CHAPTER 2

LITERATURE REVIEW

2.0 THE SAGO PALM INDUSTRY IN MALAYSIA

2.1 Sago Palm Cultivation and Uses

The sago palm exploitation started during the 19th century (Morris, 1977) in Sarawak and along the coastal belts of Johore in Batu Pahat and Muar districts (Tan, 1980). For hundreds of years, the Melanau people of Sarawak have traditionally harvested two main species of sago palms, the thorny (*Metroxylon rumphii* Martius) and the thornless (*Metroxylon sagu* Rottboll) palms for subsistence. Virtually all parts of the palm have local uses. The leaves are used as roofing material and made into attap hats, baskets and blow-pipe darts. The sticky sap is used for making gum and resins. Bark and rachis are used as firewood. Ripe fruits and the uncooked palm heart or cabbage are used as food. The sago worms (larvae of *Rhynchophorus schach*) are a highly priced delicacy obtained from the stumps of felled palms (Pearce, 1989; Kiew, 1989). However, the most important product is the starch obtained from the trunks of sago palms, after a long gestation or growth period of about 9 to 14 years, or when they are about to flower.

2.2 Sago Palm Ecology

The genus *Metroxylon* is derived from the Greek words, 'metra' meaning 'pith or heart" and 'xylon' meaning "xylem or wood". The sago palm is classified under the order of Spadicifloreae family of Palmae and sub-family of Lepidocaryoid. There are some six species under the swamp palm, *Metroxylon* which can grow in all tropical rainforests, coastal lowland and along rivers located between Latitudes 10°N and S and Longitudes 90° and 180°E where the annual precipitation is more than 2000 mm. Most *Metroxylon* spp. are found in and around New Guinea (Flach, 1977). The sago palm is an introduced crop for cultivation in South-east Asia mainly in Malaysia, Thailand and the Philippines.

Five other starch palms that are lower in productiveness and extent, favoring drier or hilly habitats are the *Arenga* (sugar palm), *Borassus*, *Caryota*, *Corypha* and *Eugeissona* (Tan, 1983). All sago palms have been recorded to thrive at altitudes up to 1000 m above sea level. The phrase "sago palms" in many reports has been quoted synonymous to *Metroxylon* spp. as it is the most economical and important genus (Cecil *et al.*, 1982).

Sago palms are hapaxanthic, meaning 'once-flowering' and soboliferous (give tillers or suckers that may grow horizontally initially and later on into erect stems) (Kiew, 1977). Sago palms go through a vegetative stage during which carbohydrates

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accumulate in the pith of the trunk. At the end of its life cycle, the starch reserves are expended on the production of an enormous inflorescence. After flowering and seed formation, the plant dies and deteriorates quickly (Flach, 1977).

Although sago palms grow best, mature earlier and yield better on mineral soils rich in organic matter, they adapt naturally on peat soils. This is of particular significance to Sarawak because about 1.5 million hectares or 12% of Sarawak's land area is under peat. Tie *et al.* (1991) reported that about 62% of sago in Sarawak is grown on peat. In the natural state, peat is usually poor in nutrients, high in acidity and possess a rather low nutrient buffering capacity, on which crops other than sago palm will grow only with much difficulty (Ahmed and Sim, 1975).

In practice, without artificial manuring or drainage, sago palms have been shown to be the only type of crop that thrives well and is capable of giving sustained economic returns on the peat swamps of Sarawak (Tie *et al.*, 1991; Tie & Lim, 1991). Diemont and Schuiling (1995) observed that sago palm in natural/wild and cultivated stands is confined to South-east Asia and Western Melanesia and their estimates of the areas and production levels of sago are shown in Table 2. In Sarawak, the hectarage under sago cultivation was about 19,720 in 1991. According to Tie and Lim (1991), from this hectarage, an estimated 2.347 million sago palms could be harvested in five years.

Table 2. Sago area in South-east Asia and production level for natural and planted stands

Stands	*Area (hectares)*	*Production (tonnes per hectares. year)*
Natural	5-6 million	2.5 - 5
Planted	210,000	10 - 25

Source : Diemont and Schuiling (1995)

2.3 Sago Starch Nature

Sago is the starch that the sago palm accumulates in its trunk as a reserve. Sago starch granules are rather larger than the other starches, ovoid and has a higher ratio of 27:73 of amylose to amylopectin (Cecil *et al.*, 1982). Some data for comparison between sago starch and starches of other plants are provided in Table 3. With advances in biotechnology and down stream processing, sago starch can be employed to produce maltodextrin (Anon, 1990), cyclodextrin (Solichien, 1995) and high fructose syrup using established methods.

Table 3. Properties of sago and other starches

	Sago	*Maize*	*Potato*	*Rice*	*Tapioca*
Granule shape	Oval	Round, polygonal	Oval	Polygonal	Oval - with indentation
Granule size (microns)	20-60	15	15-100	3-8	5-15
Amylose (%)	27	26	24	17	17
Swelling power (%)	97	24	>1000[a]	19	21
Initial pasting temperature °C[b]	69	62	56	66	58

[a] Potato starch swelling is greatly influenced by phosphate ester usually present in potato starch

[b] Estimated from graphs by Morgan (1940)

Source : Cecil *et al.*, (1982)

2.4 Sago Starch Trading

Sago starch has been a traded commodity in South East Asia for at least 800 years (Burkill, 1966). Since the 1920's, sago starch from Sarawak has been an export oriented-product. Sarawak, at present remains the world's leader in sago cultivation and is the principal exporter of sago starch (Zulphilip *et al.*, 1991).

The total world market for starch is more than 10 million tonnes per year (Flach, 1983). With sago starch accounting for less than 3% of the world market (Sanders, 1986) at F.O.B. price of US$250 per tonne, there is no reason to assume that costs will be a limiting factor for demand of sago starch in the world market. In 1992, the

state of Sarawak exported 45,780 tonnes of dry sago starch valued at more than $28 million Ringgit Malaysia or RM630/tonne (Agricultural Statistics of Sarawak, 1993).

At present, among Sarawak's palm crops, sago palm ranks second after oil palm as an agricultural export earner. For the year 1993, sago ranked fourth after oil palm, pepper and cocoa as an agricultural revenue earner for Sarawak (Agricultural Statistics of Sarawak, 1993). With the potential that it shows, this ranking may well change soon.

To remain a competitor on the world starch market, the sago industry needs large plantations to sustain constant supply. Foreseeing this, the state government of Sarawak initiated the world's largest sago palm plantation on deep peat near Mukah in 1987 (Kueh and Jong, 1993) through the Land Custody & Development Authority (LCDA) (Malaysian: Lembaga Pembangunan dan Lindungan Tanah (PELITA)), an agency of the state government of Sarawak. At present, the LCDA plantation is the only sago palm plantation in existence in the world where sago palm has been planned systematically to be managed as an estate crop (Schuiling *et al.*, 1993).

A recent newspaper report (Anon, 1995) mentioned Sarawak government's emphasis on sago research and plantations with an additional 40,000 hectares being allocated for sago cultivation in the near future. With these developments and advancements in sago palm cultivation technology coupled with the ever increasing productivity of

modern sago processing factories, the sago industry is now faced with the problem of solid and liquid waste disposal.

