ESTIMATION OF ENZYMATIC ACTIVITIES OF DIFFERENT SPECIES OF MUSHROOMS

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CERTIFICATE

This is to certify that the thesis entitled "**ESTIMATION OF ENZYMATIC ACTIVITIES OF DIFFERENT SPECIES OF MUSHROOMS**" submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by Rahul Chandra Mishra under my supervision and guidance.

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DECLARATION

I hereby declare that the thesis entitled **"Estimation of Enzymatic Activities of Different Mushroom Species"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. (Miss.) Bismita Nayak, Assistant Professor, Department of Life Science , National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:

Place: RAHUL CHANDRA MISHRA

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Place: Rourkela *Rahul Chandra Mishra*

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ABSTRACT

Mushrooms are widely spread saprophytic macro-organisms, belong to class fungi growing on dead organic matter of vegetative origin. They can utilize almost all agricultural wastes as their substrates for their growth and metabolism. During the growth of mushroom mycelia and their development into mature fruiting bodies (or sporophores), various biochemical changes are known to occur, as a result of which enzymes are secreted extracellularly to degrade the insoluble materials to simple and soluble molecules. Consequently, enzymes play a significant role in mushroom development; in addition, they also affect the food and nutrient value, flavour and shelf life of these fungi. *Pleurotus spp*, *Volvariella volvaceae* and *Ganoderma lucidium* are edible tropical fast-growing, wood degrading macro fungi that are of economic importance in India as both the sporophores and tuberous sclerotia are not only edible but also are used in medicinal preparations by native doctors. This work was undertaken to evaluate the enzymatic activities of *Pleurotus florida*, *Pleurotus sajor caju*, *Vovariella volvaceae* and *Ganoderma lucidium* and the effects of these enzymes on shelf life and food nutrients of these mushrooms.

Chapter 1

INTRODUCTION

INTRODUCTION

There are at least 12, 000 species of fungi that can be considered as mushrooms, with at least 2000 species are edible. Over 200 species have been collected from the wild vegetation and used for various traditional medicinal purposes, mostly in Far East. About 35 species have been cultivated commercially and 20 are cultivated on an industrial scale. The most cultivated mushroom worldwide is *A. bisporus* (button mushroom), followed by *Lentinus edodes* (shiitake), *Pleurotus spp* (oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom). The edible mushrooms are excellent foods that can be incorporated into well-balanced diets due to their low content of fat and energy, and high content of dietary fiber and functional compounds. Their benefit to health include; immunomodulatory, anti-tumoral, and hypo-cholesterolemic effects. In numerous molecules synthesized by macrofungi are known to be bioactive compounds such as polysaccharides, glycoproteins, terpenoids, lectins, among others.

The vegetative growth of mycelium is essential for the subsequent development of the mushroom and this mycelial growth utilizes lignocellulosic materials, such as the polysaccharides of straws and wood, for nutrient materials. Since such substrates are commonly insoluble in water, they are broken down to smaller, soluble units through the activity of enzymes excreted by the fungal cells. An active area of modern research involves the search in mushroom species for compounds that can be used in the treatment of various cancers, cardiovascular disease, viral diseases, etc. During the growth of mushroom mycelia and the development to mature fruitbodies (or sporophores), biochemical changes are known to occur, as a result of which enzymes are secreted extracellularly to degrade the insoluble materials in the substrates into simple and soluble molecules which are subsequently utilized by intracellular enzymes within the mushroom. Consequently, enzymes play significant role in mushroom development, in addition, they also affect the food nutrient, flavor and shelf life of these fungi. This work was done to evaluate the effect of substrates on the enzyme activities of mushrooms, *Pleurotus spp, Vovariella volvaceae* and *Ganoderma lucidium* and the effects of these enzymes on shelf life and food nutrients of this mushroom.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Mushrooms are the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. Like all fungi, mushrooms are not plants and do not undergo photosynthesis. The word "mushroom" is most often applied to *Basidiomycetes*, *Agaricomycete*s that have a stem (stipe), a cap (pileus), and gills (lamellae) or pores on the underside of the cap. Basidiomycetes are named for their characteristic structure or cell, the basidium**,** which is involved in sexual reproduction (fig 1). A basidium [Greek *basidion,* small base] is produced at the tip of hyphae and normally is club shaped. Two or more basidiospores are produced by the basidium, and basidia may be held within fruiting bodies called basidiocarps.

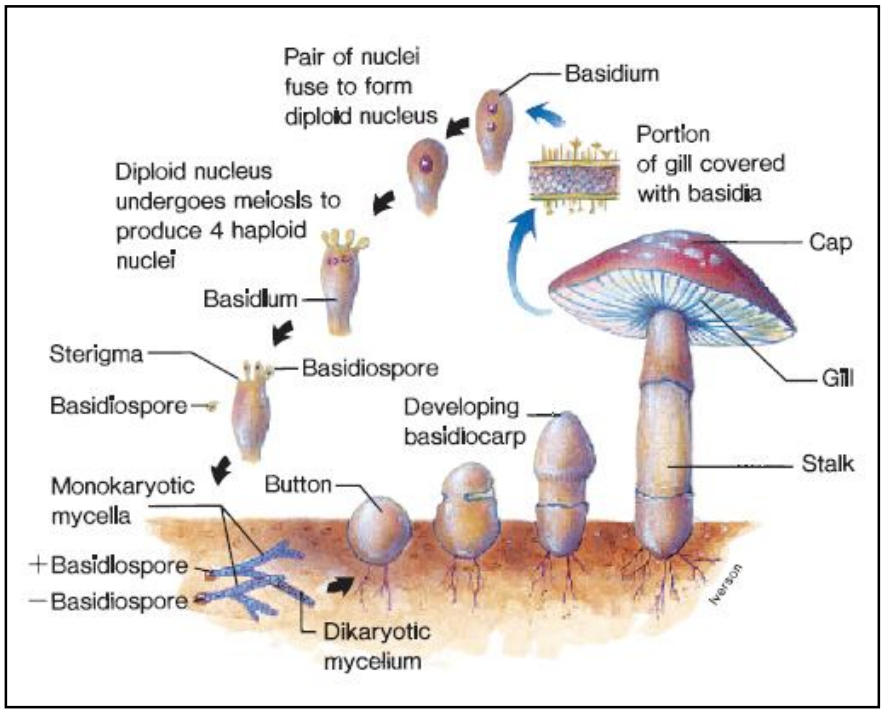


Figure 1. The life cycle of a typical soil basidiomycete

Classification

Typical mushrooms are the fruit bodies of members of the order Agaricales, whose type genus is *Agaricus* and type species is the field mushroom, *Agaricus campestris*. However, in modern molecularly-defined classifications, not all members of the order Agaricales produce mushroom fruit bodies, and many other gilled fungi, collectively called mushrooms, occur in other orders of the class *Agaricomycetes*. For example, chanterelles are in the Cantharellales, false chanterelles like *Gomphus* are in the Gomphales, milk mushrooms (*Lactarius*) and russulas (*Russula*) as well as *Lentinellus* are in the Russulales, while the tough leathery genera *Lentinus* and *Panus* are among the Polyporales, but *Neolentinus* is in the Gloeophyllales, and the little pin-mushroom genus, *Rickenella*, along with similar genera, are in the Hymenochaetales. Within the main body of mushrooms, in the Agaricales, are common fungi like the common fairy-ring mushroom (*Marasmius oreades*), shiitake, enoki, oyster mushrooms, fly agarics, and other amanitas, magic mushrooms like species of *Psilocybe*, paddy straw mushrooms, shaggy manes, etc. An atypical mushroom is the lobster mushroom, which is a deformed, cooked-lobster-colored parasitized fruitbody of a *Russula* or *Lactarius*, colored and deformed by the mycoparasitic Ascomycete *Hypomyces lactifluorum*. (*Volk T.*, 2001)

Other mushrooms are not gilled and then the term "mushroom" is loosely used, so it is difficult to give a full account of their classifications. Some have pores underneath (and are usually called boletes), others have spines, such as the hedgehog mushroom and other tooth fungi, and so on. "Mushroom" has been used for polypores, puffballs, jelly fungi, coral fungi, bracket fungi, stinkhorns, and cup fungi. Thus, the term is more one of common application tomacroscopic fungal fruiting bodies than one having precise taxonomic meaning. There are approximately 14,000 described species of mushrooms. (*Miles PG, Chang S-T*, 2004).

Morphology

A mushroom develops from a nodule, or pinhead, less than two millimeters in diameter, called a primordium, which is typically found on or near the surface of the substrate. It is formed within

the mycelium, the mass of thread like hyphae that make up the fungus. The primordium enlarges into a roundish structure of interwoven hyphae roughly resembling an egg, called a "button". The button has a cottony roll of mycelium, the universal veil that surrounds the developing fruit body. As the egg expands, the universal veil ruptures and may remain as a cup, or volva, at the base of the stalk, or as warts or volval patches on the cap. Many mushrooms lack a universal veil and therefore do not have either a volva or volval patches. Often there is a second layer of tissue, the partial veil, covering the bladelike gills that bear spores. As the cap expands, the veil breaks, and remnants of the partial veil may remain as a ring, or annulus, around the middle of the stalk or as fragments hanging from the margin of the cap. The ring may be skirt-like as in some species of *Amanita*, collar-like as in many species of *Lepiota*, or merely the faint remnants of a cortina (a partial veil composed of filaments resembling a spiderweb), which is typical of the genus *Cortinarius*. Mushrooms that lack a partial veil do not form an annulus. (Stuntz *et al*., 1978) The stalk (also called the stipe, or stem) may be central and support the cap in the middle, or it may be off-center and/or lateral, as in species of *Pleurotus*and *panus*. In other mushrooms, a stalk may be absent, as in the polypores that form shelf-like brackets. Puffballs lack a stalk but may have a supporting base. Other mushrooms, like truffles, jellies, earthstars, bird's nests, usually do not have stalks, and a specialized mycological vocabulary exists to describe their parts. The way that gills attach to the top of the stalk is an important feature of mushroom morphology. Mushrooms in the genera *Agaricus, Amanita, Lepiota* and *Pluteus*, among others, have free gills that do not extend to the top of the stalk. Others have decurrent gills that extend down the stalk, as in the genera *Omphalotus* and *Pleurotus*. There are a great number of variations between the extremes of free and decurrent, collectively called attached gills. Finer distinctions are often made to distinguish the types of attached gills: adnate gills, which adjoin squarely to the stalk; notched gills, which are notched where they join the top of the stalk; adnexed gills, which curve upward to meet the stalk, and so on. These distinctions between attached gills are sometimes difficult to interpret, since gill attachment may change as the mushroom matures, or with different environmental conditions. (Stuntz *et al*., 1978)

Microscopic features

A hymenium is a layer of microscopic spore-bearing cells that covers the surface of gills. In the non-gilled mushrooms, the hymenium lines the inner surfaces of the tubes of boletes and polypores, or covers the teeth of spine fungi and the branches of corals. In the Ascomycota, spores develop within a microscopic elongated, saclike cell called an ascus, which typically contains eight spores. The *Discomycetes*—which contains the cup, sponge, brain, and some club-like fungi—develop an exposed layer of asci, as on the inner surface of cup fungi or within the pits of morels. The *Pyrenomycetes*, tiny dark-colored fungi that live on a wide range of substrates including soil, dung, leaf litter, decaying wood, as well as other fungi, produce minute flask-shaped structures called perithecia, within which the asci develop (Ammirati *et al*., 1985). In the *Basidiomycetes*, usually four spores develop on the tips of thin projections called sterigmata, which extend from a club-shaped cell called a basidium. The fertile portion of the *Gasteromycetes,* called a gleba, may become powdery as in the puffballs or slimy as in thestinkhorns. Interspersed among the asci are threadlike sterile cells called paraphyses. Similar structures called cystidia often occur within the hymenium of the *Basidiomycota.* Many types of cystidia exist and assessing their presence, shape, and size is often used to verify the identification of a mushroom (Ammirati *et al*., 1985). The most important microscopic feature for identification of mushrooms is the spores themselves. Their color, shape, size, attachment, ornamentation, and reaction to chemical tests often can be the crux of identification. Spores often have a protrusion at one end, called an apiculus, which is the point of attachment to the basidium, termed the apical germ pore, from which the hypha emerges when the spore germinates. (Ammirati *et al*., 1985)

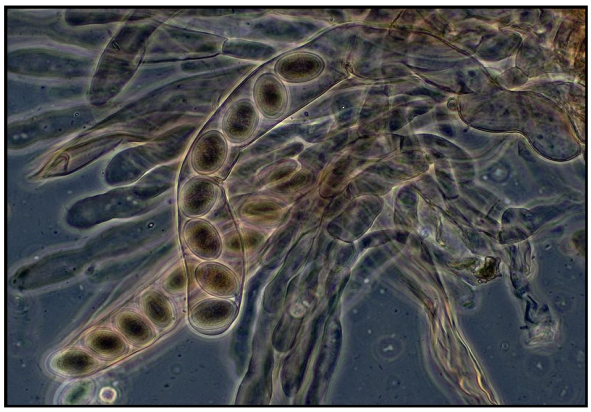


Figure 2.

