Chapter 2

Literature Review

2.1 Leachate generation and its characteristics

2.1.1 Leachate generation

Landfill leachate is high strength wastewater which contains high concentration of organic matter and ammonium nitrogen. An average fresh domestic refuse leachate can have a BOD around 15,000 mg/l. When compare to an average raw sewage BOD of 200 mg/l, it can be seen that landfill leachate is around 75 times as stronger in terms of its polluting potential (Wichitsathian, 2004).

Leachate is formed when water passes through the waste in the landfill site. The main contributor to generation of leachate can be from precipitation, rain runoff of surface drainage, groundwater percolating through the level of solid waste and extracting the dissolved and suspended materials from waste. As the liquid moves through the landfill, many organic and inorganic compounds such as heavy metals are transported in the leachate. This moves to the landfill base and collects. In addition to the leachate generation induced by precipitation, it is also produced as a result of biochemical processes that convert solid materials to liquid forms. The leachate generated by biochemical processes is characterized by high concentrations of organic and inorganic constituents. Figure 2.1 depicts a typical engineered landfill.

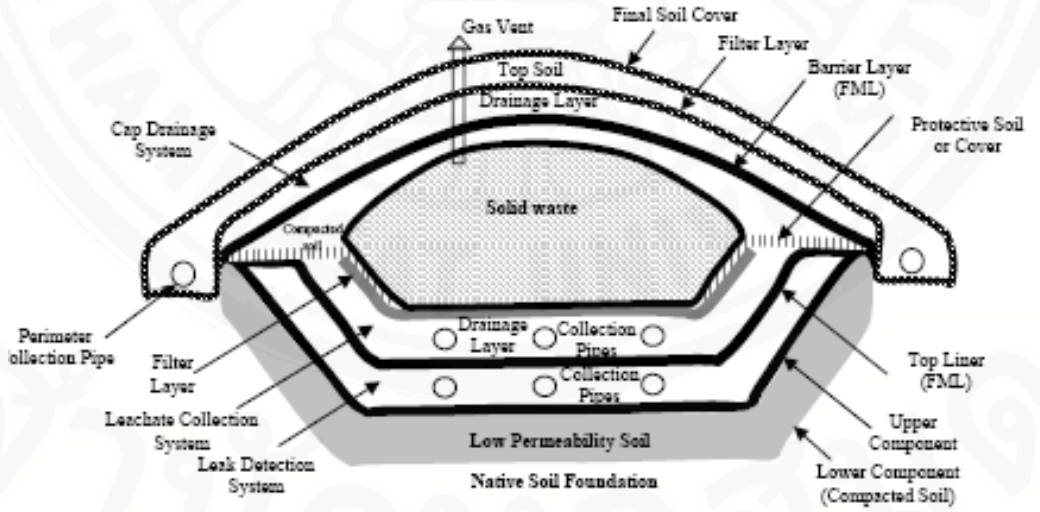

Figure 2.1 The schematic representation of a typical engineered landfill (Wichitsathian, 2004)

2.1.2 Leachate characteristics

Depending on the age and the biochemical reaction in the landfill, the characteristics of leachate varies significantly consisting of organics, ammonia-nitrogen, heavy metals and

dissolved solid to form high chemical oxygen demand (COD), nitrogen and color intensity. The leachate takes up organic and inorganic constituents by means of physical, hydrolytic and fermentative processes, thus, it contains a high concentration of organic matters and inorganic ions, including heavy metals. The presence of humic substance in leachate might enhance the transportation of heavy metals. The color of leachate from landfills appeared as dark color, while some had a light brown color. Thus, leachate is a potential hazardous waste from landfill sites. If not dealt properly, they can cause pollution to groundwater, health problem and effect environment.

The leachate quantity and quality is site specific. The major factors affecting the leachate quantity and quality can be categorized into four major divisions namely:

- Waste (type of waste, degree of decomposition, and possible seasonal variance),
- Landfill environment (phase of degradation, humidity, temperature etc.),
- Filling technique (compacting, cover, height of landfill layers), and
- Sampling (point of sampling and method of analysis).

The presence of moisture is necessary for the biological conversions within the landfill. In warmer climates, the increase in leachate production after precipitation is rapid (Lema, et. al., 1988) due to rainfall exceeding the amount which can be effectively evaporated during winter or rainy seasons.

Three types of leachates can be defined according to landfill age; young, intermediate and stabilized and characteristics of leachate vary with the age (Table 2.1). Landfill leachate from old sites is usually highly contaminated with ammonia resulting from the hydrolysis and fermentation of nitrogen containing fractions of biodegradable refuse substrates (Carley and Mavinic, 1991).