3.0 SAGO PROCESSING AND BY-PRODUCTS GENERATED

About 70% of the total sago grown in Sarawak is located in Sibu Division with Mukah and Dalat districts being the major areas. Sibu division alone accounts for about 95% of the starch from Sarawak (Chew and Shim, 1993). Almost all of the sago factories are located along the coastal belt of Mukah, Oya, Dalat and Igan in the Sibu Division (Sim, 1986). According to Chew and Shim (1990), there are about 10 multi-million dollar factories in the Sibu Division with a production capacity of 200 to 500 tonnes dry flour/factory per month. With the promotion of sago cultivation and sophistication of its processing, large quantities of solid and liquid wastes are generated creating serious pollution problems. Proper strategies for the treatment of these wastes are now required prior to safe and environmentally sound disposal.

The mature sago palm which may reach 9 - 12 m, are cut into 75 - 90 cm log sections and transported by lorries or linked up into rafts and floated by river to the factory (Tie and Lim, 1991). Debarking, to separate the pith from the bark, is the first stage of sago processing to produce starch. At the factory, debarking is done in a very crude manner with an axe. A conventional factory may generate about 8 - 15 tonnes of bark per day, for every 750 - 1200 log sections processed (Chew and Shim, 1991).

The bark which amounts to 17% of the weight of the logs, finds limited use as platforms for some factories and footpaths of nearby homes (Cecil *et al.*, 1982; Anton, 1992).

Almost two decades ago, during the First International Sago Symposium in 1976, the problem of sago waste utilization was discussed. The use of sago bark as an insulating material for air-conditioning ducts and cold-rooms, sound-proofing, etc. was suggested as it is highly termite- and fungal-resistant and retains its structure when wet. At present much of the bark is collected and burnt separately on site (Anton, 1992) as it is no longer used by some factories as fuel because its smoke is corrosive to the chimney used (Schuiling *et al.*, 1993).

The debarked pith is soft and pale pink in color. The pith contains almost all of the starch in the sago palm. The debarked pith is then split lengthwise into batons and is fed into a rasper or hammer mill where it is milled while water is added. Rotating plates then render the pith into a fine pulp which passes through several rotating drums with sieves or vibrator sieves for starch extraction. Large amounts of treated river water is mixed with the pulp to separate and wash the starch granules out of the 'repos' in the rotary sieves. At this stage 'hampas', the leftover fibrous pith residue is produced.

Two types of 'hampas' are produced in most of the factories because of the different sizes of sieves used to extract the starch from the pith residues. The coarse 'hampas' and secondly the fine 'hampas' are trapped consecutively in a screening process. The 'hampas' is usually washed off into the waterways together with the wastewater (Cecil *et al.*, 1982). Some factories, however, have introduced sieve screens to trap the 'hampas' in the effluent before discharge, which is sold locally as cattle feed supplement at a very low cost. Some larger sago factories have employed holding tanks to sediment fine 'hampas' and starch residues from the factory effluents before being discharged into rivers.

It is estimated that in an average-sized factory in Sarawak, for every 10 to 20 tonnes of sago starch produced per day, about 5 to 11 tonnes of 'hampas' and 300 to 1000 m^3 of wastewater are generated (Chew and Shim, 1990). Rasyad (1993) reported that a typical sago palm trunk contains about 29% starch and 23% sago pith residue. If one sago palm trunk yields about 150 kg of starch (Sundhagul, 1977), Sarawak would have generated about 39,240 tonnes of 'hampas' in 1992. This high quantities of 'hampas' and residual starch in the wastewater contribute to high Biological Oxygen Demand (BOD_5) of between 1,824 and 6,750 mg/L, Chemical Oxygen Demand (COD) of between 5,682 and 16,974 mg/L and particularly high suspended solids in the combined effluent (Chew and Shim, 1990; Anton, 1992). Overall, sago processing wastewater's are low in nutrients, are acidic and high in organic load. A recent study showed that sago starch processing wastewater supported the growth of purple non-

sulfur phototrophic bacterium, *Rhodopseudomonas palustris* (Getha, 1995). This organism has been found to purify polluted waters (Kobayashi, 1982).

4.0 COMPOSITION OF 'HAMPAS'

Large amounts of 'hampas' is left behind after starch extraction and is considered one of the major pollutants in rivers and streams near a sago processing factory. In literature, 'hampas' is termed as 'sago refuse', 'pith residue', 'squeezed fibers', 'sago pith meal', 'waste of pulverized sago pith' or 'ampas' (Müller, 1977; Sundhagul, 1977; Cecil *et al.*, 1982; Yahya *et al.*, 1992; Schuiling *et al.*, 1993).

Studies by Shim (1992) showed that the major constituents of 'hampas' included 66% starch, 15% crude fiber and 1% crude protein on a dry weight basis. However, studies by different research workers showed varying results of the composition of sago 'hampas' (Table 4). It can be deduced from literature that sago 'hampas' contains around 65 to 93% starch on dry weight basis, 6 to 16% crude fiber and insignificant amounts of protein and fat. The variation in the values is attributed to the differences in the processing technique, physiology of the sampled palms and variation in assay methods.

Though much effort has been made towards increasing sago palm production, very little work has been done in solid and liquid waste treatment and utilization. Sago

'hampas' having otherwise little use locally, except as dietary fiber supplement for ruminants, is posing environmental and disposal problems.

Table 4. Approximate composition of sago 'hampas' (% dry weight)

Reference	*Apparent starch*	*Crude Fiber*	*Crude Protein*	*Crude Fat*	*Ash*
Lim (1967)	92.5	1.5	1.5	0.5	4.0
Syed Jalaludin *et al.* (1970)	73.6	15.9	3.8	0.3	6.4
Sundhagul (1977)*	66.0	10.1	2.7	0.3	21.0
Cecil *et al.* (1982)					
Coarse 'hampas'	65.0	12.4	0.7	2.8	2.1
Fine 'hampas'	60.0	8.9	0.6	1.3	4.3
Horigome *et al.* (1991)	72.5	13.4	0.6	0.5	4.7
Yahya *et al.* (1992)	77.0	6.3	1.1	0.6	4.5
Shim (1992)	81.7	3.2	0.4	nd	3.0

*calculated from data, nd = not detectable

Not much work has been done on the utilization of 'hampas' as a substrate for enzyme production. Based on the physico-chemical properties of 'hampas', several enzymatic and microbial treatment strategies have been proposed by Shim (1992), Vikineswary and Nadaraj (1992) and Vikineswary *et al.* (1994).

5.0 POSSIBLE UTILIZATION OF SAGO 'HAMPAS'

Development of a suitable method for the utilization of 'hampas' would not only reduce pollution of the waterways, but also contributes to additional income for the industry. The starchy nature of sago processing waste has a resemblance to other

starchy wastes such as from cassava, potato and rice processing. Treatment strategies of sago processing waste, currently lacking but needed, could be adapted from the treatment of other starch processing wastes in use (Chew and Shim, 1993).

