
867-940	C-O-C
1025	Sulfonic Acid
2082-2134	Si:H
2188, 2231	C≡C
2262	Isocyanate
2288-2483	P-H
2545-2598	Sulfhydryl Group S-H
2622, 2639	phosphorus acid
2690	Aldehyde

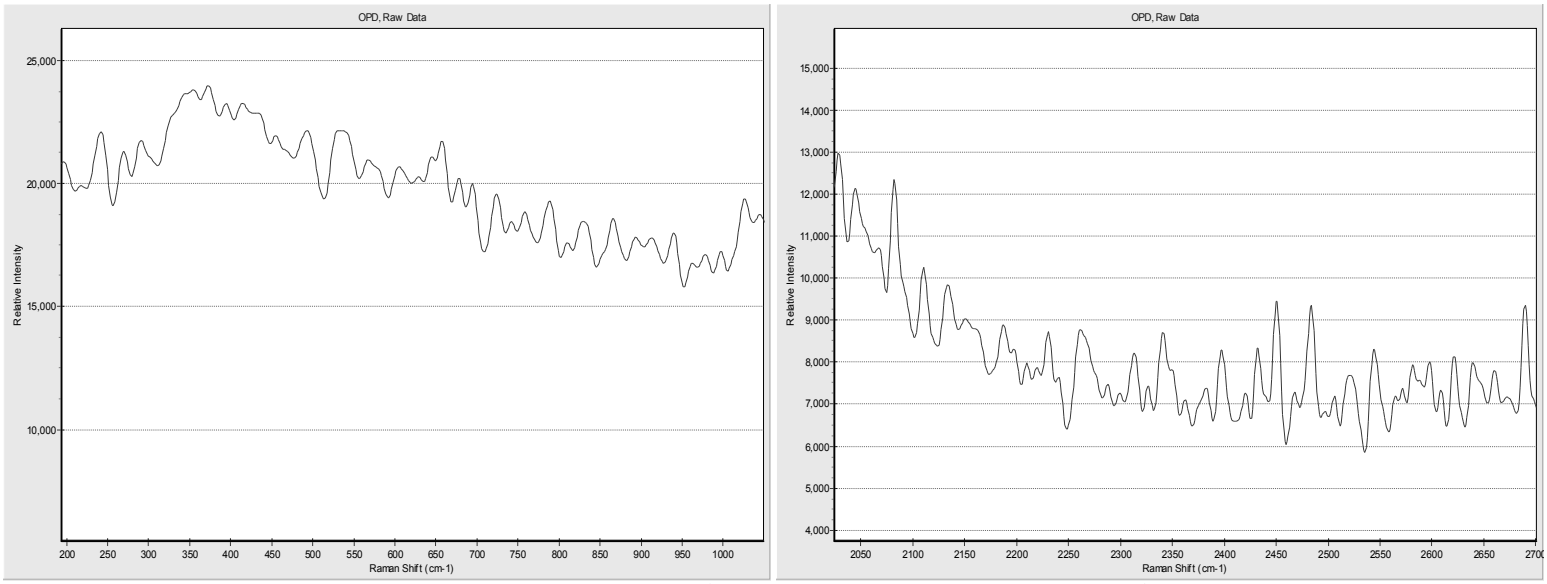

Figure 1: Raman Spectra of *Agaricus bisporus* spores (1A: Left Hand Side; 1B: Right Hand Side)

The presence of Si:H was detected at 2082 – 2134cm^{-1}and the C≡C was detected at two frequencies 2188cm^{-1} and 2231cm^{-1} respectively.[23] Whereas, a chemical species isocyanate was detected at 2262 cm^{-1}. It was observed that, P-H has a relatively broad range of Raman frequencies ranging between 2288–2483cm^{1}.[26] The S-H stretching vibrations appeared in the 2570-2580 cm^{-1} which is a characteristic region for many alkyl thiols. The presence of phosphorus acid was identified at two frequencies such as 2622 and 2639 cm^{1} and at the Raman frequency 2690 cm^{-1} aldehyde was detected.

DISCUSSION

The present investigation has revealed some important features about chemical composition of spore which are discussed below.

The first finding is presence of selenates; which are analogous to sulfates with similar chemical nature. They are highly soluble in aqueous solutions at ambient temperatures and somewhat good oxidizer. Selenate can be reduced to selenite or selenium. Furthermore, selenones (R_2SeO_2) shows peaks in the range of 320 – 390 cm^{-1}. [24] *A. bisporus* is known to contain selenium (0.77µg^{g}) which is known to exhibit medicinal property. (http://www.ncbi.nlm.nih.gov/pubmed/19153837),[28]

The spectra also revealed presence of disulfide bonds which help to provide stability to a protein molecule. Clear understanding of disulfide bonds in a protein helps to understand the structural study of proteins. Raman spectroscopy can be used for this purpose, furnishing information that other physical methods cannot. Raman spectroscopy shows a strong S-S stretching vibrational band in the region of 500-550cm^{-1}. The vibration at or near 510 cm^{-1} could be assigned to the g-g-g conformation. Many naturally occurring proteins give the disulfide stretching vibration at or near 510 cm^{-1} suggesting that the g-g-g form is the most preferred conformation.