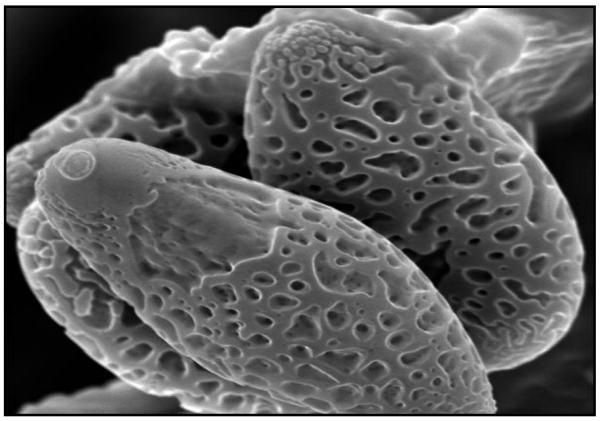


Figure 3.

Figure No. 2. Asci as viewed under atomic force microscopy and 3. Spores as viewed under Scanning electron microscope.

Mushrooms affect humans in many ways. Most are saprophytes that decompose plant debris, especially cellulose and lignin. Many mushrooms are used as food throughout the world. Many mushrooms produce specific alkaloids that act as either poisons or hallucinogens. One such example is the "destroying angel" mushroom, *Amanita phalloides.* Two toxins isolated from this species are phalloidin and amanitin. Phalloidin primarily attacks liver cells where it binds to plasma membranes, causing them to rupture and leak their contents. Alpha-amanitin attacks the cells lining the stomach and small intestine and is responsible for the severe gastrointestinal symptoms associated with mushroom poisoning. Mushrooms can be used for dyeing wool and other natural fibers. The chromophores of mushroom dyes are organic compounds and produce strong and vivid colors, and all colors of the spectrum can be achieved with mushroom dyes. Before the invention of synthetic dyes mushrooms were the source of many textile dyes. (Mussak R, Bechtold T., 2009) Mushrooms and other fungi play a role in the development of new biological remediation techniques (e.g., using mycorrhizae to spur plant growth) and filtration technologies (e.g. using fungi to lower bacteria levels in contaminated water).

Enzymes and Proteins from Mushrooms

Mushrooms are saprophytic, growing on dead organic matter of vegetative origin. Therefore they can utilize almost all agricultural wastes as substrates (Miles and Chang, 1997). During the

growth of mushroom mycelia and the development to mature fruitbodies (or sporophores), biochemical changes are known to occur, as a result of which enzymes are secreted extracellularly to degrade the insoluble materials in the substrates into simple and soluble molecules which are subsequently utilized by intracellular enzymes within the mushroom. Consequently, enzymes play significant role in mushroom development; in addition, they also affect the food nutrient, flavour and shelf life of these fungi (Baardseth, 1979; Paranjpe and Chen, 1979; Wang, 1989; Zadrazil *et al*., 2004). A variety of proteins with interesting biological actions is elaborated by mushrooms and many of these proteins have potentially applicable activities. They include ribosome inactivating proteins, antifungal proteins, ribonucleases, ubiquitin- like proteins and peptides, lectins, cellulases, xylanases, laccases, invertases and phosphatases.

Proteases from Mushrooms

Pleureryn is a small and novel aspartic protease with a molecular mass of 11.5 kDa from the edible mushroom *P. eryngii* (Wang HX, Ng TB., 2001). However, its N-terminal sequence resembles DNA replication licensing factor more than aspartic proteases. It also exhibits some inhibitory activity against HIV-1 reverse transcriptase. This is reminiscent of a suppressive action of HIV-1 protease, also an aspartic protease, on its homologous reverse transcriptase. Pleureryn exhibits a pH optimum of 5 and a temperature optimum of 45^{o} C, with considerable activity remaining at high temperatures and at pH 4 and 12. Pleureryn is unique in that it is relatively stable to changes in pH or temperature (Wang HX, Ng TB., 2001). Other mushroom proteases tend to be less stable. The metalloproteases from *Armillariella mellea* and *Tricholoma saponaceum* (Kim JH, Kim YS., 1999, 2001) are, by contrast, thermolabile. An aspartic protease from another mushroom, *I. lacteus*, has a molecular mass of 35 kDa (Kobayashi H., *et al* 1989). Metalloendopeptidases from *A. mellea*, *P. ostreatus* and *T. saponaceum* have a molecular mass in the range 18.5 – 20 kDa (Nonaka T., et al 1995). A thermostable lysine-specific zinc-metalloendopeptidase has been isolated from fruiting bodies of the mushroom *Grifola frondosa* (Nonaka T., *et al* 1995). The protease has a molecular mass of 20 kDa, a p*I* of 7.46 and a pH optimum of 9–10. It demonstrates high affinity for β -d-glucan and chitin. Prolylendopeptidases

from *A. bisporus* and *Lyophyllum cinerascens* have molecular masses close to 78 kDa (Sattar AKMA., *et al* 1990, 1990). An alkaline serine protease (Pen ch 13, also known as Renn 13) has been identified as the major allergen from airborne *Penicillium chrysogenum (P. notatum)*. It has a molecular mass of 28 kDa. It exhibits 83 and 49% amino acid sequence identity with its counterparts from *P. citrinum* and *Aspergillus fumigatus*, respectively (Chou H., *et al* 2001). An alkaline serine proteinase with a molecular mass of 28.7 kDa has been isolated from *Fusarium culmorum* (Pekkarinen AI, *et al* 2002). A pH of 8.3–9.6 and a temperature of 50^0 C are required for its maximal activity. A subtilisin-like serine protease with a molecular mass of 36 kDa has been purified from *Podospora anserine* (Paoletti M., 2001). An aspartic proteinase from *A. fumigatus* demonstrates a molecular mass of 39 kDa and a broad range of activity from pH 2.0 to 7.0 (Reichard U., *et al* 2001). It exhibits 88% sequence identity with aspartic proteinase from *A. niger* and 64% identity with the vacuolar proteinase A of *S. cerevisiae*. A protease with a molecular mass of about 30 kDa has been isolated from *Candida caseinolytica* (Poza M., *et al* 2001). Its action is demonstrable over a broad pH range. In summary, proteases with different molecular masses, optimum pH values and optimum temperatures are produced by different fungal species.

Xylanases

Xylanases (1,4-β-d-xylan xylanohydrolases) catalyze the random hydrolysis of the xylan backbone of heteroxylans. As a result the cellulose fibrils are exposed and susceptible to attack of side-chain cleaving enzymes such as α –arabinofuranosidases and acetylxylanases. Xylanases occur in diverse organisms. Bacterial and fungal xylanases are produced inductively or constitutively in response to the carbon source on which they are grown. A cellulose-free xylanase has been purified from *Aspergillus niger* (Qy Y, Gao P, *et al*., 1996). Maximum xylanase activity is induced by xylan, followed by lignocellulose. The enzyme exhibits the highest activity at 45–50^0 C, but 70–95% of the activity disappears within 5 min. Cellulase free xylan degrading enzymes from *Acrophialophora nainiana*, *Humicola grisea* var. *thermoides* and two *Trichoderma harzianum* strains have been employed to bleach the pulp of *Eucalyptus kraft* before a chlorine dioxide and alkaline bleaching sequence. The *T. harzianum* enzyme

preparations are slightly more effective in decreasing pulp viscosity and chlorine chemical consumption and enhancing the brightness of the kraft pulp. *A. nainiana* xylanase is the most potent in reducing pulp viscosity (Medeiros RG, *et al*., 2002). The activities of some hydrolytic enzymes (cellulase, endo-1,4-β-xylanase, β-glucosidase and amylase) and reductase enzymes (monophenol monooxygenase and peroxidase) in strains of *Fusarium oxysporum* (Schlecht snyd. and Hans) isolated from different habitats (plant substrates, cultivated soil and non-cultivated soil) have been examined by Kurchenko *et al.* (Kruger *et al*., 2002). It is found that strains isolated from plant substrates display the highest activity of hydrolytic enzymes, followed by strains from cultivated soil, whereas strains isolated from non cultivated soil exhibit the lowest activity. The reverse trend occurs regarding the redox enzymes. A purified xylanase from *F. verticillioides* exhibits a molecular mass of 24 kDa, an optimum temperature of 50^0C, an optimum pH of 5.5, a pH stability range of 4.0–9.5, and thermal stability up to 50^0C (Saha BC., 2001). *F. oxysporum f.* sp. *melonis* produces an endo-1,4-β- xylanase with a molecular mass of 80 kDa. Its optimum pH and temperature are 5.0 and 50^0C, respectively (Alconada TM, Martinez MJ, 1994). *P. purpurogenum* produces two immunologically distinct xylanases. Xylanase A possesses a molecular mass of 33 kDa and an isoelectric point at pH 8.6. Xylanase B, the other major form, manifests a molecular mass of 23 kDa and an isoelectric point at pH 5.9 (Trinci APJ *et al.*, 1994). The xylanase from the thermophilic fungus *Humicola lanuginose* is abundant in acidic amino acids. It is unusual in that inactivation, probably due to aggregation, occurs after storage at -20^0 C in the dry state for over 2 months (Anand L *et al.*, 1990). From another thermophilic fungus, *Thermoascus aurantiacus*, a xylanase, a glucosidase, an exocellulase and an endocellulase have been purified. Two structurally similar xylanases from *T. reesei* and their genes have been characterized (Torronen A *et al., 1992*). They have a molecular mass of 19 and 21 kDa and an isoelectric point of 5.2 and 9.0, respectively. A 66-kDa xylanase has been purified from the rumen anaerobic fungus, *Neocallimastix patriciarum*. The large N-terminal reiterated regions consisted of distinct catalytic domains which displayed similar substrate specificities to the full-length enzyme (Gilbert HJ *et al., 1992*). Thus it appears that xylanes from different species may differ in molecular mass, isoelectric point, optimum pH, pH stability range and optimum temperature

Cellulases

The cellulolytic enzymes of anaerobic fungi have been studied because of their potential value in biotechnology (Gilbert HJ, 1992; Wallace RJ, 1994;) including use as enzyme supplements for live stock, and in food and beverage, detergent, textile and pulp and paper industries (Campbell GL, 1992). Their nutritional function is evident by the degradation of plant fiber serving as carbon sources. In general, cellulolytic fungi produce a large number of cellulases (Slomczynsky D *et al., 1995*); many use them for degradation of the plant cell wall polysaccharides (Akin DE *et al*., 1990). Endoglucanases (endo-1,4-β-glucanases), cellobiohydrolases (CBH, exo-1,4-β-glucanase), and β-glucosidases are three major types of cellulolytic enzymes. Endoglucanases randomly hydrolyze 1,4-β- bonds along the interior of the cellulose chain. Cellobiohydrolases cleave cellobiosyl units from non-reducing ends of the cellulose chains. Glucosidases cleave glucosyl units from non-reducing ends of cello-oligosaccharides. Cellobiohydrolase Cel7A from *Trichoderma reesei* has been expressed from *Pichia pastoris*. The thermostability, k(cat), *K*m and pH optimum are not affected by heteroglycosylation (Boer H *et al., 2000*). A cellulase, with a molecular mass of 58 kDa, a pH optimum of 5.5, and a temperature optimum at 40^0 C has been purified from the ruminal fungus *O. joyonii* and cloned in *Escherichia coli*. The *O. joyonii* cellulase exhibits strong activity on carboxy methyl cellulose (CMC), lichenan and barley β-glucan. CMC is a water soluble long-chained cellulose with carboxymethyl substitutions. It is commonly used as a model substrate for detecting β-1,4-endoglucanases. Digestion of lichenan and barley β-glucans (mixtures of β-1,3- and β-1,4 linkages) may be mainly attributed to random cleavage of β-1,4 linkages in the substrates because of the inability of the *O. joyonii* cellulase to digest laminarin and pachyman which have β-1,3-glucans as the main components. The enzyme has no activity on avicel (crystalline cellulose) and pullulan (α-1,6 glucan). *O. joyonii* cellulose is able to cleave *p*-nitrophenyl-β-d-cellobioside but not glucopyranoside, suggesting that it possesses cellobiohydrolase but not glucosidase activity. Its activity on *p*-nitrophenyl-β-d-cellotrioside, -cellotraoside and -cellopentaoside indicates its cellodextrinase activity. The enzyme has activity over a broad pH range (pH 5–7) with the highest activity at pH 5.5. It is stable at temperatures up to 50^0 C with a temperature optimum of 40^0 C. However, it has to be borne in mind that all of the enzyme activities were measured under anaerobic conditions in this

investigation and the results might have been different had the experiments been conducted in an anaerobic setting [200]. Despite the fact that many cellulases contain a cellulosebinding domain and a catalytic domain, *O. joyonii* cellulose is unable to bind Avicel, a microcrystalline cellulose and a search for cellulose-binding domain sequences has met with no success. The absence of cellulose-binding domains and the presence of reiterated scaffold binding sequences in *O. joyonii* CelB2 cellulase suggest the immobilization of the enzyme to cellulosome, a cellulose hydrolytic complex. Cellulases, glucanases and xylanases from anaerobic rumen fungi have been cloned in *E. coli* and expressed. These fungal enzymes increase the efficiency of feedstuff digestion in monogastric animals by promoting breakdown of polymers in the plant cell wall and hence are potential enzyme supplements for livestock. The enzymes are being used or considered for use by pulp and paper, textile, detergent and food and beverage industries. Three exo-glucanases, two endo-glucanases and two beta-glucosidases, have been isolated from the culture medium of *A. nidulans*. The optimal pH for all forms of cellulase components ranges from pH 5.0 to 6.0 and the optimum temperature is 50 and 65^0 C for exo-glucanases and endo-glucanases but 35 and 65 ^{0} C for beta-glucosidases. All cellulase components are stable for 10 min at 40–50^0 C. Exo-II and Exo-III exhibit a higher affinity for the substrate than Exo-1. The *K*m values of Endo-1 and Endo-II and their maximum reaction velocities are comparable. The beta-glucosidases exhibit *K*m values of 0.24 and 0.12 mmol and Vmax values of 8.00 and 0.67 IU/mg protein. The molecular masses for various enzyme forms are: Exo-1, 29 kDa; Exo-II, 72.5 kDa; Exo-III, 138 kDa; Endo-1, 25 kDa; Endo-II, 32 kDa; beta-Gluco-1, 14 kDa and beta-Gluco-II, 26 kDa. Exo- and endo-glucanases but not beta-glucosidases require metal ions as co-factors. $Hg2^+$ ions inhibit the activity of all cellulase components (Bagga PS *et al., 1990*). In summary, cellulases with a variety of molecular masses, and temperature and pH optima are known.