The pith of the sago trunk which acts as the starch reserve, closely resembles cassava roots (Table 5). According to Lim (1967) the pith of the sago trunk could possibly be used for human food, preparation of industrial starches and also as animal feed. Bintoro (1995) noted that sago pith residue decomposed faster when mixed with soil, organic matter or inorganic fertilizer. The decomposed sago pith residue could be used as an organic fertilizer to improve crop growth and the residue was found to decrease the negative effect of over dosage of inorganic fertilizer.

Table 5. Comparison of cassava roots and sago pith composition

Component (%)	*Tapioca Root*		*Sago Pith*	
	fresh	*dry matter*	*fresh*	*dry matter*
Moisture	62.4	-	80.0	-
Soluble carbohydrates	35.5	94.4	18.5	92.5
Protein	0.4	1.1	0.3	1.5
Fat	0.2	0.5	0.1	0.5
Fiber	0.8	2.1	0.3	1.5
Ash	0.7	1.9	0.8	4.0

Source : Lim (1967)

Preliminary solid-state and submerged fermentation studies carried out by Shim (1992) and Vikineswary and Nadaraj (1992), respectively showed that 'hampas' is amenable to fungal degradation. Tuen (1994) and Pongsapan *et al.* (1984) proved that sago 'hampas' could be incorporated with commercial feed in the diet of ruminants. This is being practiced in Malaysia and Indonesia by cattle and goat farmers in the vicinity of sago factories.

Other potential use of sago 'hampas' are as glue extender/filler in the adhesive mix used in plywood manufacturing (Paridah, 1992) and for hydrolysis to sugars by enzymes like α-amylase and amyloglucosidase, as described by Kunlal *et al.* (1981). The glucose liberated from the starch can then be used as feedstock for ethanol production as shown by Kim *et al.* (1988 and 1992) on a pilot-scale semi-batch simultaneous saccharification and fermentation process of sago starch using *Zymomonas mobilis*. Ethanol so produced can be used as fuel and for other industrial purposes. Other practical options for the utilization of sago 'hampas' are discussed below.

5.1 Biogas production

Anaerobic digestion is a process which converts biomass or organic matter into biogas containing typically 50 - 65% methane (CH_4) and 35 - 50% carbon dioxide (CO_2) (Bailey and Ollis, 1986). Digestion is carried out by mixed population of

methanogenic bacteria like *Methanobacterium*, *Methanobacillus*, *Methanococcus* and *Methanosarcina*.

In villages throughout India, Indonesia, Thailand, Uganda, Bangladesh, China and Taiwan biogas plants using cow dung as raw material are a common sight. Tanticharoen and Lerttriluck (1986) reported biogas production from tapioca starch wastewater in Thailand. Biogas (methane) produced by anaerobic digestion of cassava solid waste in Indonesia is used for heating, home cooking, lighting and also to run internal combustion engines (Industry Information Report, 1985). Considering the fact that cassava roots closely resembles sago pith, the same could possibly be exploited for sago processing slurries supplemented with animal wastes rich in nitrogenous by-products.

Müller and Trösch (1986) have studied the possibilities of utilizing spent wheat straw biodegraded by the fruiting fungi for production of biogas. Twenty-two basidiomycetes, including *Pleurotus ostreatus* and *Pleurotus ostreatus* var. Florida, were employed to degrade wheat straw in sterile, solid-state cultures. Results showed that biogas yield produced from these "myco-straws" was twice that produced from untreated ones. In another study utilizing spent agro-residues from *P. sajor-caju* cultivation as seed for biogas production, there was an increase in biogas yield which varied from 21.5% in the case of spent bagasse to 38.8% in the case of spent paddy straw compared to untreated ones (Bisaria *et al.*, 1990). Increased susceptibility due

to biodegradation and favorable C/N ratio as a consequence of fungal growth seemed to be the main cause for enhanced biogas production.

This procedure using white rot fungi, firstly causing microbial delignification followed by biogas production, offers complete utilization of a waste product. The useful by-products from these processes are mushrooms for human consumption and methane as a fuel.

5.2 Animal feed

Most of the important domestic animals in South-east Asia are cattle, pigs, poultry, goats and sheep (FAO, 1985) and much of the traditional animal feed resources in the tropics are crop residues which are normally poor in protein but rich in fiber. At the first International Sago Symposium held in 1976, by-product utilization technologies were discussed. It was observed that sago together with other starchy swamp plants showed potential for livestock nutrition, because feed energy (and not protein) is the main limiting factor responsible for optimum performance of all livestock species in the tropics.

Müller (1977) recommended that sago refuse containing more than 12% fiber be excluded from pig and poultry diets and used for ruminants as a forage substitute. In this respect it should be realized that fiber in sago refuse is free from hydrocyanic acid

in contrast to cassava wastes (Kunlal *et al.*, 1981). The content of protein, lipids, minerals and vitamins in sago refuse are insignificant. Müller (1977) also highlighted the limiting amino acids, essential fatty acids, basic minerals and microelements in sago refuse and noted that sago starch has a higher ratio of amylose to amylopectin but rather low level of amylase, which may prove unsuitable for young animals with undeveloped enzymatic system.

Subsequent studies by Yahya *et al.* (1992) supported the suitability of sago pith meal as a potential energy feed for ruminants. Studies by Tuen (1994) and Pongsapan *et al.* (1984) showed that sago 'hampas' could replace up to 45% of the commercial concentrate or native grass in the diet of ruminants without severely affecting feed intake, live weight gain and growth rate.

Various pretreatment and upgrading options may render sago 'hampas' more digestible and palatable. Hydrothermal (steam and hot water) and chemical pretreatment using dilute acids (H_2SO_4), alkalis (NaOH), ammonia, urea, peroxides (H_2O_2) and organosolvents (ethylenediamine) that disrupt both the macro- and the microscopic structures of lignocellulosic materials serve as an option by increasing pore size, solubilizing lignin and/or hemicellulose, and/or increasing surface area (Weil *et al.*, 1994). Müller (1977) recommended ensiling of sago 'hampas' for improving its palatability and also as a means of preserving it.

The process of bioconversion using fungi, would be a potentially good option to improve the utilization of sago 'hampas' and also to produce value-added products such as enzymes and proteins. Biological processing offers many potential advantages over conventional chemical and/or physical processing methods. The advantages are (a) greater substrate and reaction specificity, (b) lower energy requirements, (c) cost effectiveness, (d) lower risk of pollution, (e) higher yields of desired products, and (f) opportunities for transformations not feasible with chemical reagents (Ghosh and Singh, 1993).

Bioconversion has received much attention as it emulates nature where lignocellulosics are recycled on a vast scale by various microorganisms, notably by the lignin-degrading basidiomycete fungi, thus increasing the dry matter digestibility (Zadrazil, 1977, 1980; Reade and McQueen, 1983; Bakshi *et al.*, 1985; Moyson and Verachtert, 1991).

5.3 Production of edible mushroom

It has been reported by many research workers that mushroom cultivation is the most economical means of utilizing lignocellulosic wastes (Bisaria *et al.*, 1987; Zadrazil and Reiniger, 1988). Mushroom cultivation integrates the principles of microbiology, environmental technology, and solid state fermentation in the transformation of waste materials into food for humans. Lignocellulosics can be recycled efficiently by

mushroom cultivation (Figure 3) which offers a practical option for producing food rich in protein and vitamins from wastes.