Organobromine compounds are produced naturally by marine creatures (sponges, corals, sea slugs, tunicates, sea fans) and seaweed, plants, fungi, lichen, algae, bacteria, microbes, and some mammals. Many of these organobromine compounds are used in chemical defense, to facilitate food gathering, or as hormones.[29] This investigation detected the presence of C-Br in the spores of *Agaricus* however, role of C-Br in spore is presently indistinct.

Further to add to the discussion about C-S, the C-S stretching frequencies of a methionine residue depend on its conformation. The 655 cm^{-1} and 724 cm^{-1} bands are for the trans form of the methionine side chain, and the 700 ± 5 cm^{-1} band is for the gauche form. Similarly, in cysteine and cystine, the C-S stretching vibration is related to the internal rotation along the C-C axis of X-C-CH2-S-S. It depends upon the atom X at the trans site with respect to the sulfur atom adjacent to the C-C bond. It was observed that when X is a hydrogen atom (expressed by the symbol, P_H) the C-S stretching vibration lies between 630 and 670 cm^{-1}. When X = carbon (P_C) it is at 720 cm^{-1} for the conformation and at 700 cm^{-1} when X = nitrogen (P_N). A correlation between the C-S stretching frequencies and the internal rotation about the C-S bond in methionine and isobutyl methyl sulfide has been suggested. Furthermore, the C-S was detected between 679 – 694 cm^{-1} i.e. near to 700cm^{-1} suggesting the gauche form of the methionine side chain and X being nitrogen.[22]

It is well known that due to the C=O stretching vibration, the fluorides absorb at about 50 cm^{-1} higher region than that of Acid bromides and iodides. Our findings are in accordance with Socrates.[24] The peaks observed in 723 – 789 cm^{-1} could be due to bending vibration. The cooling system is often required to control the temperature during cultivation of *A. bisporus*. It could be therefore, predicted that the fluorine from CFC generated by these cooling systems might be accumulated by the fruit bodies and has got incorporated into the spores. Other source of the fluorine would be the water that is used during the cultivation.

A C-O-C bond is present in carbohydrates. The vibrational modes of carbohydrates are complex, and the interpretation of Raman spectra is often difficult. Many different carbohydrates are actually isomers; sometimes the only difference is the position of a hydroxyl group. Being polyhydroxyl compounds, carbohydrates give extremely complex Raman spectra involving different types of OH, C-H, C-C, C-C-O, C-O, C-O-C, and C-O-H vibrations.[22] It is generally accepted that several absorption bands are characteristic for α-and β-anomers, and they have been grouped as type 1, 2a, 2b, and 3. Of which type 2 bands originate from the anomeric C-H deformation (bending vibration): equatorial C-H vibration in a-D-anomers in the $^4C_1$ (D) conformation and only type 2 bands can be used to differentiate the anomeric glycosidic linkages. The type 2 bands differentiate α- or β-anomers in monosaccharides and disaccharides or the glycosidic linkages in polysaccharides.[22] The rule is equally applicable to the normal glycosidic linkage (C-O-C). The cell wall of mushrooms contain high amounts of substances like chitin (a polymer of β-(1,4)-branched N-acetylglucosamine) and glucans (specially β-glucans) with glycosidic linkage, which can be used for medical purposes and nutrient matter (http://en.wikipedia.org/wiki/Sulfonic_acid).

Furthermore, the cell wall components of *A. bisporus* have been investigated and was concluded that glucose being main sugar followed by galactose, mannose and xylose.[31] The peaks for glycosidic bonds observed in the present investigation could therefore; not because of one type of carbohydrate but, due to presence of C(6)-OH sugars such as Glucuronic acid (910, 943 cm^{-1}), D-Glucose (916 cm^{-1}), Deuterated D-Glucose (890, 962 cm^{-1}), Glucosamine HCL (896, 916, 943 cm^{-1}), Deuterated Glucosamine HCL (905, 950 cm^{-1}), N-Acetyl Glucosamine (916, 936, 983 cm^{-1}) and Deuterated N-Acetyl Glucosamine (902, 936, 983cm^{-1}).