Laccases

Lignin ranks second, after cellulose, in abundance in the biosphere as a renewable organic compound. The biodegradation of lignin is a rate-limiting step in the carbon cycle. Ligninolytic enzymes are highly non-specific on account of the complex structure of lignin, and can be used in the degradation of structurally different environmental pollutants (Bumpus JA, Aust SD, 1987). Laccases (benzenediol: oxygen oxidoreductase) form a class of ligninolytic enzymes that

are phenol oxidases capable of catalyzing one-electron oxidation of aromatic substrates and the concomitant reduction of oxygen to water. Laccases can also act on non-phenolic lignin subunits in the presence of readily oxidizable primary substrates which are electron-transfer mediators (Bourbonnais R, Paice MG, 1990). Combinations of laccase/glucose oxidase and laccase/ manganese peroxidase, rather than laccase or peroxidase alone, have been considered to be the minimal enzyme requirement for effective lignin degradation. The combined action of laccase and FAD-dependent aryl alcohol oxidase significantly reduces the molecular mass of soluble lignosulfonates. Laccases are multicopper blue oxidases, widely distributed in plants and fungi. These enzymes are either monomeric or multimeric glycoproteins. Heterogeneity may be present owing due to variable carbohydrate contents or differences in copper content. These enzymes demonstrate a rather low degree of specificity with regard to the reducing substrate: they catalyze the oxidation of *ortho*and *para*-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to generate a free radical. Structural information about the metal sites of laccase has been gathered by spectroscopic studies. The biological function of laccase is correlated to lignin biodegradation in combination with either manganese peroxidase and/or lignin peroxidase. Laccase can also catalyze the oxidative polymerization of the phenolic compounds originating from lignin, which are then easily eliminated. Laccases oxidize phenolic units in lignin to phenoxy radicals, which can lead to the degradation of lignin-related structures. In the presence of appropriate redox mediators, such as 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1 hydroxybenzotriazole (1-HBT), laccases catalyze the oxidation of nonphenolic lignin model compounds, depolymerize kraft lignin, and degrade polycyclic aromatic hydrocarbons, which are not substrates for laccase alone. Due to the aforementioned characteristics, its efficacy as an agent for selected detoxification (Dec J *et al., 2003*), pollutant degradation, and catalyst for regiospecific biotransformation, and its possible utilization as electrode for organic phase enzymatic assay, laccase may play an important role for biotechnological applications. Veratryl alcohol oxidase can act together with laccase to prevent polymerization of phenolic compounds and reduce molecular mass of lignosulfates (Marzullo L *et al., 1995*). Extracellular laccases have been purified from submerged cultures of *Coriolus versicolor, Panus tigrinus, Phlebia radiata* and *Phlebia tremellosa*, and from cultures of *P.*

tigrinus, P. radiata and *A. bisporus* grown on wheat straw (solid-state fermentation). A laccase from *Marasmius quercophilus* has been characterized, in addition to laccases from *A. bisporus, Polyporus anceps, Pycnoporus cinnabarinus*. *Rigidoporus lignosus*. *Trametes trogii* and *C. hirsutus*. They are glycoproteins with molecular masses close to 60 kDa. However, *Tricholoma giganteum* laccase has a molecular mass of only 43 kDa while *C. cibarius* laccase is composed of two 46-kDa subunits. Laccases have also been identified and characterized from *A. nidulans*, *Botrytis cinerea,* and *N. crassa*. A laccase gene homologous to the laccase gene of *N. crassa* has been isolated and characterized from *P. anserine*. The promoter region of the laccase gene from *P. anserine* contains two sequences identical to the eukaryotic xenobiotic responsive element and another two sequences homologous to the eukaryotic antioxidant responsive element (Fernandez-Larrea J *et al.,* 1996). The activity and stability of laccase from *Pleurotus ostreatus* are enhanced by copper but reduced by mercury. Laccases from different strains of *P. ostreatus* differ in their Kcat and *K*m values for springaladazine, ABTS and guaiacol. Many fungal species examined secrete more than one laccase isoenzyme. Different conditions of growth may produce different patterns of isoenzymes.

Amylases

Enzymes that participate in the hydrolytic degradation of starch are collectively referred to as amylolytic enzymes or amylases. Specific enzymes classified within this group include α amylase, β-amylase, gluco-amylase (also known as amyloglucosidase), pullulanase and inso amylase. Amylases are, classified into two categories, endoamylases and exoamylases (Gupta *et al*., 2002). Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successfully resulting in short end products. A large array of amylases, are involved in the complete breakdown of starch. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars. Also, enzymatically - mediated isomerisation of glucose yields high-fructose syrups. Abundant supplies of starch may be obtained from seeds and tubers, such as corn, wheat, rice tapioca and

potato. The widespread availability of starch from such inexpensive sources, coupled with large-scale production of amylolytic enzymes, facilitates the production of syrups containing glucose, fructose or maltose, which are of considerable importance in the food and confectionery industry. Furthermore, they may be produced quite competitively when compared with the production of sucrose, which is obtained directly from traditional sources such as sugar-beet or sugar-cane (Gupta *et al.*, 2002). Starch may be hydrolysed by chemical or enzymatic means.

Table 1. Characteristics of amylases produced by fungi

Microbial origin	Activity	Substrate	MW	T° opt.	pH opt.	Application	Reference
A. oryzae	6583 U/g	spent brewing grains	ND	30	5	optimisation increase yield	Francis *et al.*, 2003
T. lanuginosa	193 U/mg	corn flour, soybean meal	ND	50	5.7	hyperproduction	Rubinder *et al.*, 2002
A. wentii	6 mg/ml	soluble starch	ND	20	6.0	optimization studies	Sinha and Chakrabarty, 1978
Nocardiopsis sp.	1130 U/mg	yam	ND	70	5.0	industrial liquefaction	Stamford *et al.*, 2001
H.orenii	22.32 U/mg	starch	ND	65	7.5	purification and characterization studies	Mijts and Patel, 2002
T. curata	0.39 mg/ml	starch	62	60	5.5	characterization studies	Glymph and Stutxenberger, 1977
L. kononenkoae	0,80 g/l	cross linked starch	76	70	4.5 5.0	characterization studies	Prieto *et al.*, 1995
A. oryzae	11 U/ml	soluble starch	ND	60	8	control of fermentation cycle	Gigras *et al.*, 2002
A. oryzae	ND	starch	52	50	4.5	production of maltohepataose	Chang *et al.*, 1996
A. foetidus	ND	rice starch	42	45	50	waste water treatment	Michelena *et al.*, 1984
A. flavus	50 U/ml	tapioca	53	55	6.0	starch liquefaction	Khoo *et al.*, 1994
T. lanuginosus	45.19 U/mg	soluble starch	61	70	6.6	substrate homologue	Nguyen *et al.*, 2002
S. alluvius	364 mg/ml	starch, pullulan	62	40	6.3	industrial ethanol	Wilson an Ingledew, 1982

ND = Not Determined
RT = Room Temperature

α –Amylases

α-Amylase activity is widely distributed in nature. α-Amylase is an endo-acting enzyme, catalyzing the random hydrolysis of internal α-1,4 glycosidic linkages present in the starch substrate. However, α - amylases which are in most demand hydrolyses the α- 1,4 glycosidic bond in the interior of the molecule (Gupta *et al*., 2002). These enzymes are incapable of hydrolyzing α-1,6 glycosidic linkages present at branch points of amylopectin chains. One exception to this is the α-amylase produced by *Thermactinomyces vulgaris,* which can hydrolyse both α-1-6 and α-1-4 glycosidic linkages. The α-amylase family consists of a large group of starch hydrolases and related enzymes, currently known as glycosyl hydrolases family 13 (Henrissat, 1991). Thermostable α-amylases have been characterised from *Pyrococcus woesei, Pyrococcus furiosus* (Koch *et al.,* 1991) and *Thermococcus profundus* (Chung *et al*., 1995; Kwak *et al*., 1998; and Lee *et al*., 1996). The optimum activity of these enzymes is 100^0 C and 80^0 C respectively. The gene encoding an extracellular α-amylase from *P. Furiosus* has been recently cloned and the recombinant enzyme expressed in *Bacillus subtilis* and in *E. coli* (Dong *et al*., 1997; Jorgenesn *et al*., 1997). The high thermostability of the pyrococcal extracellular α-amylase (thermal activity) even at 130^0 C in the absence of metal ions, together with its unique product pattern and substrate specificity, makes this enzyme an interesting candidate for industrial application (Niehaus *et al.,* 1999).

Two of the more commonly used bacterial α-amylases are those isolated from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. *Bacillus* amylases exhibit a pH optimum close to neutrality and are stabilized by the presence of calcium ions. α-Amylase produced by *Bacillus licheniformis* is particularly suited to industrial applications because of its thermal stability. This enzyme consists of 483 amino acids and has a molecular weight of 55.2 kDa. Its pH optimum is 6.0 and its temperature optimum is 90oC. Most other α-amylases, including those produced by *B. amyloliquefaciens*, are rapidly inactivated at temperatures above 40oC (Niehaus *et al.,* 1999). Several thermostable α-amylases have already been characterised (Koch *et al*., 1991). The most thermostable α-amylase to date is from *Pyrococcus woesei*. It remained active after autoclaving for 4 h at 120^0 C (Antranikian, 1987).

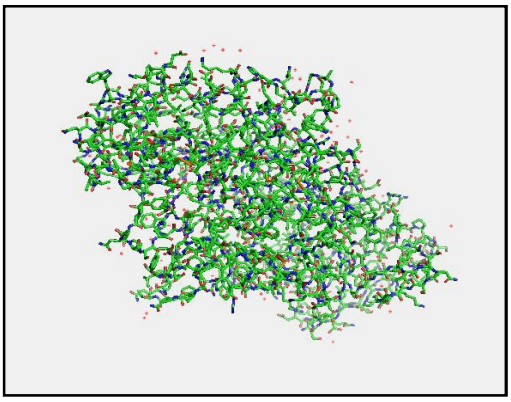


Figure 4. 3-Dimensional representation of the molecular structure of α-amylase

Lipases

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface (Martinelle et al., 1995) and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Lipases display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show normal Michaelis–Menten kinetics in solution. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues. How lipases and lipids interact at the interface is still not entirely clear and is a subject of intense investigation (Balashev et al., 2001). Because of their wide-ranging significance, lipases remain a subject of intensive study (Alberghina et al., 1991; Bornscheuer, 2000). Research on lipases is focussed particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance (Alberghina et al., 1991; Bornscheuer, 2000). In comparison with this effort, relatively little work has been done on

development of robust lipase bioreactor systems for commercial use. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification (Chowdary et al., 2001; Hamsaveni et al., 2001; Kiran et al., 2001a; Kiyota et al., 2001; Krishna and Karanth, 2001; Krishna et al., 2001; Rao and Divakar, 2001), transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Ducret et al., 1998; Zhang et al., 2001) and other chemicals (Therisod and Klibanov, 1987; Weber et al., 1999; Bornscheuer, 2000; Berglund and Hutt, 2000; Liese et al., 2000; Azim et al., 2001). The expectation is that lipases will be as important industrially in the future as the proteases and carbohydrases are currently.

Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes (Liese et al., 2000). One limiting factor is a shortage of lipases having the specific required processing characteristics. An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases (Liese et al., 2000). The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Lesser amounts of lipases are used in oleochemical transformations (Bornscheuer, 2000).