World-wide production of *Pleurotus* spp. (Oyster mushroom), *Auricularia* spp. (Wood ear), *Tremella fuciformis* (White jelly fungus or "Silver Ear") and *Pholiota nameko* ("Nameko" or Viscid mushroom) has increased at an accelerated rate of 112 - 438% over 1986 - 1990 (Chang and Miles, 1991). The percentage of button mushroom (*Agaricus bisporus*), Shiitake or oak mushroom (*Lentinus edodes*), straw mushroom (*Volvariella volvacea*) and winter mushroom (*Flammulina velutipes*) recorded a 16-43% increase over the same period. This upward trend of world production of the cultivated edible mushrooms could be due to the advances in both basic knowledge and practical technology of 'mushroomology' (Chang and Miles, 1991). Sago 'hampas', an underutilized agro-industrial residue, could be used as a substrate for growing edible mushrooms which are in great demand throughout the world. The high starch content of 'hampas' could serve as a co-substrate for the basidiomycete to derive energy for lignin degradation (Eriksson and Kirk, 1985). Schuiling and co-workers (1993) observed that in Indonesia, a certain mushroom (*Volvariella volvacea*?) grew on 'hampas' that is not discharged directly into the waterways. The growth of mushrooms on 'hampas' indicates the presence of starch in 'hampas' if one goes by the studies of Deinum and Setijoso (1932) who pointed out that "... these mushrooms only thrive on refuse from which the flour has not been

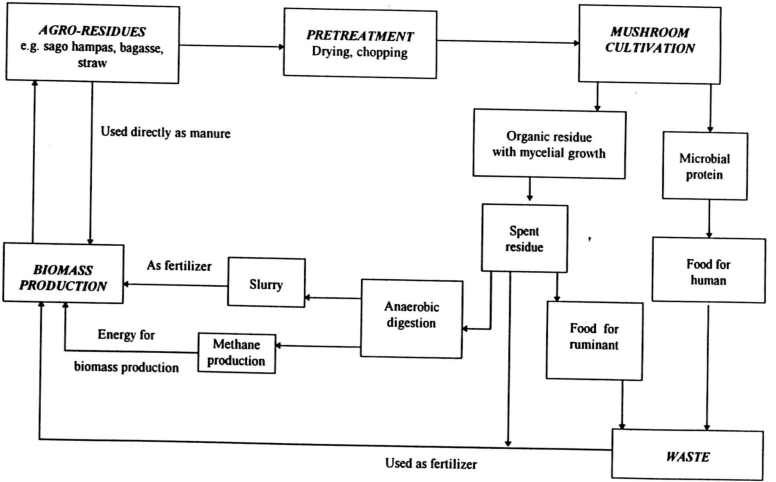

Figure 3. Recycling of agro-residues (Adapted from Madan *et al.* 1987)

completely washed out". These observations show that sago 'hampas' would undoubtedly be a suitable substrate for mushroom cultivation.

5.4 Enzyme production by fermentation

The term "enzyme", literally 'in yeast' was coined by Kühne in 1876 (Gerhartz, 1990). Perhaps the earliest examples of a bioprocess, is the fermentation of sugar to alcohol by yeast. Many enzymes are being produced using microorganisms on an industrial scale using different bioprocesses (Sastry, 1995) and majority are extracellular (Table 6). Industries using enzymes on a commercial scale include brewing, baking, dairy, starch processing, leather, textiles and recently waste treatment (Gacesa and Hubble, 1987). The world market for industrial enzymes in 1988 was approximately US$600 million (sales value). The relationship between product concentration and selling price for a broad range of products classifies enzymes as low volume high value products (Arbige and Pitcher, 1989; Ward, 1989).

The exploitation of microbial enzymes by solid state fermentation dates back to Jokichi Takamine, who in 1894 patented a method of fungal amylase preparation, marketed under the name Takadiastase from *Aspergillus oryzae* (Ward, 1989). The 'koji' method of growing mold on the surface of solid substrate, such as wheat or bran, undoubtedly reflected his insight into similar processes for preparation of oriental

Table 6. Sources and applications of industrial enzymes

Enzyme	*Source*	*Application/function*
Fungal α-amylase [EC. 3.2.1.1]	*Aspergillus niger* *A. oryzae, A. awamori*	Maltogenic saccharification. Limited hydrolysis of starch
Bacterial α-amylase [EC 3.2.1.1]		Starch conversion.
Mesophilic	*Bacillus subtilis* *B. amyloliquefaciens*	Limited hydrolysis of starch
Thermophilic	*B. licheniformis* *B. stearothermophilus*	High-temperature liquefaction of starch
Amyloglucosidase or glucoamylase [EC 3.2.1.3]	*Aspergillus niger*	Starch syrups, dextrose, foods. Saccharification, primarily 1,4-bond degradation
β-Glucanase from fungi	*Aspergillus niger* *Penicillium emersonii*	Brewing and food processing
from bacteria	*Bacillus subtilis*	
Bacterial proteinase		
alkaline[EC 3.4.21.14]	*Bacillus licheniformis*	Detergent enzymes.
neutral [EC 3.4.24.4]	*Bacillus subtilis*	Leather industry, brewing, flavoring.
Cellulases		Breakdown of cellulose to glucose.
Exocellulase or Exobiohydrolase[EC 3.2.1.91]	*Trichoderma* sp., *Aspergillus* sp.	Removes cellobiose from the nonreducing end of glucan chain.
Endocellulase or endoglucanase [EC 3.2.1.4]	*Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp. and bacteria.	Hydrolyzes β-1,4-glucan link in cellulose.
β-Glucosidase or cellobiase [EC 3.2.1.21]	*Aspergillus niger, Trichoderma* sp.	Hydrolysis of terminal non-reducing β-D-glucose residues with release of β-D-glucose.
Invertase or β-Fructosidase [EC 3.2.1.26]	Yeast	Confectionery industry. In inversion products of sucrose - glucose and fructose
Pectinases Pectin transeliminase [EC4.2.2.3] Polygalacturonase [EC 3.2.1.15] Pectin methylesterase[EC3.1.1.11]	*Aspergillus niger*	Processing of fruit and vegetables in making juices, wine production.
Rennet enzymes Chymosin or rennin [EC 3.4.4.3] Pepsin [EC 3.4.4.1]	*Mucor* spp., *Bacillus subtilis*	Dairy industry. Milk coagulant, cheese making
Lipase [EC 3.1.1.3]	*Aspergillus niger, A. oryzae, Rhizopus,* sp. *Mucor* sp., *Pseudomonas* sp.	Dairy and detergent industry, in leather production for fat splitting.
Laccase [EC 1.10.3.2]	*Polyporus versicolor, Myceliophtora thermophyla Pleurotus* sp.	Juice stabilization and oxidation of polyphenols. Color increase in apple juice, wastewater treatment, phenolic removal from must and wine.
Xylanase [EC 3.2.1.8]	*Aspergillus* sp., *Trichoderma* sp.	Hemicellulose hydrolysis and Kraft pulp bleaching

Source: Gerhartz (1990), Arbige and Pitcher (1989), Olsen (1995), Platt *et al.,* (1985)

fermented foods such as soy sauce, sake (rice wine, a traditional alcoholic drink) and miso (a paste used as a breakfast food or a soup base).