Table 2.1 Relations between landfill age, leachate characteristics and efficiency of systems

Type of leachate	**Young**	**Intermediate**	**Stabilized**
Age of landfill (years)	<5	5-10	>10
pH	<6.5	6.5–7.5	>7.5
BOD/COD	<0.5	0.1–0.5	<0.1
COD (mg/L)	>10,000	<10,000	<5,000
COD/TOC	<2.7	2.0-2.7	>2
VFA (%TOC)	>70	5-30	<5
Process	**Treatment efficiency**		
Biological treatment	Good	Fair	Poor
Chemical oxidation	Fair-poor	Fair	Fair
Chemical precipitation	Fair-poor	Fair	Poor
Activated carbon	Fair-poor	Good-fair	Good
Coagulation-flocculation	Fair-poor	Good-fair	Good
Reverse osmosis	Fair	Good	Good

(Source: Amokrane et. al., 1997)

The change of BOD/COD ratios of leachate depends greatly on the age of landfill (Kylefors, 1997). During the initial stages, the landfill is aerobic rich in biodegradable

organic content. As the landfill age increases, the microorganism present in the landfill tend to degrade these organic compounds into inorganic components. When anaerobic phase begins, the COD starts increasing causing a decrease in BOD/COD ratio (Table 2.2). This decrease observed suggests the change in biodegradability of the leachate with time. For young landfill, the ratio is around 0.5-1.0 while it reaches almost 0.1 in the stabilized landfill. The reason for low biodegradability in the stabilized landfill could be due to the presence of humic and fluvic acids.

Table 2.2 Variation of COD, BOD and BOD/COD with increasing landfill ages

Age (year)	1	2	3	4	5	6	7	8	9	10	11
BOD (mg/l)	25,000	10,000	290	260	240	210	190	160	130	100	80
COD (mg/l)	35,000	16,000	1,850	1,500	1,400	1,200	1,200	1,150	1,100	1,050	1,000
BOD/COD	0.71	0.60	0.17	0.17	0.16	0.16	0.14	0.13	0.10	0.08	0.08

(Source: Ragle, 1995)

Nitrogen concentration is another indicator which signifies the age of landfill leachate as presented in Table 2.3. The ammonia concentration in leachate is high due to hydrolysis, decomposition and fermentation of biological substrate. In the first few years, the ammonia concentration tends to increase slightly over time and then decreased as the landfill age increases. The ammonium concentration in the leachate also varies with the landfill age.

Table 2.3 Nitrogen concentrations from various sources

Sample	Age (year)	NH_3-N (mg/l)	Organic-N (mg/l)	NO_3-N (mg/l)
Sewage[1]	-	15	10	0
Young leachate[1]	1	1,000-2,000	500-1,000	0
Pillar point (Hong Kong)	6	2,563	197	2.5
Ma Yau Tong (Hong Kong)[2]	10	1,156	24	1.1
Several sites (Germany)[1]	12	1,100	-	-
Du Page Co. (Illinois)[1]	15	860	-	-
Rainham (UK)[1]	24	17	-	-
Waterloo (Canada)[1]	35	12	-	-

(Source: [1]McBean, et.al., 1995. [2]Robinson and Luo, 1991)

The differences in leachate quality can be due to seasonal variation. Table 2.4 shows the physiochemical characteristics of wet and dry season of landfill leachate in Nigeria. Conductivity, total solids, suspended solids, total dissolved solids, alkalinity, sulfate, DO, BOD, and COD are higher in wet season. While in dry season, the color and turbidity are higher. The reason may due to no dilution from precipitation in rainy season.

Table 2.4 Physiochemical characteristics of wet, dry leachate samples of landfill in Nigeria

Parameters	Wet Season	Dry season
Temperature (°C)	25.66	25.76
pH	8.28	8.03
Color (CU)	423.60	434.71
Turbidity (FTU)	83.40	139.0
Conductivity (μS/cm)	5562	4,807
Total Solids (mg/l)	4,819.6	3,883.43
SS (mg/l)	213.60	148.71
TDS (mg/l)	4,606	3,735
Alkalinity (mg/l)	2,208.40	1,421.43
Chloride (mg/l)	1,606	1,271.29
Sulfate (mg/l)	111.18	65.33
DO (mg/l)	2.09	1.87
BOD (mg/l)	990.60	675.57
COD (mg/l)	3,066.6	2,802.14

(Source: Oyoh et al., 2008)

Table 2.5 shows the typical leachate quality from large landfills worldwide. Among the leachate from Hong Kong, Southeast Asia, South Africa and UK, Southeast Asia has the highest COD, TOC, Chloride, alkalinity, conductivity, sulfate, magnesium, potassium, calcium, chromium, manganese, copper and cadmium. In UK, it has the highest BOD, sodium, iron, and nickel. Hong Kong has the highest nitrate and nitrite and phosphate. For South Africa, arsenic and mercury are found to be high.