Lipases can play an important role in the processing of g-linolenic acid, a polyunsaturated fatty acid (PUFA); astaxanthine, a food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of g-decalactone, a fruit flavor; dicarboxylic acids for use as prepolymers; interesterification of cheaper glycerides to more

valuable forms (e.g., cocoa butter replacements for use in chocolate manufacture) (Undurraga et al., 2001); modification of vegetable oils at position 2 of the triglyceride, to obtain fats similar to human milkfat for use in baby feeds; lipid esters including isopropyl myristate, for use in cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications. The increasing awareness of the importance of chirality in the context of biological activity has stimulated a growing demand for efficient methods for industrial synthesis of pure enantiomers including chiral anti - inflammatory drugs such as naproxen (Xin et al., 2001) and ibuprofen (Lee et al., 1995; Ducret et al., 1998; Xie et al., 1998; Arroyo et al., 1999; Chen and Tsai, 2000); antihypertensive agents such as angiotensin-converting enzyme (ACE) inhibitors (e.g., captopril, enalapril, ceranopril, zofenapril, and lisinopril); and the calcium channel blocking drugs such as diltiazem. Lipases are used in synthesis of these drugs (Berglund and Hutt, 2000).

Applications of Lipases

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse et al., 2001) and polyurethane (Takamoto et al., 2001). Major applications of lipases are summarized in Table 2. Most of the industrial microbial lipases are derived from fungi and bacteria (Table 3).

Table 2: Some commercially available microbial lipases (Jaeger and Reetz, 1998)

Type	Source	Application	Producing company
Fungal	*C. rugosa*	Organic synthesis	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme, Sigma
	C. antarctica	Organic synthesis	Boehringer Mannheim, Novo Nordisk
	T. lanuginosus	Detergent additive	Boehringer Mannheim, Novo Nordisk
	R. miehei	Food processing	Novo Nordisk, Biocatalysts, Amano
Bacterial	*Burkholderia cepacia*	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	P. alcaligenes	Detergent additive	Genencor
	P. mendocina	Detergent additive	Genencor
	Ch. viscosum	Organic synthesis	Asahi, Biocatalysts

Table 3: Industrial applications of microbial Lipases (Vulfson, 1994)

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

L – Asparginase

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an enzyme that primarily catalyzes the conversion of L-asparagine to L-aspartic acid and ammonia. Arima *et al*., showed that a number of bacteria and fungi produced true extracellular asparaginase activity, i.e., the active enzyme could be isolated from culture filtrates. A discovery that the anti-leukemic activity of guinea pig serum is associated with its L-asparaginase activity (Broome JD; 1961, 1968) and isolation of this enzyme from *Escherichia coli* (Mashburn, L. T., 1964) brought considerable attention to bacterial L-asparaginases, leading to identification of related enzymes in several other sources. The basis of their clinical activity is attributed to the reduction of circulating L-asparagine in blood. Since some neoplastic cells depend on extracellular supplies of this amino acid, they are selectively killed by L-asparagine deprivation. It was shown that some of the

vaccine preparations had high L-asparaginase activity and this enzyme has been used for their potency control during the production process (Beumer-Jachmans, M. P. 1973). The therapeutic effect of vaccines used for immunotherapy of cancers such as lymphoblastic leukemia and breast cancer (Hortobagyi, G. N, *et al.*, 1980) was attributed to their asparaginase activity. L-Asparaginases, includes the enzymes that primarily utilize L-asparagine as a substrate. The enzymes belonging to the second class, also referred to as glutaminase asparaginases, catalyze the hydrolysis of both L-asparagine and L-glutamine with comparable efficiency. Although the anti-cancer properties were demonstrated for enzymes belonging to both classes, their practical applications were highly restricted by the side effects associated with therapy. Thus, antitumor activity of L - Asparginase demonstrated in studies with mice was accompanied by a variety of side effects, linked (at least partially) to the L-glutaminase activity of this enzyme. L-asparaginases, with their high specificity for L-asparagine and low-to-negligible activity against L-glutamine, are reported to be less troublesome during the course of anti-cancer therapy. Numerous studies of L asparaginases have been conducted in order to understand the catalytic mechanism and the substrate specificity of these enzymes. Studies of the dependence of activity on pH, conducted for various Lasparaginases, confirmed that the enzymes are stable and active in the pH range of 4-9 (Wehner *et al*., 1992; Distasio J.A *et al*., 1976). It has been shown that the enzymatic reaction proceeds according to a two step ping-pong mechanism similar to the mechanism of serine proteases, except that the attacking nucleophile is a threonine. Two threonine residues, Thr15ErA and Thr95ErA, are located in the active site of L-asparaginase. The crystal structure of the acyl-enzyme intermediate of Thr89ValEcA mutant provided the first experimental evidence that Thr15ErA is the nucleophile. The specificity toward a variety of substrates of L-asparaginases was assessed in multiple kinetic studies.

Phosphatase

Phosphate solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Application of PSMs in the field has been reported to increase crop yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have

been reported to play a role in phosphate solubilization by PSMs (Abd-Alla, 1994; Whitelaw, 2000). Species of *Aspergillus, Penicillium* and yeast have been widely reported solubilizing various forms of inorganic phosphates (Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). In the present study fungal strains having potential to solubilize insoluble phosphates were isolated. The fungal isolates were checked for the ability to solubilize different insoluble phosphates. The major part of soil P (sometimes as much as 90%) is sequestered in the organic compounds phosphomonoesters and phosphodiesters (Nygren 2008). The phosphatase enzyme capabilities of ectomycorrhizal fungi are continuously distributed between species rather than restricted to a particular taxonomic group (Nygren 2008). Sheathing mycorrhizal fungi have been shown to possess phosphatase enzymes which can hydrolyze inositol hexaphosphate.

Phosphatase production by basidiomycete fungi in liquid culture is independent of P in the medium. Saprophytic basidiomycetes tend to incorporate hydrolysed phosphate into their biomass. In contrast mycorrhizal fungi release more hydrolysed phosphate into solution than they absorb (Dighton 1983). Fungi are able to secrete hydrolytic enzymes involved in the degradation of organic matter (Abuzinadah & Read 1986, Burns & Dick 2002, Lindahl *et al.* 2005). Acid phosphatases solubilize insoluble forms of P not readily available to uninfected plant roots (Tibbett *et al.* 1998a). These enzymes are generally bound to the outer cell walls (Rast *et al.* 2003, Alvarez *et al.* 2004). Phosphatase activities of ectomycorrhizal fungi can vary between species, resulting in different efficiency of P utilization of host plant (Ho & Zak 1979, Dighton 1983). These enzymes are in direct contact with soil environment but are able to adapt to various soil conditions and maintain activity. However, soil components, pH and trace elements can modify the conformation of enzymes and affect their activities (Eivazi & Tabatabai 1977, Geiger *et al.* 1998). Activities of acid phosphatase are found to differ significantly amongst ectomycorrhiza synthesized with different fungi and among different species of the same fungi (Antibus *et al.* 1986, Buée *et al.* 2005, 2007, Courty *et al.* 2006). Ectomycorrhizal phosphatases generally have a pH and temperature optimum approaching that of their native soil.

Ribosome Inactivating Proteins from Mushrooms

Ribosome-inactivating proteins (RIPs) are a group of proteins that share the property of damaging ribosomes in an irreversible manner, acting catalytically, i.e. enzymatically. RIPs were initially detected in plants, mostly in Angiopermae, both mono- and dicotyledons, and also in mushrooms (Yao et al., 1998; Lam and Ng, 2001), and in an alga, Laminaria japonica (Liu *et al*., 2002). *Lyophyllum shimeij* fruiting bodies, (lyophyllin), *Volvariella volvacea* fruiting bodies (*V. volvacea* RIP) Other proteins described as type 1 RIPs are not included because of some there is only the deposited amino acid sequence (euserratin from *Euphorbia serrata*, a 'PAP' from *Phytolacca acinosa*) and of others, from mushroom fruiting bodies, there is no stringent evidence for their RIP activity (pleuturegin from *Pleurotus tuberregium*, velutin, flammulin from *Flammulina velutipes*, hypsin from *Hypsizygus marmoreus*). The properties of RIPs raised hopes of utilizing them for various purposes. Possible applications in medicine and in agriculture were envisaged. In medicine, they have been studied as immunotoxins or as antiviral agents, mainly against HIV, with the difficulties outlined above. Possibly, realistic hopes might be their use for the ex vivo purging of bone marrow or other cell suspensions and for the therapy of topical tumours, for instance of bladder cancer, as suggested by in vitro studies (Thiesen *et al*., 1987; Battelli *et al*., 1996) and initial clinical trials (Yu *et al*., 1998; Zang *et al*.,2000). In agriculture, RIPs are tested to increase resistance against viruses and possibly other parasites. Again, their toxicity to transfected plants is a limit to their use. Immunotoxins could be used for experimental purposes, but, surprisingly, their use is still scarce, with the noticeable exceptions of the immunotoxins against various cells of the nervous system. Probably their use in this field has been greatly facilitated by the availability of the appropriate immunotoxins. Hopefully, immunotoxins against other cells types will be prepared and used in other fields.

Antifungal Proteins from Mushrooms

Proteins with suppressive effects on fungal growth are produced by mushrooms and other fungi. It has been demonstrated that both angiosperm ribosome inactivating proteins and antifungal proteins exert anti-fungal activity. The same occurs in the mushroom L. shimeiji [88]. A 14-kDa antifungal protein designated Lyophyllum antifungal protein (LAP) has been isolated from

fruiting bodies of L. shimeji. Its antifungal potency is higher than that of lyophyllin, a ribosome inactivating protein from the same mushroom. Lyophyllin is 30 times more potent than LAP toward the fungus *P. piricola*. LAP suppresses cell-free translation with a low potency (IC50 = 70 μM) but inhibits HIV-1 reverse transcriptase with a high potency (IC50 = 5 nM). The chromatographic behavior of LAP in general resembles that of lyophyllin. It is eluted from a Mono S column slightly earlier than lyophyllin . Thaumatin-like proteins from the mushrooms *Lentinus edodes* and *Irpex lacteus* and the fungus *Rhizoctonia solani* inhibit the growth of *Saccharomyces cerevisiae*, and are capable of hydrolyzing polymeric carboxymethylated-pachyman in an in-gel β-1,3-glucanase assay. An endo-1,3-β-glucanase from *Agaricus bisporus* was characterized with regard to substrate specificity, optimum temperature and optimum pH. Antifungal activity was not investigated, however. Erygin is an antifungal peptide from the mushroom *Pleurotus erygii* with a molecular mass of 10 kDa and inhibitory activity toward *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

Ubiquitin-like Peptides and Proteins

An 8-kDa ubiquitin-like peptide has been isolated from the mushroom *Calvatia caelata* .Asimilar ubiquitin-like protein and a peptide have been purified from *Pleurotus ostreatus* and *P. sajor-caju* cv hsiu tseng, respectively. All three of them inhibit cell-free translation, and demonstrate ribonuclease and N-glycosidase activities, albeit with different potencies. A ubiquitin-like peptide with ribonuclease activity against various polyhomoribonucleotides has been purified from the yellow mushroom *Cantharellus cibarius*. Their sequences are distinct from those of ribonucleases discussed in the following. The peptide from *C. caelata* demonstrates antimitogenic activity toward mouse splenocytes and antiproliferative activity toward human breast cancer cells. The ubiquitin-like peptide from *Agrocybe cylindracea* exerts immunostimulating and antiproliferative activities. The ubiquitin-like peptides and proteins are unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and Mono S. Ubiquitin-conjugated proteins including cell cycle regulatory proteins, p53 tumor suppressor, the transcriptional regulator NF-kB and its inhibitor, many transcription factors, and the *mos* protooncogene, are targets for degradation by the 26S proteasome. The ubiquitin-meidated

pathway regulates cell-cycle progression, signal transcription regulation, receptor down-regulation, endocytosis, immune response, development, and apoptosis. Defects in ubiquitin-mediated events may be involved in the development of pathological conditions including malignant transformation. Whether ubiquitins have similar significance in the life of mushrooms remain to be elucidated, but it is likely in view of the conserved sequence exhibited by ubiquitins.