Since then, the solid-state method has been used for the production of many enzymes, like cellulase (Muniswaran and Charyulu, 1994; Shamala and Sreekantiah, 1987), amylase (Frost and Moss, 1987), xylanase (Kitpreechavanich *et al.*, 1992) and lipase (Gao and Breuil, 1995). Production of lignin degrading enzymes using solid state fermentation has also been reported (Bonnen *et al.*, 1994; Valmaseda *et al.*, 1991).

Microbial conversion of an abundant supply of lignocellulosic wastes (both from agriculture and forest) has become a subject of considerable interest as a renewable source of materials for fine chemical production, animal feedstuff, waste treatment and pharmaceutical manufacture (Agosin and Odier, 1985; Buchholz *et al.*, 1980; Detroy and Hesseltine, 1978; Schiesser *et al.*, 1992). Lignin, present in most lignocellulosics, acts as an obstacle to microbial utilization of cellulose and hemicellulose in such materials, and may be removed by microbial conversion in solid state fermentation (Agosin and Odier, 1985; Platt *et al.*, 1984; Zadrazil and Brunnert, 1982). Enzyme production through solid state fermentation is often simpler and requires less processing energy than through submerged fermentation (Moo-Young *et al.*, 1983).

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5.4.1 Applications of ligninolytic enzymes

Enzymes are natural biocatalysts that speed up a chemical reaction without being affected in the process and are biodegradable. As they work best in mild conditions, they require less energy than many synthetic chemicals do. This explains why in 1993, Novo Nordisk - producer of biotechnology products sold US$230 million worth of enzymes to the US$25 billion detergent industry (Flynn *et al.*, 1994).

The white-rot fungi, as ligninolytic agents, have been used for biological waste treatment (Kirk and Farrell, 1987) even though the role of ligninolytic enzymes in the oxidation of organopollutants is not fully understood. A review on the biopotentialities of white-rot fungi, which includes *Coriolus, Cyathus, Fomes, Ganoderma, Panus, Pleurotus, Phlebia, Phanerochaete* and *Trametes* as biodegraders of lignocellulosic materials is presented by Ghosh and Singh (1993).

The ligninolytic enzymes, lignin peroxidase (LiP; EC 1.11.1.14) and manganese-dependent peroxidase (MnP; EC 1.11.1.13) are produced by *Phanerochaete chrysosporium* (Kirk and Farrell, 1987). Recently Srinivasan *et al.* (1995) demonstrated that *Phanerochaete chrysosporium* BKM-F1767 did produce extracellular laccase (EC 1.10.3.2) in a defined medium, contrary to the widely held belief that this fungus does not produce laccase (Kirk and Farrell, 1987; Thurston, 1994).

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Lignin peroxidase has not been reported in *Pleurotus* spp., but these fungi produces laccase and Mn-dependent peroxidase (Gutiérrez *et al.*, 1994). In addition, both *Phanerochaete* and *Pleurotus* secrete different extracellular H_2O_2-producing oxidases (Guillén *et al.*, 1992). In particular, the ability of laccase to oxidize a wide range of substituted phenols (Shuttleworth and Bollag, 1986) and to detoxify phenolic pollutants has received much attention (Bollag *et al.*, 1988). The natural function of laccase from white-rot fungi is not known although it seems to have a role in fruiting (Wood, 1980a). It is possible that laccase renders phenolic compounds less toxic through the polymerization reaction. The primary goal of enzymatic treatment systems of wastes would be to decrease chemical consumption/dependence and reduce environmental loading. The recent discovery of these ligninolytic enzymes, that are thought to have significant role in degradation of lignin, has led lignin biodegradation research into practical industrial applications.

5.4.2 Biopulping and biobleaching processes

A possible area of application for ligninolytic enzymes would be in the pulp and paper industry. Kraft pulping (chemical method) is the major pulping method used where about 90% of the lignin is removed from the raw material. Biopulping would eliminate the pollution load associated with chemical pulping processes. Eriksson and Kirk (1985) reported that *Sporotrichum pulverulentum* has been successfully used to pretreat wood chips. Another example of a white-rot fungi, *Phlebia brevispora*, when

inoculated on wood chips, decreased the energy requirements by 47% compared to untreated chips (Boominathan and Reddy, 1992). The use of this fungus for treating chips also increased the tensile strength of pulp.

After Kraft pulping process, the remaining 10% lignin in the pulp normally comprises of various conjugated structures, including quinones and catechols which are responsible for the brown color characteristics of Kraft pulp. Chlorination of the pulp would normally remove most of the lignin and lignin-derived compounds as chlorolignins. Thus, toxic and carcinogenic organic chlorine compounds are released during chlorination or chemical bleaching. Many studies have been carried out to substitute this chlorination step by other less harmful methods (Eriksson, 1989; Eriksson and Kirk, 1985). Biological bleaching of Kraft pulp using white rot fungi (*Phanerochaete chrysosporium* and *Trametes versicolor*) was found to be good for this purpose. Biobleaching not only increased the pulp brightness and reduces the lignin content, but immobilization of these fungi during biobleaching resulted in bleached pulp free from the fungal mycelium (Kirkpatrick *et al.*, 1989).

Faced with market, environmental and legislative pressures, the pulp and paper industry is compelled to incorporate changes in its pulping, bleaching and effluent treatment methods in order to minimize the environmental impact of mill effluents. Xylanases, a hemicellulolytic enzyme, is found to substantially reduce chlorine usage. Although the xylanolytic mechanism in pulp bleaching is still not clear, it appears that a

partial hydrolysis of xylan improves the susceptibility of lignin to removal by the subsequent chlorination and alkali extraction stages. Viikari *et al.* (1990), Buchert *et al.* (1994) and Bajpai *et al.* (1994) reported that the direct use of cellulase-free hemicellulases, mainly endo-β-xylanases for bleaching of Kraft pulp, rendered the structure of the fibers more permeable, and allowed enhanced extraction of residual lignin from the fibers. They also reported large-scale application of xylanase for improving bleachability in more than 10 pulp mills throughout Europe since 1992. The totally chlorine free bleaching sequences in use is based on bleaching of oxygen-delignified pulps with enzymes and hydrogen peroxide. Enzyme-aided bleaching is thus both environmentally and economically advantageous.

5.4.3 Detoxification of phenolic pollutants

Phenolic pollutants are produced by many industries. Although chlorophenols, cresols and other aromatic compounds are biodegradable (Steiert and Crawford, 1985), biological transformation and chemical reactions can also alter the toxicity of some of these compounds. Apart from that, the color of phenolic wastes is not effectively removed by conventional treatment methods such as aerated lagoons and activated sludge plants. Physical and chemical treatment techniques are also ineffective. Techniques like ultrafiltration and ion exchange are expensive. Therefore, alternative biotreatment processes are needed (Eriksson and Kirk, 1985).