The spores also revealed presence of sulfonic acid. A sulfonic acid (or sulphonic acid) refers to a member of the class of organosulfur compounds with the general formula $RS(=O)_2–OH$, where R is an organic alkyl or aryl group and the $S(=O)_2–OH$ group a sulfonyl hydroxide.[25] *A. bisporus* is commercially grown on compost which consist mainly plant derived polydaccharides comprising of many different monosaccharides such as glucose, xylose, and arabinose, while smaller amounts of galactose, rhamnose, mannose and glucuronic acid (http://hdl.handle.net/2066/33138) or *A. bisporus* can be readily grown on protein as a sole source of carbon, nitrogen and sulfur.[33] Glucuronic acid is a sulfated derivative found mainly in the cell wall of spores of fungi.[34] Furthermore, in an investigation the presence of glucuronic acid and uronic acid in compost on which *A. bisporus* is grown was detected.[32] It could be therefore concluded that the mudhroom might have bio accumulated these sulfur derivatives. Moreover, *A. bisporus* also contain sulfur containing amino acids such as Cysteine and Methionine along with other amino acids[35] and the peaks obtained could be assign to these amino acids. The possible source for Si:H peak could be the water or the compost formula used during cultivation.

Another important finding of the study is the presence of isocyanate in the spores. Occurrence of contaminating mould in the mushroom bed is a serious problem causing the yield loss (http://www.gesundheitsschutzbs.ch/files/berichte/165014_Trockenpilze2009%20_%20erl.pdf) and often these competitor fungi are completely inhibited by spraying of fungicides. Furthermore, a secondary action can also be attributed to butyl isocyanate, a co-product of methyl benzimidazole carbamate (MBC). It is therefore, revealed that the sample collected for the present study have traces of fungicides and consumption of such fruitbbodies is questionable.

The reason for the appearance of P-H peak could be that, the mushroom or the growing room could have been fumigated with Phosphine containing fumigant which is a common practice in mushroom cultivation.

The Raman spectra of proteins normally originate from C-C, C=C, C-N, C=N, C-H, C-S, C-O, C=O, N-H, O-H, and S-H vibrations. Among these vibrational modes, only the S-H vibration appears in the 2500-2600 cm^{-1} region. Therefore, little interference or ambiguity is involved in the detection of a sulfhydryl group. In the present study, sharp peaks were observed in the range of 2545 – 2598 cm^{-1} clearly suggesting the presence of sulfhydryl group (-SH).

A peak for Phosphorus acid was also obtained in the study. Phosphorus acid or Phosphorus oxoacids are oxoacids of phosphorus. Phosphorus exhibits

oxidation states from +1 to +5 whereas oxygen may be in oxidation state +2 or +1, depending on whether a compound contains the peroxide group. There are a large number of such compounds, some of which cannot be isolated and are only known through their salts (http://en.wikipedia.org/wiki/Phosphorus_acid). Phosphorus oxoacids containing P in oxidation state +3: H_3PO_3 (or $HPO(OH)_2$), Phosphorous acid or phosphonic acid, a diprotic acid and Phosphorus oxoacids containing P in oxidation state +5 H_3PO_4 (or $PO(OH)_3$), Phosphoric acid, a tribasic acid. Nasiri *et al.* [9] detected the presence of phosphorus and potassium phosphorus in *Agaricus* mushroom. Moreover, to remove the casing soil and to increase shelf life the fruit bodies of the mushroom are washed using various wash additives such as sodium hypochlorite, trisodium phosphate, hydrogen peroxide, chlorine dioxide, sodium hypochlorite, hydrogen peroxide, potassium sorbate, sodium salts of benzoate, EDTA, and phosphoric acids.[7] It is therefore, revealed that the fruit bodies used in the current investigation may have been washed with phosphoric acid giving characteristic peak for it.

As far as the aldehydes are concerned, 65 aromatic compounds from *A. bisporus* including aldehydes were reported (http://phd.lib.uni-corvinus.hu/584/2/Geosel_Andras_ten.pdf) similarly, aldehydes are commonly reported in ligninolytic basidiomycetes; and *Agaricus* cultivation is commonly done on lignocellulosic material. Lankinen[18] reported various ligninolytic enzymes from *A. bisporus* and one of the enzymes Aryl alcohol Oxidase (AAO) oxidize aromatic alcohols (anisyl, veratryl alcohol) to aldehydes and H_2O_2.

Hence, we, for the first time demonstrated the identification of chemical species in the spores of *Agaricus bisporus* using highly sensitive Raman spectroscopy and we believed that this study has opened a new avenue for interdisciplinary research approach to work on filamentous and higher fungi.

CONCLUSION

The conventional procedures to detect the biomolecules and compounds involve complicated and time consuming extraction and purification procedures, solubility tests, absorption spectroscopy and chemical analyses. This urged a need to develop new sensitive technologies to obtain information from biological materials including fungi. A great deal has been achieved in this area using Raman or Surface Enhanced Raman Spectroscopy. The current work has clearly demonstrated that various types of biomolecules can be identified using surface enhanced Raman spectra obtained from a suspension of spores of *Agaricus bisporus*. The method is relatively simple and fast compared with conventional methods.

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