Table 4: Extracellular enzymes produced from mushrooms

ENZYME ACTIVITY DETECTED	ROLE	NUTRIENT IN GROWTH SUBSTRATE (COMPOST)	PRODUCT OF ENZYME ACTIVITY FOR ASSIMILATION BY FUNGUS	STAGE OF LIFE CYCLE WHERE ACTIVITY UTILIZED
Laccase	Broad role in lignin biodegradation	Phenols or lignin	Lower molecular weight aromatic compounds	Mycelial growth on compost
Endocellulase, Exocellulase, β-Glulosidase } Cellulase Complex	Cellulose degradation Replenish carbohydrate levels	Cellulose	Sugars	Fruiting body development
Xylanase	Xylan degradation	Xylan (hemicellulose)	Sugars	Mycelial growth on compost
Protease	Protein degradation	Protein	Amino acids	Mycelial growth on compost
Phosphatase	Liberation of phosphate ion	Phosphate esters	Free phosphate	Not known
Lipase	Lipid degradation			Mycelial growth on compost
DNAase	DNA degradation	DNA	Sugars, nucleic acid bases, phosphates	Mycelial growth on compost
RNAase	RNA degradation	RNA	Sugars, nucleic acid bases, phosphates	Mycelial growth on compost
Laminarinase	Glucan degradation	Glucans	Sugars	Mycelial growth on compost
β-N-Acetylmuraminase (Lysozyme)	Peptidoglycan degradation	Bacterial cell wall polymers	Peptidoglycan fragments	Mycelial growth on compost
β-N-Acetylglucosaminase	Peptidoglycan degradation	Bacterial cell wall polymers	Peptidoglycan fragments	Mycelial growth on compost

Edible Mushrooms

Edible mushrooms are excellent foods that can be incorporated into well-balanced diets due to their low content of fat and energy, and high content of dietary fiber and functional compounds (Breene, 1990; Manzi, Aguzzi, & Pizzoferrato, 2001). Their benefits to health include immunomodulatory, anti-tumoral, and hypocholesterolemic effects (Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006). In numerous molecules synthesized by macrofungi are known to be bioactive, such as polysaccharides, glycoproteins, terpenoids, lectins, among others (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007).

Pleurotus florida

Pleurotus is an important genus of edible basidiomycetes which are commonly called oyster mushroom: all known species are edible, with several being commercially cultivated (Ragunathan, Gurusamy, Palaniswamy, & Swaminathan, 1996). The popularity of this genus is on the increase, especially because of its flavor and texture (Kim et al., 2007). The species are also rich in minerals, are good sources of protein, and have short life cycle (Yildiz, Yildiz, Gezer, & Temiz, 2002). Among oyster mushrooms, the best known Pleurotus spp. are *Pleurotus citrinopileatus, Pleurotus ostreatus, Pleurotus ostreatoroseus, Pleurotus pulmonarius, Pleurotus eryngii,* and *Pleurotus florida* (=*P. ostreatus* var. *florida*) (Ku¨ es & Liu, 2000).

Figure 5. ***Pleurotus florida***

Pleurotus sajor caju

The genus Pleurotus (Jacq: Fr.) Kumm. (Pleurotaceae, higher Basidiomycetes) comprises a group of edible ligninolytic mushrooms with medicinal properties and important biotechnological and environmental applications. The evolutionary connection among species in the genus Pleurotus is still not clear and many taxonomic issues remain controversial. The cultivation of Pleurotus spp is an economically important food industry worldwide which has expanded in the past few years. P. sajor caju is the third most important cultivated mushroom for food purposes. Nutritionally, it has unique flavor and aromatic properties; and it is considered to be rich in protein, fiber, carbohydrates, vitamins and minerals. Pleurotus spp are promising as medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypocholesterolic and immunomodulation activities. The bioactive molecules isolated from the different fungi are polysaccharides. One of the most important aspects of Pleurotus spp is related to the use of their ligninolytic system for a variety of applications, such as the bioconversion of agricultural wastes into valuable products for animal feed and other food products and the use of their ligninolytic enzymes for the biodegradation of organopollutants, xenobiotics and industrial contaminants.

Figure 6: ***Pleurotus sajor caju***

Volvariella volvaceae:

Volvariella volvacea is a popular cultivable mushroom of the tropics and subtropics and known from ancient times, is commonly called paddy mushroom, straw mushroom, banana mushroom or Chinese mushroom. It prefers to fruit on paddy straw substrate. Paddy straw is comprised mainly of cellulose and lignin that are to be degraded enzymatically for efficient substrate utilization by the fungus. It is the third most important mushroom cultivated in the world with an annual production of 287 million tones (Thakur et al 2003). The importance of paddy straw mushroom is reflected by the steady increase in considerable pressure now a days to develop processes for the rational treatment and/or disposal of the vast quantities of waste lignocellulosic materials generated annually through activities of the agricultural, forestry and food processing industries. The most significant approach in terms of producing a higher value product from the waste is the cultivation of edible mushrooms by solid-state fermentation (Chang and Miles, 1991). The cultivation of edible mushrooms is a prime example of the bioconversion of many types of low value lignocellulosic wastes, primarily from agricultural practices, into a higher value commodity. *Vovariella volvacea* utilizes cellulosic materials more effectively than any other cultivated mushroom (Kaur, 2002) . This mushroom has successfully been cultivated on a variety of lignocellulosics like cereal straw, sugar-cane baggase, oil palm pericarp and banana leaves. This indicates that V. volvacea has a potential to secrete several extracellular enzymes to degrade such lignocellulosics.

Figure 7: *Volvariella volvaceae*

Medicinal Mushroom - *Ganoderma lucidium*

Ganoderma lucidum (Ling Zhi) is a basidiomycete white rot macrofungus which has been used extensively as "**the mushroom of immortality**" in China, Japan, Korea and other Asian countries for 2000 years. A great deal of work has been carried out on therapeutic potential of *Ganoderma lucidum*. The basidiocarp, mycelia and spores of *Ganoderma lucidum* contain approximately 400 different bioactive compounds, which mainly include triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides and trace elements which has been reported to have a number of pharmacological effects including immunomodulation, anti-atherosclerotic, anti-inflammatory, analgesic, chemopreventive, antitumor, chemo and radio protective, sleep promoting, antibacterial, antiviral (including anti-HIV), hypolipidemic, anti-fibrotic, hepatoprotective, anti-diabetic, anti-androgenic, anti-angiogenic, anti-herpetic, antioxidative and radical-scavenging, anti-aging, hypoglycemic, estrogenic activity and anti-ulcer properties. *Ganoderma lucidum* has now become recognized as an alternative adjuvant in the treatment of leukemia, carcinoma, hepatitis and diabetes. The macrofungus is very rare in nature rather not sufficient for commercial exploitation for vital therapeutic emergencies; therefore, the cultivation on solid substrates, stationary liquid medium or by submerged cultivation has become an essential aspect to meet the driving force towards the increasing demands in the international market.

Figure 8: ***Ganoderma lucidium***

Table 5. Therapeutic Effects and Bioactive Compounds of *Ganoderma lucidum* Reported in the Literature Until 2009

Therapeutic Effects	Bioactive Compound	References
Immunomodulation: Mitogenic activity, Stimulation of immune effector cells and complement system	Protein LZ-8, β-D-glucan, Ganoderic acid	Kim *et al.*, [30]; Zhang *et al.*, [36]; Wang *et al.*, [38]; Won *et al.*, [41]; Lee *et al.*, [40]; Han *et al.*, [42], Li *et al.*, [43], Oh *et al.*, [44], Tasaka *et al.*, [18]; Yang and Pai, [52]; Kino *et al.*, [50]
Anti-Cancer, Anti-Tumour, Chemo and Radio Prevention	β-D-glucans, heteropolysaccharides, glycoproteins, lanostanoid, 3β-hydroxyl-26-oxo-5α-lanosta-8,24-dien-11-one, and steroid, ergosta-7,22 diene 3β,3α,9α triol	Miyazaki and Nishijima, [53]; Usui *et al.*, [54]; Usui *et al.*, [55]; Sone *et al.*, [56]; Kishida *et al.*, [57], Zhang *et al.*, [58], Cheong *et al.*, [59], Wasser and Weis, [19]
Anti-HIV-1 And Anti-HIV-1-Protease	Triterpenoids: Lucidenic acid O; Lucidenic lactone; Ganoderiol; Ganodermanontriol and Ganoderic acid	Sahar, [27]; Hobbs, [7]; McKenna, [8]; Gao, [10]; Min, [29]
Anti-Diabetic	Glycans: Ganoderans B and D	Mohammad, *et al.*, [80]; Wasser, [4]; Hobbs, [7]; McKenna, [8]; Gao, [9]
Hepatoprotective	Ganoderic acids R and S and ganosporeric acid A	Hirotani, [81]; Chen, [82]; Wang, [83]; Gao, [11]
Anti-Inflammatory	Ganoderic Acid C, 3-oxo-5α-lanosta-8,24-dien-21-oic acid	Joseph *et al.*, [84]; Horng-Huey Ko *et al.*, [85]; Lin *et al.*, [86]
Anti-Allergic	ganoderic acids C and D	Zhou, [20]; Liu, [18]; Smith, [16]
Anti-Androgenic	ganoderol B	Liu *et al.*, [92]; Fujita *et al.*, [93]; Shimizu *et al.*, [94]
Anti-Angiogenic activity	Ethanol extract (Compound not reported)	Song, *et al.*, [95]
Anti-Herpetic	Acidic protein bound polysaccharides	Kim *et al.*, [96]; Eo *et al.*, [97]; Liu *et al.*, [98]; Oh *et al.*, [99]
Anti-Oxidant	Chloroform extract (Compound not reported)	Karaman *et al.*, [100]; Joseph *et al.*, [84]
Anti-Microbial: Anti-Viral, Anti-Bacterial, Anti-Fungal	Neutral protein bound polysaccharide, Acidic protein bound polysaccharide, ganodermin	Wasser, [4]; Stamets, [101]; Hobbs, [7]; McKenna, [8]; Gao, [10]; Smith, [16]; Suay, [102]; Yoon, [103]; Sugiura and Ito, [104]; Kim *et al.*, [105]; Eo *et al.*, 2000 [107]; Eo *et al.*, [106]; Wang and Ng, [108]
Estrogenic	Ethanol extract (Compound not reported)	Shimizu *et al.*, [94]
Anti-Mutagenic	Methanol extract (Compound not reported)	Lakshmi *et al.*, [109]
Anti-Ulcerogenic	Polysaccharides	Gao *et al.*, [12]
Anti-Proliferative activity	Ganoderic acid T	Hong, [110]; Jiang, [111]; Hu, [112]; Muller *et al.*, [113]; Tang *et al.*, [114]
Cardiovascular and Circulatory Functions	Powdered mycelium and water extract of mycelium (Compound not reported)	Kabir, [115]; Soo, [116]; Lee, [117]; Jin *et al.*, [118]

Chapter 3

MATERIALS & METHOD

MATERIALS & METHOD

Mushroom strains:

Four Strains of Mushrooms, i.e. fruiting body of mushrooms were obtained from the following sources:

Volvoriella volvaceae – Sector 19 Market, Rourkela.

Pleurotus florida, - Ispat Mushroom House, Rourkela.

Pleurotus sajor caju – Ispat Mushroom House, Rourkela.

Ganoderma lucidium – Ispat Mushroom House, Rourkela.

Preparation of Malt Agar Spawn Culture Bottles:

Spawn Culture glass bottles of 250 ml capacity were used for culturing mycelia of the mushrooms.

Preparation of Mycelial Cultures of the Mushrooms:

Freshly obtained mushroom strains (*Volvoriella volvaceae*; *Pleurotus florida*, *Pleurotus sajor caju*; and *Ganoderma lucidium*) were surface sterilized with distilled water and 0.2% $HgCl_2$ solution (0.2 grams of $HgCl_2$ in 100 ml distilled water). Then the mushrooms were cut into small pieces and the transverse section of the untouched tissues were cut into 3×3 cm and were soak dried using filter paper. These tissues were then centre inoculated in the pre prepared spawn culture bottles. The spawn bottles containing the mycelial cultures were plugged tightly with cotton and incubated at 25±3^oC for 8 to 10 days.

Enzymatic Screening of Mushrooms using Agar Plates:

Mushrooms were screened for enzymes using agar plate method with the following compositions:

Starch Agar Medium (For Amylase Test)

Ingredient	gram/litre
Starch	20.0
Peptone	5.0
Beef extract	3.0
Agar	15.0
pH	7.0 ± 0.2

Gram's iodine solution was used to detect the amylase production. A clear zone surrounding the colony results in positive test for amylase.

Gelatin Agar Medium (For Protease Test)

Ingredient	gram/litre
Gelatin	20.0
Caesin	10.0
Sodium Chloride	10.0
Sodium taurocholate	5.0
Sodium bicarbonate	1.0
Agar	15.0
pH	8.5 ± 0.2

Mercuric chloride solution was used to detect the protease production resulted as clear zone surrounding the colony.

Composition of the mercuric chloride reagent:

20% hydrochloric acid was mixed in 15% mercuric chloride solution.

Lipase Test Medium

Ingredient	gram/litre
Peptone	10.0
Sodium chloride	5.0
Calcium chloride	0.1
Agar	20.0
Tween 20	10.0ml
pH	6.0 ± 0.2

Tween 20 was sterilized separately and added to the medium at the time of pouring. Fragmentation of the media resulted in the lipase production.