Successful decolorization of polymeric dye containing wastewaters (Platt *et al.*, 1985) and phenolic effluents (Davis and Burns, 1990) by *Pleurotus ostreatus* sp. '*florida*' and by both soluble and immobilized laccase was reported. The researchers indicated that the color removal is possibly linked to laccase synthesis and reflects one of the many complex processes in lignin degradation.

Sago 'hampas', being a starchy fibrous lignocellulosic material, has been shown to be degraded by *Myceliophthora thermophila* and *Trichoderma harzianum* (Shim, 1992; Vikineswary and Nadaraj, 1992). They reported the presence of appreciable amounts of cellulase, α-amylase and xylanase during the growth of those organisms and proposed that sago 'hampas' can be used as a cheap substrate for enzyme production. Further studies were found to be necessary on optimization of enzyme production using sago 'hampas' as substrate probably with an edible fungi such as *Pleurotus* sp. which would upgrade the nutritive value of the spent substrate and which can be used as a feed for ruminants after enzyme extraction through simple solid substrate fermentation technique.

6.0 SOLID SUBSTRATE FERMENTATION FOR ENZYME PRODUCTION

Solid state cultivation is characterized by the growth of microorganisms on water insoluble substrates in the absence of free water, while solid substrate fermentation (SSF) is characterized by the growth of microorganisms on solid substrates in the

presence of free water (Doelle, 1994). In literature, the two terms, solid-state and solid-substrate fermentation have been loosely used to refer to the same process, as one in which the fermentation medium (substrate) is 'solid-like and water insoluble', i.e. is relatively low in water compared with the more standard commercial liquid fermentation. The distinction between a solid state cultivation and a solid substrate fermentation is difficult to define precisely as free water content of the solids varies widely (Doelle, 1994), e.g. free water in maple bark is 40% as moisture, in sago 'hampas' is 90% as moisture, etc.

During the past several decades, research for the production of microbial metabolites in industrial fermentation technology has largely been focused on submerged fermentation. Until recently, traditional SSF has not generated much interest, even though it was used from a long time in oriental food fermentations, production of yeast-leavened bread, mold-ripened cheese and composting of solid waste (Cannel and Moo-Young, 1980).

During the period of 1940 - 80's, the neglect of SSF was not only caused by the popularity of the submerged culture process, but also because of the difficulties associated with the quantification of parameters such as microbial biomass, substrate consumption, concentration of end-products formed in SSF. Measurement of fungal growth rate in solid culture where the fungal mycelia are intricately interwoven with the substrate, is more difficult than in liquid culture (Moo-Young *et al.*, 1983).

However, in the past 10 to 15 years, there has been a resurgence of interest in research in SSF, as demonstrated by the production of unconventional proteins (Chahal, 1982), enzymes from lignocellulosic wastes (Platt *et al.*, 1984) and by the production of ethanol from cassava processing residue using SSF (Kunlal *et al.*, 1981). This renewed interest in SSF was due to the low manufacturing costs by utilizing unprocessed or partially processed raw materials. In addition, the simpler SSF requires lower pre-processing energy compared to submerged fermentation. However, the barriers imposed by the solid nature of the fermenting mass makes SSF usually slower in nature (Cannel and Moo-Young, 1980; Aidoo *et al.*, 1982). However, for utilizing lignocellulosic agroindustrial wastes, SSF has numerous advantages over submerged fermentation (Table 7) in addition to producing a concentrated solutions of enzyme (Macris and Kekos, 1989). Prasertsan and Oi (1992) showed that oil palm cake and fiber were suitable as substrates in SSF using *Aspergillus niger* ATCC 6275 for the production of carboxymethyl-cellulase, xylanase and β-glucosidase.

Traditionally, most of SSF's are carried out in stationary shallow trays (Fig. 4) where the substrate with inoculum is evenly distributed in 5 - 15 cm deep layers. A few examples of the commonly used reactor types are the stationary trays and the tunnel fermenters which are basically batch systems, whereas the paddle fermenter, rotary drum and tower type systems are ideal for either batch or for continuous operation (Ward, 1989; Weiland, 1988).

Table 7. Comparison between solid substrate fermentation (SSF) and submerged fermentation (SMF)

Characteristics	*SSF*	*SMF*
Microorganism and substrate	Generally static	Agitated
Water usage	Limited	Unlimited
Oxygen supply	Diffusion	Aeration
Substrate	Simple with minimal addition of nutrients	Complex and rich in nutrients
Sterilization of substrate	May be unnecessary	Necessary
Liquid waste produced	Negligible	Significant volume
Physical energy	Low	High
Human energy	High	Low
Capital investment	Low	High
Process control	Not rigorous	Rigorous
Space requirement	Less	Large
Productivity	High	Low
Solid waste produced	High	Nil
Volume of fermentation mash	Smaller	Larger

Source: adapted from Huang *et al.*, (1985)

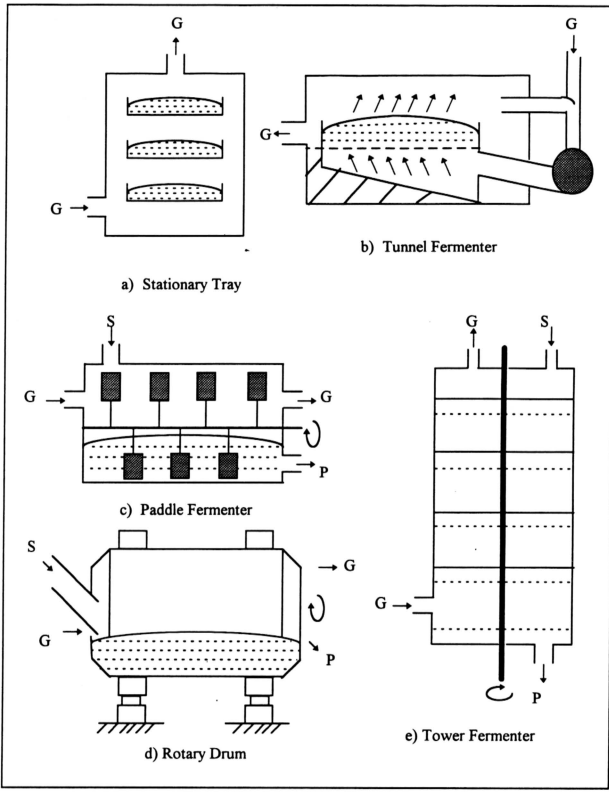

Figure 4. Examples of reactor types for solid substrate fermentation.
Source: Weiland (1988)

With the many advantages of SSF and lower overheads, the choice of a suitable microorganism becomes crucial for bioconversion of lignocellulosic materials. The white-rot fungi and related basidiomycete fungi are probably the most 'ideal microorganisms' capable of utilizing most of the polymers in lignocellulosic residues (Kirk, 1983). They are known to excrete a range of hydrolytic and oxidizing enzymes into the lignocellulosic substrate to depolymerize the lignocellulose polymers which can be assimilated by the fungi (Wood *et al.*, 1988).

7.0 *Pleurotus* sp.

White rot fungi, the most extensive biodegrader can degrade lignocellulosic materials in two distinctive ways: (a) the simultaneous degradation of cellulose, hemicellulose and lignin; and (b) the selective degradation of lignin and hemicellulose.

Pleurotus sp. (Fr.) Quél., order Agaricales (Singer, 1986) is one of the most potent biolignolytic organisms that possesses characteristic features of rapid growth and extensive degradation of lignin than the other plant substrate components resulting in increased *in vitro* digestibility of the fermented substrate (Zadrazil, 1984). *Pleurotus* spp. is of increasing economic interest, accounting for approximately 7% of the total world production of edible mushrooms (Chang and Miles, 1991).

Pleurotus sp., a type of oyster mushroom has been reported to be suitable for upgrading the nutritive value of various agricultural by-products including sugar cane bagasse (Ortega *et al.*, 1992, 1993), wheat straw (Calzada *et al.*, 1987; Zadrazil, 1977), cotton waste (Chang *et al.*, 1981), rubber wood sawdust (Lee, 1992), cocoa shell waste (Pettipher, 1987) and rice straw (Rajarathnam *et al.*, 1987).

The spent residue left after mushroom cultivation has been reported to be free of toxic compounds and has a good savory taste (Chang, 1980). In a study on the suitability of spent rice straw after *Pleurotus sajor-caju* cultivation, for cattle feed, it was found that spent straw did not contain any aflatoxin B_1, B_2, G_1, G_2, ochratoxin A, B, C, sterigmatocystin, zearolenone, patulin, penicillic acid or citrinin (Bano *et al.*, 1986). They also found that feeding the spent *P. sajor-caju* straw to either albino rats or mice did not cause any toxic hazard as confirmed by histopathological examinations.

The gray oyster mushroom, *Pleurotus sajor-caju* has many added advantages. It is easy to culture, has a very high saprophytic colonizing ability, grows at ambient room temperatures of 19° to 30°C and can colonize on sterilized, pasteurized (60° to 90°C), fermented (55°C, 120 days) and unsterilized plant residues (Bisaria, *et al.*, 1987; Chang *et al.*, 1981; Singh, 1983).

The ability of this fungus to grow on a wide range of natural lignocellulosic wastes is mainly due to their ability to excrete hydrolyzing and oxidizing enzymes (Bano *et al.*,

1993; Rajarathnam *et al.*, 1987). It was demonstrated that *Pleurotus* spp. grown in SSF on lignocellulosic substrates produced cellulase (Madan and Bisaria, 1983), xylanase (Madan and Bisaria, 1983; Valmaseda *et al.*, 1991), phenol oxidase (Bollag and Leonowicz, 1984) and peroxidase (Platt *et al.*, 1984). Madan and Bisaria (1983) studied, endoglucanase, FPase, β-glucosidase and xylanase activities of *P. sajor-caju* during SSF of rice straw. There are indications that the ligninolytic system of *Pleurotus* spp., being inadequately defined, is different from that of other white rot fungi (Kimura *et al.*, 1990; Waldner *et al.*, 1988). The production of enzymes; laccase (polyphenol oxidase), Mn-peroxidase, H_2O_2-producing enzymes and ligninases, which have been postulated to have a role in lignin degradation by the white rot fungi has intensified research towards the breakdown of this complex molecule and also utilization of lignocellulosic residues for commercial exploitation.

8.0 LIGNOCELLULOSICS AND THEIR EXPLOITATION

The term 'lignocellulose' implies materials that are comprised of mainly cellulose, hemicellulose and lignin. Biopolymers (termed biomass in aggregate) of carbohydrate and polyphenolic nature represent the largest reservoirs of organic carbon fixed in plants. They are available for exploitation as potential sources of food, fuel and chemical feedstocks (Grohmann and Himmel, 1991).

Enzymatic conversion of this large reservoir of raw materials would play an increasingly important role because enzymes achieve relatively high catalytic activities and high selectivity. They are non-hazardous and therefore, have a low impact on the environment (Kresse, 1995). The knowledge of the various degradative enzymes secreted by basidiomycetes during growth on lignocellulosic residues can provide information that permits more effective use or improvement of the microbial systems (Leatham *et al.*, 1991). For example, the edible mushroom, *Pleurotus* spp. cultured on lignocellulosic substrates in SSF was found to secrete a range of important degradative enzymes such as cellulases, xylanases, phenol oxidase and peroxidase (Bollag and Leonowicz, 1984; Madan and Bisaria, 1983; Platt *et al.*, 1984) acting to depolymerize the lignocellulose polymers into compounds of lower molecular weight which can be assimilated by the fungus.

The production rates of these enzymes closely correlate fungal growth and colonization. They are also subject to a variety of regulatory controls including catabolite repression, induction and feed back inhibition and inactivation (Wood *et al.*, 1988). The mode of action of the various hydrolytic and oxidative enzymes produced by basidiomycetes fungi are tabulated in Table 8.

Table 8. Mode of action of extracellular enzymes of basidiomycetes in lignocellulose degradation

Enzyme	*Reactions*
Laccase, Phenol oxidase	Oxidizes *o*- and *p*-phenols and aromatic amines to quinones.
Ligninase, Lignin peroxidase	Cα-Cβ cleavage of propyl side chains of lignin, lignin model compounds, partially depolymerize lignin.
Endocellulase, 1,4-β-D-glucan-4-glucanohydrolase	Cleavage of internal 1,4 β-D glycosidic bonds in cellulose chains.
β-glucosidase, glucohydrolase	Cellobiose hydrolysis to glucose units. Glucose removal from non-reducing ends of cellulose chains.
Xylanase, Endo-1,4 β-xylanase	Cleavage of internal 1,4-β-D glycosidic bonds in xylans.
Xylosidase, β-xylosidase	Xylobiose hydrolysis to xylose units. Xylose removal from non-reducing ends of xylan chains.

Source: Wood *et al.* (1988)

9.0 CELLULOSE DEGRADING ENZYMES

Cellulose forms the basic structural material of the cell walls of all higher plants. The main characteristics of cellulose are its great strength, fibrous nature, insolubility, and inertness. Cellulose is basically composed of long, linear chains of β-1,4 linked glucose units (Wood, 1989).

Cellulases are enzyme complexes, which in stepwise, breakdown native cellulose or derivatives of cellulose to glucose. According to Wood (1989) and Gerhartz (1990), cellulose depolymerization needs a synergistic reaction of three hydrolytic enzymes, and the first attack is simultaneously effected by at least two of them. The three enzymes involved in cellulose breakdown are (a) exocellulase or exobiohydrolase (EC 3.2.1.91), (b) endocellulase or endoglucanase (EC 3.2.1.4) and (c) β-glucosidase or cellobiase (EC 3.2.1.21).

The primary step in cellulose hydrolysis is the degradation of some amorphous regions in the cellulose fibers by endoglucanase. New free end chains of cellulose are produced which then become the substrate for exocellulase (also called C_1 *activity*). This enzyme removes cellobiose from the non-reducing end of the polyglucan chain. By this way, the substrate for the action of endoglucanase (often called C_x *activity*) is formed. The third enzyme, β-glucosidase hydrolyses cellobiose released during this sequence of reactions. This step is crucial because the accumulation of cellobiose inhibits cellulose degradation by the other enzymes. C_x cellulase degrades soluble fragments or derivatives of cellulose (carboxymethyl cellulose or hydroxyethyl cellulose), forming cellobiose. It is reported that the effect of enzymatic hydrolysis of cellulose depends very much on the pretreatment (temperature, alkaline oxidation and mechanical degradation) of the substrate (Olsen, 1995).