L- Asparaginase Test Medium (Modified Czapex Dox Medium)

Ingredient	gram/litre
Glucose	2.0
L-asparagine	10.0
K_2HPO_4	1.52
Potassium chloride	0.52
Magnesium sulphate	0.52

Cupric nitrate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Phenol red	0.009
Agar	20.0
pH	7.2 ± 0.2

Nessler's reagent was used as the reagent for L- Asparginase plate test to detect clear zone surrounding the colonies

Phosphate Solubilizaton Test (Pickovaskaya Medium)

Ingredient	gram/litre
Glucose	10.0
Tri-calcium phosphate	5.0
Ammonium sulphate	0.5
Sodium chloride	0.2
Magnesium sulphate	0.0001
Ferrous sulphate	0.001
Yeast Extract	0.5
Agar	15.0
pH	7.2 ± 0.2

Medium for Xylanase test

Ingredient	gram/litre
Xylan	5.0
Peptone	5.0
Yeast Extract	5.0
K_2HPO_4	1.0
Magnesium sulphate	0.2
Agar	20.0
pH	7.0 ± 0.2

Reagent:
0.1% Congo red solution – 0.1 gm congo red powder in 100 ml distilled water.
1 molar NaCl solution
The culture plates were flooded with Congo red solution. After 30 minutes they were washed with NaCl solution and observation was done for visualizing clear zone surrounding the colony.

Estimation of Enzymes:

Estimation of L – Asparginase activity

Estimation of L – Asparginase activity was carried out using modified Czapex Dox's media. Conical flasks (150 ml) containing 50 ml each of the appropriate medium was inoculated with each of the test organism. The flasks were incubated at 30^{o}C at 240 rpm per minute in a controlled environment incubator – shaker. Uninoculated media served as controls. The fungal cultures were harvested by filtration through Whatman No. 1 filter paper. The enzyme activity was estimated in culture filtrates by Nesselarization (*Imada et al. 1973*) and expressed as IU per ml. One international unit of L – Asparginase activity is defined as that amount of enzyme which catalyses the formation of 1 μmol of ammonia per unit under the conditions of assay.

Estimation of Available Phosphorous:

Composition of Reagents for Phosphate solubalization estimation

1. Extraction Solution: 0.55gm NH_4F was mixed in 1.04ml conc. HCl, and volume was made up to 500 ml with distilled water.

2. Standard stock solution: 0.109 gm KH_2PO_4 was mixed in 125 ml distilled water and 6.25 ml 7 Normal H_2SO_4. Then the volume was made up to 250 ml. 10 ml of this standard Solution was added in 490ml distilled water to get a 10 ppm solution, i.e. 50 times dilution.

3. REAGENT A: 6gm of Ammonium Molybdate was added in 125ml distilled water in one flask, and 0.145gm Antimony potassium Tartarate was added in 50 ml distilled water in another flask. These two solutions were then added to 500 ml of 2.5M H_2SO_4 solution, mixed thoroughly and volume was made up to 1000ml.

4. REAGENT B: Dissolve 1.056gm ascorbic acid in 200ml reagent A and mix. Do not keep for more than 24 hours.

While preparing curve for standard, the standard stock solution will be taken from 1 to 9 ml, accordingly, the volume of water decreases and increases to keep the constant volume 24 ml.

The volume of extraction solution and reagent B remains constant in all the tubes. After adding reagent B the sample were left for 15 minutes incubation. The colour slowly develops. Then, the readings were taken in UV Vis Spectrophotometer at 730 nm.

TABLE FOR STANDARD:

Stock Solution	Extraction Solution	Distilled Water	Reagent B
Blank	5 ml	15 ml	4 ml
1 ml	5 ml	14 ml	4 ml
2 ml	5 ml	13 ml	4 ml
3 ml	5 ml	12 ml	4 ml
4 ml	5 ml	11 ml	4 ml
5 ml	5 ml	10 ml	4 ml
6 ml	5 ml	9 ml	4 ml
7 ml	5 ml	8 ml	4 ml
8 ml	5 ml	7 ml	4 ml
9 ml	5 ml	6 ml	4 ml

For estimating the concentration of phosphorous in sample broth, the broth was filtered through Whattman Filter Paper No.1. The filtrate was kept in vials and the biomass was weighed. Then 500 µl of sample was added in 5 ml of extraction solution and 14.5 ml of distilled water with 4 ml of reagent B. After 15 minutes of incubation the readings were taken. The concentration of phosphorous in 1 ml of stock solution was found out. Then the values were put in X axis and OD in Y axis and the graph was plotted.

TABLE FOR SAMPLE:

Sample (500 µl)	Extraction Solution	Distilled Water	Reagent B
P F	5 ml	14.5 ml	4 ml
P S C	5 ml	14.5 ml	4 ml
V V	5 ml	14.5 ml	4 ml
G L	5 ml	14.5 ml	4 ml

PF – *Pleurotus florida*

PSC – *Pleurotus sajor caju*

VV – *Volvariella volvaceae*

GL – *Ganoderma lucidium*

Chapter 4

RESULTS & DISCUSSION

RESULTS & DISCUSSION

Culture of Mushroom Mycelium in Vitro conditions

Mycelial cultures of Mushrooms were obtained after 8 – 10 days of incubation at 25 ± 3 ^{o}C. These mycelial cultures obtained were further used for the rest of the experiments.

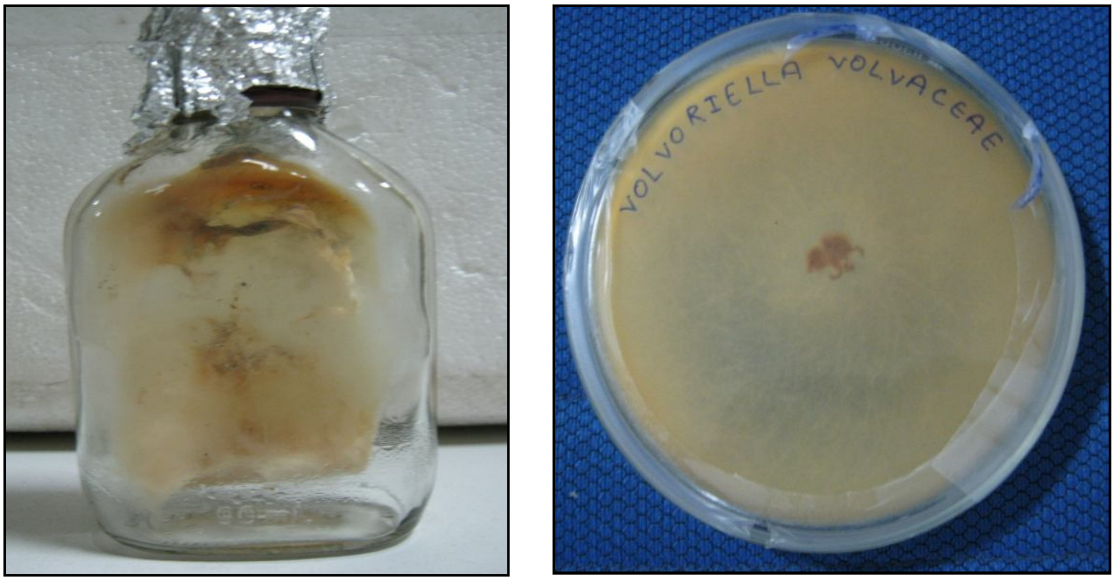


Figure 9: ***Volvariella volvaceae***

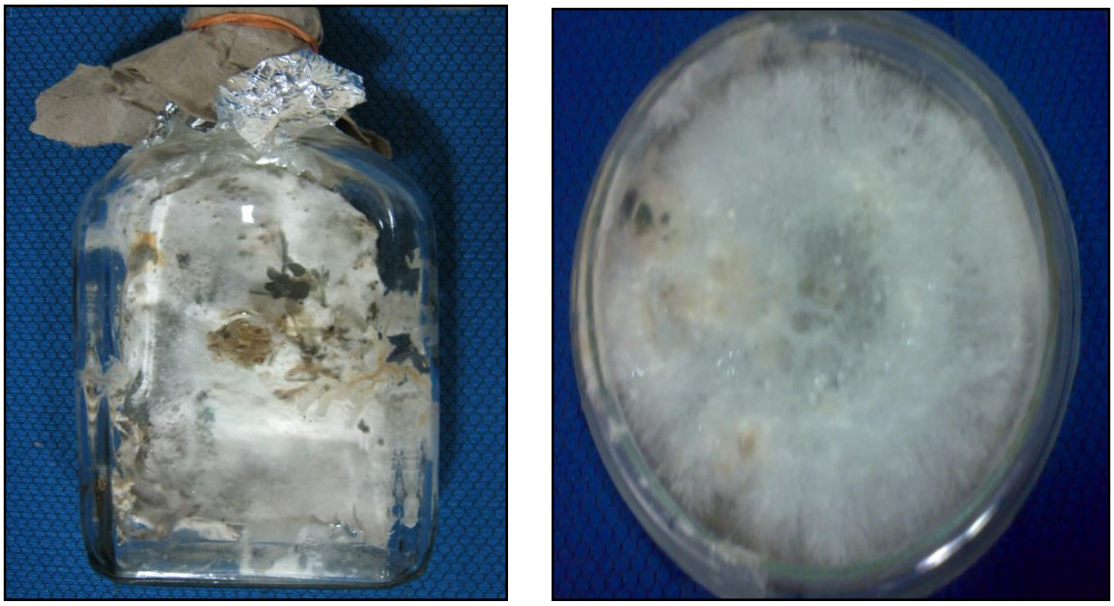


Figure 10: ***Ganoderma lucidium***

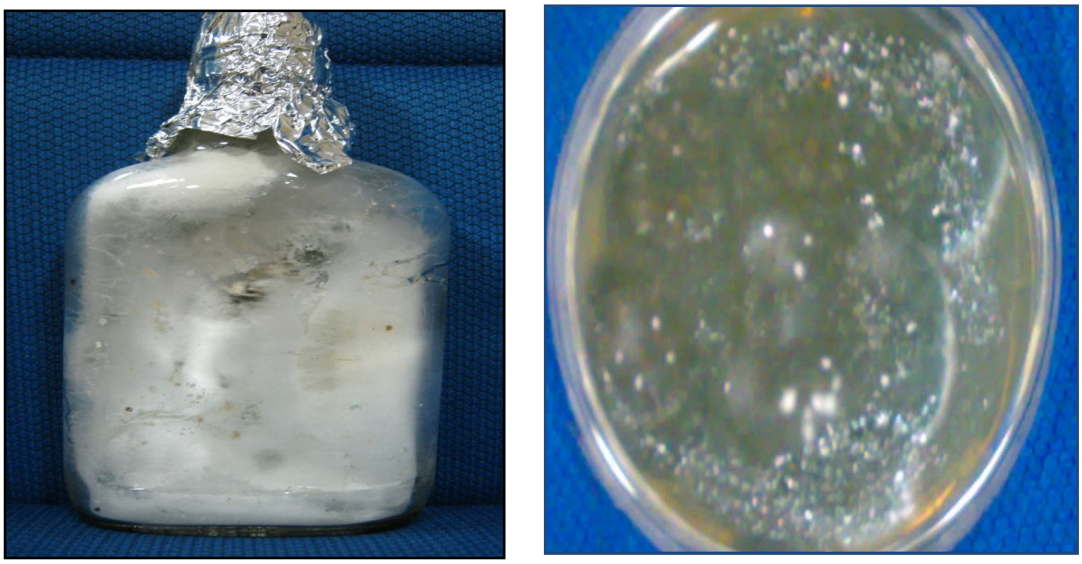


Figure 11: ***Pleurotus sajor caju***

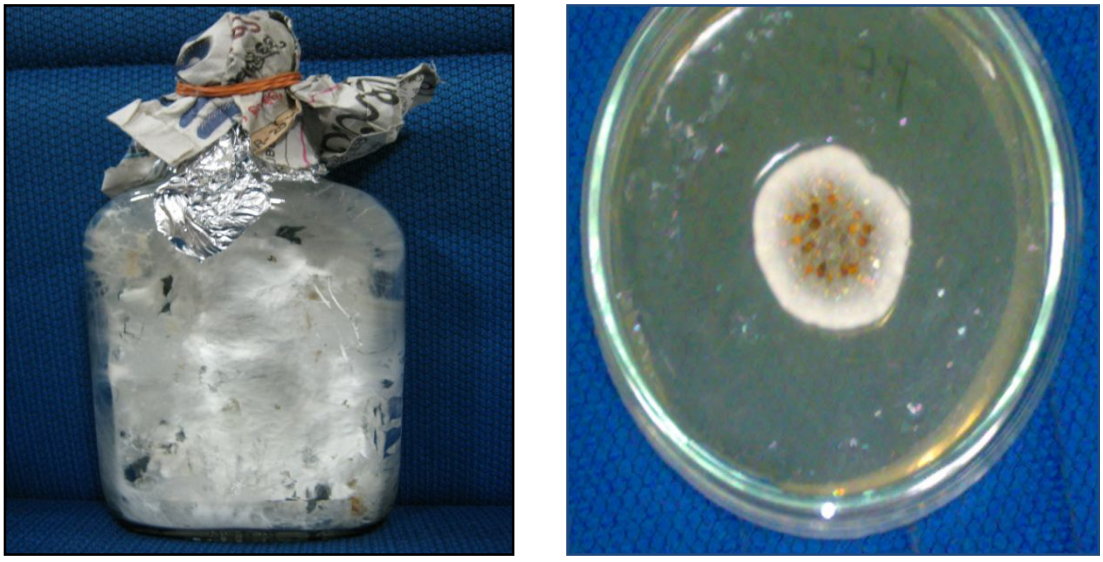


Figure 12: ***Pleurotus florida***

Screening of Potential Strain with Enzymatic Activitiy

After incubation of Agar plates for 6 – 7 days at 25 ± 3^{o}C for the enzymatic tests the following results were obtained:

	Pleurotus florida	***Pleurotus sajor - caju***	***Volvariella volvaceae***	***Ganoderma lucidium***
Amylase	+ + +	+ +	+ +	+ + +
Protease	+	+	+	+
Lipase	+ +	+ +	+ +	+ +
L – Asparginase	+ +	+ + +	+ +	+ + +
Phosphate Solubalization	+ +	+ +	+ + +	+ + +
Xylanases	+ +	+ +	+ +	+ +

It was seen that among the four different species i.e. *Vovariella volvaceae*, *Pleurotus florida*, *Pleurotus sajor caju* and *Ganoderma lucidium*; *Ganoderma lucidium* was the most potent strain which showed the best enzymatic activities among the four species. All the four species showed very less protease activity.

Figure 13: Plate Test for Amylase

CONTROL **RESULTS**

Volvariella volvaceae

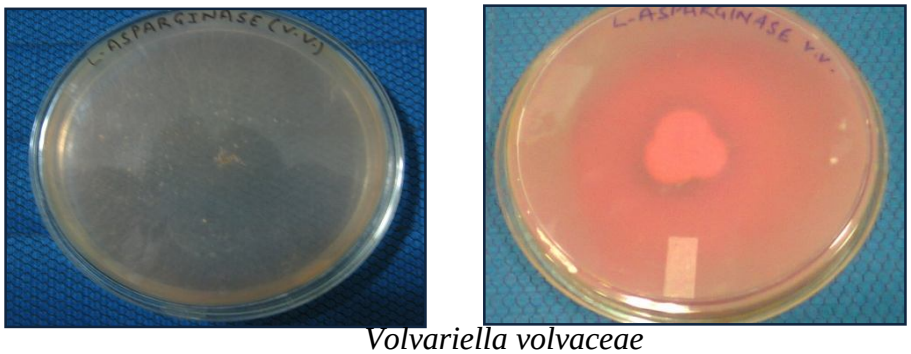
Pleurotus sajor caju

Pleurotus florida

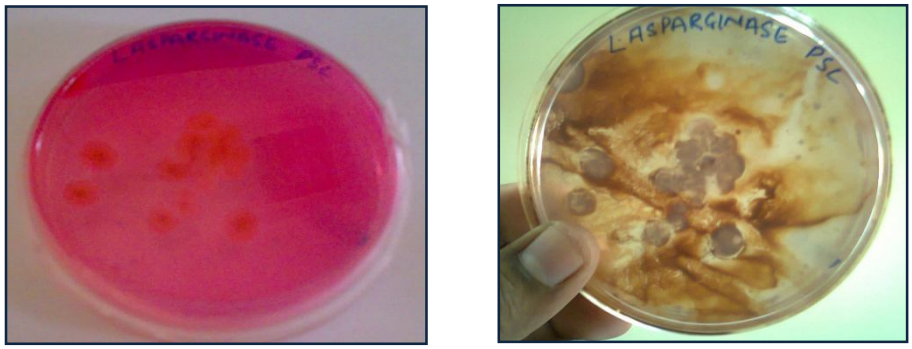
Ganoderma lucidium

Figure 14: Plate Test for L – Asparginase

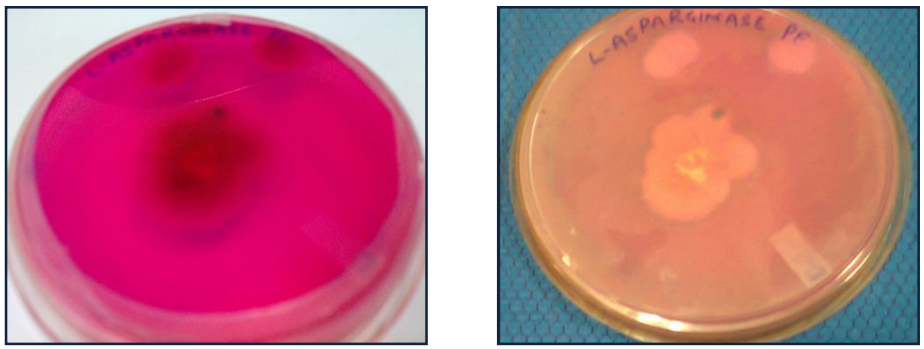
CONTROL **RESULTS**



Volvariella volvaceae



Pleurotus sajor caju

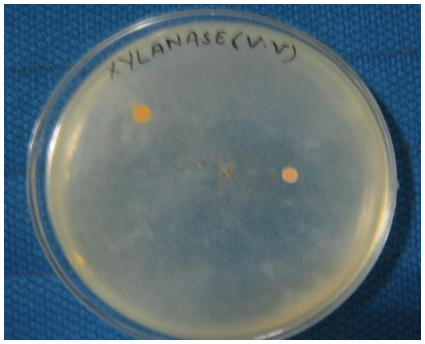


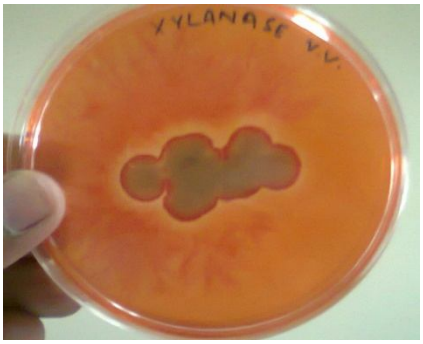
Pleurotus florida

Ganoderma lucidium

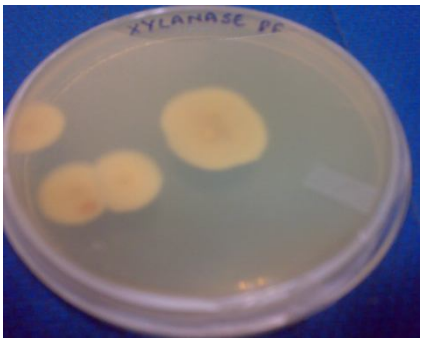
Figure 15: Plate Test for Xylanase

CONTROL **RESULTS**

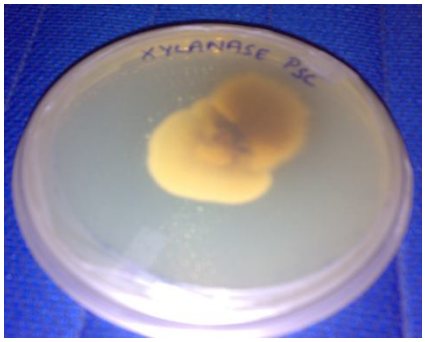




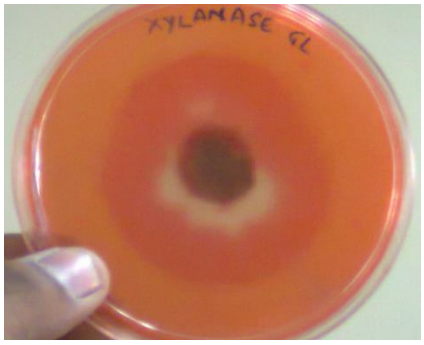
Volvariella volvaceae



Pleurotus florida

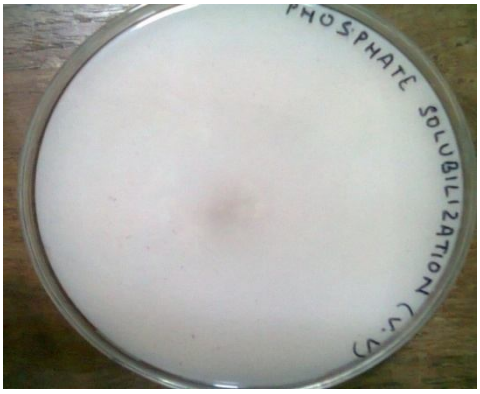


Pleurotus sajor caju



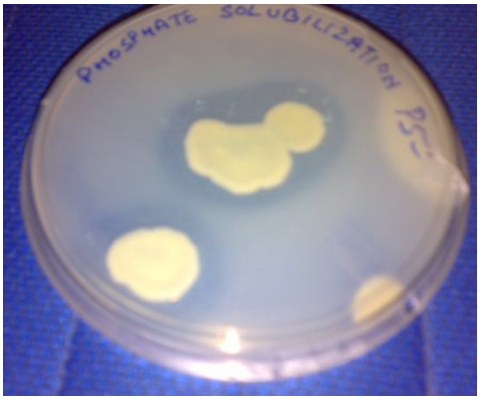
Ganoderma lucidium

Figure 16: Plate Test for Phosphate Solubalization

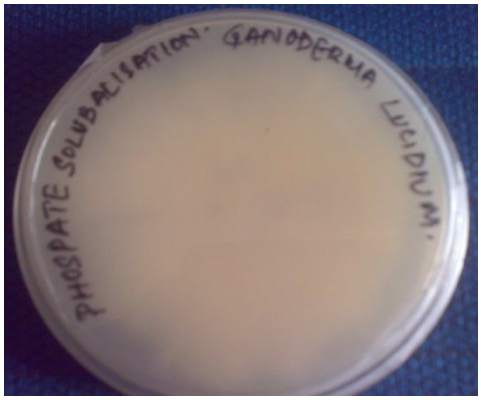


Volvariella volvaceae

Pleurotus florida



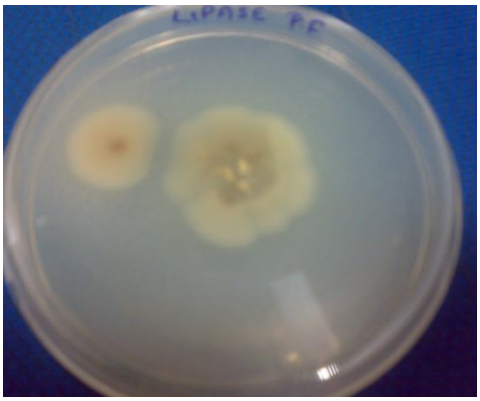
Pleurotus sajor caju



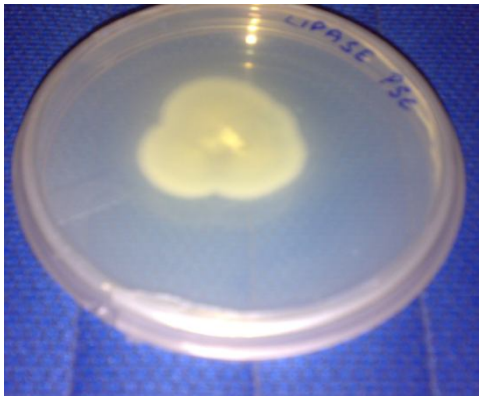
Ganoderma lucidium

Figure 17: Plate Test for Lipase

Volvariella volvaceae



Pleurotus sajor caju



Pleurotus sajor caju

Ganoderma lucidium

Estimation and Selection of Strain with Enzymatic Activity

L – Asparginase Activity

The L – Asparginase activity of different mushroom species after 96 hrs of incubaton and Nesslerization were found out spectrophotometrically at 450 nm in IU / ml and was found out to be,

Mushroom	**Enzyme activity (IU / ml)**
Pleurotus florida	0.95
Pleurotus sajor caju	0.73
Volvariella volvaceae	1.76
Ganoderma lucidium	2.15

Among the four mushroom species i.e. *Vovariella volvaceae, Pleurotus florida, Pleurotus sajor caju* and *Ganoderma lucidium;* it was found out that *Ganoderma lucidium* is the most potent strain showing L- Asparginase activity showing 2.15 IU/ml, whereas the other strains i.e. *Pleurotus florida, Pleurotus sajor caju and Volvariella volvaceae* gave L – Asparginase activity as 0.95, 0.73 and 1.73 IU / ml respecttievely.

Phosphate Solubilization Activity

The phosphate solubalization activity of different mushroom species were found out from the culture filtrates after 6 – 7 days of incubation spectrophotometrically at 730 nm and the graph was plotted by taking concentration on X – axis and OD on Y – axis. Among the four strains the most potent strain showing phosphate solubilization activity was found out to be *Volvariella volvaceae* at 0.4882 ppm concentration at 10% dilution. The other strains, i.e. *Pleurotus sajor caju, Pleurotus florida and Ganoderma lucidium* showed phosphate solubilization activity at 0.082 ppm at 30 % dilution, 0.0435 ppm at 50% dilution and 0.0568 at 0% dilution.