10.0 XYLAN DEGRADING ENZYMES

Xylan is the major hemicellulose component, ranking second to cellulose in nature (Bastawde, 1992). Unlike cellulose, xylan is a complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds (Gilbert and Hazlewood, 1993).

Due to the heterogeneity of xylans, microbial enzymes act cooperatively to convert xylan to simpler sugars. These enzymes include β-1,4-endoxylanases (xylanases : EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55) which hydrolyses arobinose side chains; α-glucoronidase which removes glucoronic acid side-chains from the xylose units; xylan esterases (EC 3.1.1.6) which release acetate groups and β-xylosidase (xylobiase : EC 3.2.1.37) which hydrolyses xylobiose to xylose (Wong *et al.*, 1988; Poutanen *et al.*, 1991). The presence of xylan degrading enzymes has been found to expose more surface area of cellulose for the action of cellulase enzymes (Shamala and Sreekantiah, 1986) and hence both of the groups of enzymes behave in considerable synergy.

Most fungal species produce xylanase as well as cellulase complex during growth on pure cellulose or agricultural residues as the primary carbon sources (Ryu and Mandels, 1980; Wood, 1971). Xylanases have potential applications mainly in the

pulp and paper industries (Viikari *et al.*, 1991) and agricultural- and forestry-residue utilization.

11.0 LIGNIN DEGRADING ENZYMES

The white-rot fungi decomposes almost all components of the wood, including lignin (Schubert, 1965). The processes involved in lignin degradation revolves around three main classes of ligninolytic enzymes, i.e. lignin peroxidase, manganese-dependent peroxidase and laccase (Higuchi, 1993; Kirk and Farrell, 1987). Two or more of these three families of enzymes are quite widely distributed in the Basidiomycetes that cause white-rot decay of wood (Coll *et al.*, 1993; Kantelinen *et al.*, 1989; Maltseva *et al.*, 1991). Recently, laccase and other lignocellulose modifying enzymes have been reported in marine fungi (Ascomycetes) which are often found on decaying branches, leaves and driftwood (Raghukumar *et al.*, 1994).

There is considerable information available on the ligninolytic activities of selected fungi, particularly *Phanerochaete chrysosporium* (Kirk and Farrell, 1987), which appear in response to starvation of the fungi for nutrients such as nitrogen and carbon.

The blue copper oxidase (laccase) catalyzes the one-electron oxidation of phenols to phenoxy radicals, eventually transferring four electrons to O_2 (Reinhammar, 1984). Laccase partially depolymerizes methylated lignin and also causes cleavage between

the α- and β-carbons of aromatic side chains (Tien and Kirk, 1983). Laccase activity of fungi is, therefore, considered to be of equal importance for selective delignification and lignin transformations as the generalized ligninase-type reactions.

Laccase is a type of copper-containing polyphenol oxidase discovered in 1883 (Yoshida, 1883) in the exudates of the Japanese lacquer tree *Rhus succedanea* and *Rhus vernicifera*. A few years later, Bertrand (1896) obtained an enzyme from the Indo-Chinese lacquer tree which he called 'laccase' and subsequently it was demonstrated that it is present in many fungi and plants, including potatoes, beets, apples, cabbages and several mushroom fungi. In the basidiomycete fungi, it is claimed that laccase has a part in the enzymatic machinery capable of mineralizing lignin. Recent studies by De Jong *et al.* (1992) and Peláez *et al.* (1995) on white rot fungi showed that manganese peroxidase in combination with either laccase or lignin peroxidase may be the necessary (minimum) complement for lignin degradation.

12.0 INDUCTION OF LACCASE

Research on ligninolytic microorganisms and their enzymes, particularly laccase, was intensified in recent years because of its potential industrial applications in detoxification of phenolic pollutants (Bollag and Leonowicz, 1984; Bollag *et al.*, 1988). Both inducible and constitutive laccases have been reported from various fungi, including *Pleurotus* (Bollag and Leonowicz, 1984; Leonowicz and Trojanowski,

1975a, 1975b, 1978). Fåhraeus *et al.* (1958) studying the influence of various culture conditions for laccase production found that early addition of the inducer may retard mycelial growth and too late an addition could stop enzyme production at a low level owing to exhaustion of necessary energy-yielding or building materials. Gigi *et al.* (1980) reported that the inducing effect became operative at zero time and later addition of inducers did not cause an increase in laccase production.

Various phenolics have been used successfully to induce the production of laccase. Exolaccases of *Pleurotus* sp. have been induced by ferulic acid (Leonowicz *et al.*, 1972; Leonowicz and Trojanowski, 1975a) and 2,5-xylidine (Fåhraeus and Reinhammar, 1967; Bollag and Leonowicz, 1984). Laccases from other white rot fungi, *Botrytis cinerea*, *Neurospora crassa* and *Polyporus versicolor* have been induced by gallic acid (Gigi *et al.* 1980, 1981), cycloheximide (Froehner and Eriksson, 1974) and 2,5-xylidine (Fåhraeus and Reinhammar, 1967), respectively. Generally, induction for laccase excretion was better studied in defined liquid media.

13.0 PURIFICATION OF LACCASE

Several purification methods for laccase have been described which varied according to the enzyme source and the degree of purity to be attained. The purification methods for the copper containing blue oxidases have been reviewed (Malmström *et al.*, 1975) and most often they included precipitation with ammonium sulfate

$(NH_4)_2SO_4$ of different concentration, gel chromatography on Sephadex G-100, and ion exchange chromatography on the anion exchangers DEAE-cellulose, or combinations of some of these methods, together with dialysis to remove low molecular impurities. Ultracentrifugation, ultrafiltration and electrophoresis have also been shown to produce high degree of homogeneous laccase protein (Gigi *et al.* 1980, 1981; Leatham and Stahmann, 1981; Wood, 1980b).

Although the purification steps may lead to laccase concentrations, this enzyme may show considerable heterogeneity after purification. This is an inevitable consequence of the extracellular location of these proteins. A typical purified laccase may contain 15 to 20% carbohydrate with a molecular weight of 60 to 80 kDa and the copper content may vary between two and four atoms per enzyme molecule (subunit) (Thurston, 1994).

In the natural state, Basidiomycetes thrive on wood or plant litter and come in contact with various phenols of plant origin. Fungal laccase, can oxidize these compounds present at low concentrations. However, oxidation of phenolics at higher concentrations may require additional biosynthesis of a more active form of the enzyme such as the inducible form.

Thus, the production of laccase as a value-added product through fermentation using edible fungi may enhance the possible function of this enzyme in the detoxification of phenolic pollutants. A cheap and suitable organic waste such as 'hampas' as a substrate for laccase production may later render it suitable for animal feed supplement or for mushroom cultivation, after enzyme harvesting.