Table for Standard

	OD	OD'	Avg OD	Concentraion (ppm)
Blank	1.032	1.037		0
1	0.048	0.082	0.065	0.416667
2	0.112	0.099	0.1055	0.833333
3	0.134	0.183	0.1585	1.25
4	0.218	0.243	0.2305	1.666667
5	0.271	0.279	0.275	2.083333
6	0.307	0.315	0.311	2.5
7	0.41	0.399	0.4045	2.916667
8	0.43	0.426	0.428	3.333333
9	0.466	0.476	0.471	3.75

Graph – Concentration vs OD

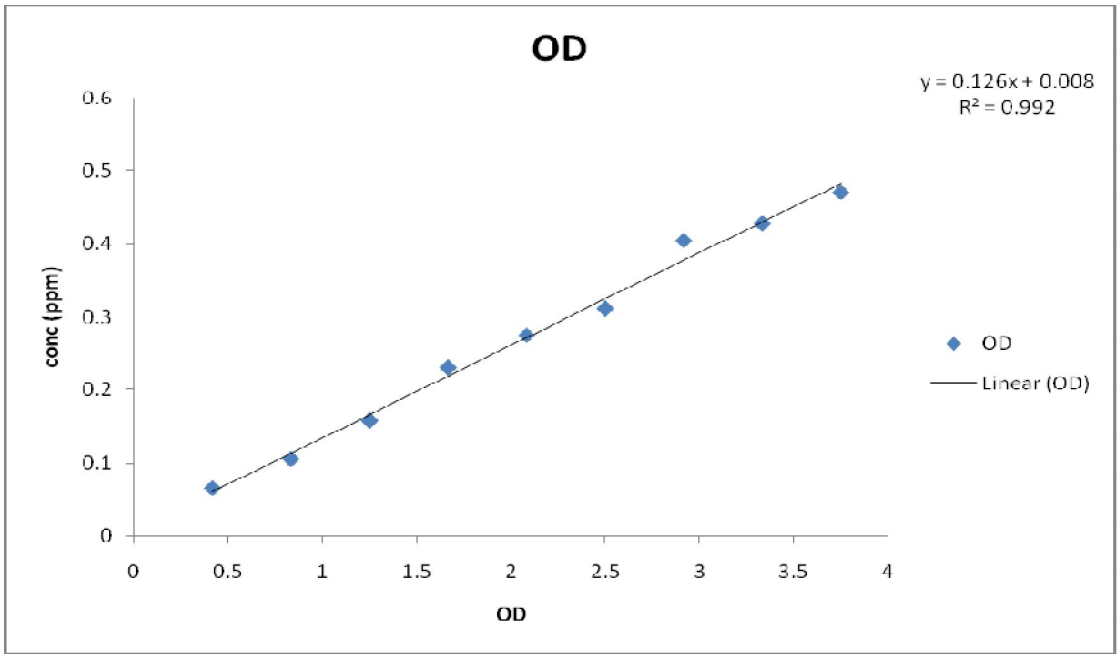


Table for Samples

	OD	**OD'**	**Avg. OD**	**Conc (ppm)**	**FinalConc (ppm)**
PF	0.101	0.118	0.1095	0.0217	0.0435
PSC	0.126	0.14	0.133	0.0217	0.0824
VV	0.316	0.332	0.324	0.0488	0.4882
GL	0.402	0.373	0.3875	0.0568	0.0568

Table showing Biomass of the four Strains of Mushroom after Phosphate Solubilization Activity

Mushroom Strain	**Biomass (in gms)**
PF	0.9
PSC	0.9
VV	1
GL	4.1

PF – *Pleurotus florida*

PSC – *Pleurotus sajor caju*

VV – *Volvariella volvaceae*

GL – *Ganoderma lucidium*

Chapter 5

CONCLUSION

CONCLUSION

In this work we studied, the enzyme activities of *Pleurotus spp*, *Volvariella volvaceae* and *Ganoderma lucidium* grown on Malt Extract Agar media. Higher activities of Amylase, L – Asparginase, Xylanase, Phosphate Solubalization, and Lipase were observed in Ganoderma lucidium, where as very less Protease activity was observed in all the four strains of mushroom.

Phosphate solubilization and L – Asparginase values were higher in *Volvariella volvaceae* and *Ganoderma lucidium* respectievely. *Volvariella volvaceae* showed phosphate solubilization activity of 0.4882 ppm concentration at 10% dilution at 730 nm and *Ganoderma lucidium* showed L- Asparginase activity of 2.15 IU/ml.

In summary in this work, we observed that during the growth of mushroom mycelia and the development to mature fruitbodies (or sporophores), biochemical changes are known to occur, as a result of which enzymes are secreted extracellularly to degrade the insoluble materials in the substrates into simple and soluble molecules which are subsequently utilized by intracellular enzymes within the mushroom. Consequently, enzymes play significant role in mushroom development, in addition, they also affect the food nutrient, flavour and shelf life of these fungi. Thus, *Ganoderma lucidium* which shows higher activity of L – Asparginase can be used for the production of this enzyme or as a potent source therapeutic agent.

Chapter 6

REFERENCES

REFRENCES

1. Alconada TM, Martinez MJ., 1994, Purification and characterization of an extracellular endo-1, 4-xylanase from *Fusarium oxysporum f.* sp. melonis. FEMS Microbiol Lett, 118, 305–10.

2. Akin DE, Borneman WS., 1990, Role of rumen fungi in fiber degradation. J Dairy Sci; 73, 3023–32.

3. Ammirati *et al*., 1985, 25–34.

4. Anand L, Krishnamurthy S, Vithayathil PJ., 1990, Purification and properties of xylanase from the thermophilic fungus, *Humicola lanuginose* (Griffon and Maublanc) Bunce. Arch Biochem Biophys; 276, 546–53.

5. Bagga PS, Sandhu DK, Sharma S., 1990, Purification and characterization of cellulolytic enzymes produced by *Aspergillus nidulans*. J Appl Bacterial; 68, 61–8.

6. Beumer-Jachmans, M. P. (1973) *Ann. Microbiol. 124*, 289- 292

7. Boer H, Terri TT, Koivular A., 2000, Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. Biotechnol Bioeng; 69, 486–94.

8. Bourbonnais R, Paice MG., 1990, Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation. FEBS Lett; 267, 99–102.

9. Broome, J. D. (1961) *Nature 191*, 1114-1115.

10. Broome, J. D. (1968) *Br. J. Cancer 22*, 595-602.

11. Bumpus JA, Aust SD., 1987, Biodegradation of new environmental pollutants by the white rot fungus *Phanerochaete chrysoporium*: involvement of the lignin degrading system. BioEssays; 6, 166–70.

12. Campbell GL, Bedford MR., 1992, Enzyme application for monogastric feeds: a review. Can J Anim Sci; 72:449–66.

13. Chou H, Lai HY, Tam MF, Chou MY, Wang SR, Hon SH, et al., 2001, cDNA cloning, biological and immunological characterization of the alkaline serine protease major allergen from *Penicillium chrysogenum*. Int Arch Allergy Immunol; 127, 15–26.

14. Dec J, Haider K, Bollag JM., 2003, Release of substitutents from phenolic compounds during oxidative coupling reactions. Chemosphere; 52, 549–56.

15. Distasio, J. A., Niederman, R. A., Kafkewitz, D., and Goodman, D. (1976) *J. Biol. Chem. 251*, 6929-6933.

16. Fernandez-Larrea J, Stahl U., 1996, Isolation and characterization of a laccase gene from *Podospora anserine*. Mol Gen Genet; 252, 539–51.

17. Gilbert HJ, Hazelwood GP, Laurie JI, Orpin CG, Xue GP., 1992, Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. Mol Microbiol; 6, 2065.

18. Gomes J, Gomes I, Steiner W., 2000, Thermolabile xylanase of the Antartic yeast *Cryptococcus adeliae*: production and properties. Extremophiles; 4:227–35.

19. Gupta R., Gigras P., Mohapatra H., Goswami V.K. and Chauhan B. 2003. Microbial α-amylase: a b α-amylase biotechnological perspective. Journal of Process Biochemistry. 20, 1-18.

20. Hortobagyi, G. N., Yap, H. Y., Wiseman, C. L., Blumenschein, G. R., Buzdar, A. U., Legha, S. S., Gutterman, J. U., Hersh, E. M., and Bodey, G. P. (1980) *Cancer Treat. Rep. 64*, 157-159.

21. Kim JH, Kim YS., 1999, A fibrinolytic metalloprotease from the fruiting bodies of an edible mushroom *Armillariella mellea*. Biosci Biotechnol Biochem; 63, 2130–6.

22. Kim JH, Kim YS., 2001, Characterization of a metal enzyme from a wild mushroom, *Tricholoma saponaceum*. Biosci Biotechnol Biochem; 65, 356–62.

23. Kimura T, Ito J, Kawano A, Makino T, Kando H, Karita S, et al., 2000, Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. Biosci Biotechnol Biochem; 64, 1230–7.

24. Kobayashi H, Sokibata S, Shibuya H, Yoshida S, Kusakabe I, Murakami K., 1989, Cloning and sequence analysis of cDNA for *Irpex lacteus* aspartic proteinase. Agric Biol Chem; 53, 1927–33.

25. Kruger RP, Winter HC, Simonson- Leff N, Stuckey JA, Goldstein IJ, Dixon JE., 2002, Cloning, expression and characterization of the gal-1, 3-gal high affinity lectin from the mushroom *Marasmius oreades*. J Biol Chem; 277, 15002–5.

26. Marzullo L, Cannio R, Giardina R, Santini MT, Sannice G., 1995, Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. J Biol Chem; 270, 3823–7.

27. Mashburn, L. T., and Wriston, J. C. (1964) *Arch. Biochem. Biophys. 105*, 450-452.

28. Miles PG, Chang S-T. (2004). *Mushrooms: Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact*. Boca Raton, Florida: CRC Press. ISBN 0-8493-10431

29. Mussak R, Bechtold T. (2009). *Handbook of Natural Colorants*. New York: Wiley. pp. 183–200. ISBN 0-470-51199-0.

30. Nonaka T, Ishikawa H, Tumuraye Y, Hashimoto Y, Dohmae N., 1995, Characterisation of a thermostable lysine-specific metalloendopeptidase from the fruiting bodies of a basidiomycete *Grifola frondosa*. J Biochem (Tokyo); 118:1014–20.

31. Paoletti M, Castroviejo M, Begueret J, Clave C., 2001, Identification and characterization of a gene encoding a subtilisin-like serine protease induced during the vegetative incompatibility reaction in *Podospora anserine*. Curr Genet; 39, 244–52.

32. Pekkarinen AI, Jones BL, Niku-Paavola ML., 2002, Purification and properties of an alkaline protease of *Fusarium culmorum*. Eur J Biochem; 269:798–807.

33. Poza M, ke Miyuel T, Sievro C, Villa TG., 2001, Characterization of a broad pH range protease of *Candida caseinolytica*. J Appl Microbiol; 91:916–21.

34. Qy Y, Gao P, Wang D, Zhao X, Zhang X., 1996, Production, characterization and application of the cellulase-free xylanase from *Aspergillus niger*. Appl Biochem Biotechnol; 57, 375–81.

35. Reichard U, Cole GT, Ruchel R, Monod M., 2000; Molecular cloning and targeted deletion of PEP2 which encodes a novel aspartic proteinase from *Aspergillus fumigatus*. Int J Med Microbiol 290, 85–96.

36. Roberts, J., Holcenberg, J. S., and Dolowy, W. C. (1972) *J. Biol. Chem. 247*, 84-90.

37. Saha BC., 2001, Xylanase from a newly isolated *Fusarium verticillioides* capable of utilizing cornfiber xylan. Appl Microbiol Biotechnol; 56:762–6.

38. Sattar AKMA, Yamamoto N, Yoshimoto T, Tsuru D., 1990, Purification and characterization of an extracellular prolyl endopeptidase from *Agaricus bisporus*. J Biochem (Tokyo); 107, 256–61.

39. Slomczynsky D, Nakas JP, Tanenbaum SW., 1995, Production and characterization of laccase from *Botrytis cinerea*. Appl Environ Microbiol; 61, 907–12.

40. Stuntz *et al*., 1978, pp. 12–13.

41. Stuntz *et al*., 1978, 28–29

42. Torronen A, Mach RL, Messner R, Gonzalez R, Kalkkinen N, Harkk A, et al., 1992, The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Biotechnol; 10, 1461–5.

43. Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Richers A, et al., 1994, Anaerobic fungi in herbivorous animals. Mycol Res; 98:129–52.

44. Volk T. (2001). "*Hypomyces lactifluorum*, the lobster mushroom". *Fungus of the Month*. University of Wisconsin-La Crosse, Department of Biology. Retrieved 2008-10-13.

45. Wallace RJ., 1994, Ruminal microbiology, biotechnology and ruminant nutrition: progress and problems. J Anim Sci; 72:2992–3003.

46. Wang HX, Ng TB., 2001, Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom *Pleurotus eryngii*. Biochem Biophys Res Commun; 289, 750–5.

47. Wehner, A., Harms, E., Jennings, M. P., Beacham, I. R., Derst, C., Bast, P., and Ro¨hm, K. H. (1992) *Eur. J. Biochem. 208*, 475-480.

48. Yoshimoto T, Sattar AKMA, Hirose W, Tsuru D., 1990, Purification and characterization of an extracellular prolyl endopeptidase from *Agaricus bisporus*. J Biochem (Tokyo); 107, 256–61.