Table 2.5 Typical leachate quality data from large landfills worldwide

Determinant	Units	Hong Kong	South-east Asia	South Africa	UK
pH	–	7.4–8.6	6.0–8.4	7.5–8.3	7.4–8.5
COD	mg/l	650–2,800	1,600–13,000	1,400–6,000	2,600–8,500
BOD_5	mg/l	45–400	–	300–700	90–3,000
Total organic carbon (TOC)	mg/l	–	400–10,000	–	400–3,400
Ammoniacal-N	mg/l	1,200–3,000	1,200–3,000	900–3,000	1,100–2,500
Chloride	mg/l	500–3,000	2,500–6,300	1,200–4,000	1,700–5,200
Alkalinity (as $CaCO_3$)	mg/l	3,000–12,000	8,000–40,000	3,000–12,000	7,000–17,000
Conductivity	µS/cm	14,000–30,000	14,000–42,000	10,000–30,000	14,000–30,000
Nitrate-N	mg/l	< 0.1–22	< 1–12	< 0.1–1.3	< 0.3–2
Nitrite-N	mg/l	< 0.1–3.6	< 1 – < 2	< 0.1–1.4	< 0.1–2
Sulphate (as SO_4)	mg/l	–	< 5–1,200	< 0.6–400	< 5–150
Phosphate (as P)	mg/l	3–125	2–50	1–25	4–20
Sodium	mg/l	200–2,100	1,500–3,000	800–2,500	1,700–4,000
Magnesium	mg/l	18–50	60–500	75–400	17–150
Potassium	mg/l	375–1,200	1000–3,000	550–1,600	750–1,700
Calcium	mg/l	20–45	50–3,000	50–200	40–420
Chromium	µg/l	–	200–2,500	80–300	40–2,200
Manganese	µg/l	–	250–17,500	12–900	70–2,200
Iron	µg/l	5,000–9,000	1,000–20,000	2500–20,000	3000–72,000
Nickel	µg/l	–	400–1,500	80–120	150–3,000
Copper	µg/l	–	< 50–400	< 50	20–80
Zinc	µg/l	200–2,200	150–1,500	30–200	30–330
Cadmium	µg/l	–	< 50	< 1 – < 10	< 10–20
Lead	µg/l	–	< 300 – <1,000	< 4–56	< 40–600
Arsenic	µg/l	–	–	7.5–62	2–24
Mercury	µg/l	–	–	< 50	< 0.1

* The < symbol means that in a number of cases the determinant was not detectable above the limit of detection for a given analytical technique (Source: Oo et.al, 2003)

Table 2.6 provides comparison of leachate characteristics of landfills surveyed in Asia, Europe and America. It is shown that the pH of the leachate in Thailand, Malaysia, Hong Kong and Europe are slightly alkaline while in USA is slightly acidic. Chloride is found to be high in Pathumthani landfill site (1,220-5,545 mg/l) while in USA, the lowest one is 70 mg/l. USA has the lowest total solids while On-Nuch landfill site has the highest total solids in Thailand but this value is still much less than in Malaysia. Phitsanulok landfill site has the highest suspended solids (1,950 mg/l), COD (4,900-11,000 mg/l) but this is still much less than COD in the new landfill site in USA (10,000-40,000 mg/l). BOD is found to be high in Nakhonpathom (3,000-7,150 mg/l) while in USA and Europe, COD is much higher than in Thailand (28,000 and 90,000 mg/l, respectively).

TKN is high in Europe (5,000 mg/l) while in Thailand the highest one is about 1,260 mg/l). Ammonia is high in new Hong Kong landfill site (2,700 mg/l) while the highest one in Thailand is about 1,250 mg/l). The highest Nickel in Thailand is found at Phitsanulok landfill site (1.56 mg/l) while this is still lower than in Europe (2.05 mg/l). Cadmium is not

the problem in Thailand and USA while in Air Hitam, Malaysia, and Europe is found to be as high as 0.23 and 0.14 mg/l, respectively. Pb in On-Nuch landfill site is as high as 0.52 mg/l but still less than Pb in Europe (1.02 mg/l) and Malaysia (5.37 mg/l in Air Hitamand and 3.45 mg/l in Taman Beringin). Chromium is as high as 2.9 mg/l in Nakhonpathom landfill site while in Malaysia, Europe and USA. Mercury is found to be high (1.7 mg/l in Phitsanulok landfill site compared to Malaysia, Europe and USA.

However, these leachate characteristics of each landfill are site specific and depend on many factors such as type of waste, degree of decomposition, and possible seasonal variance, humidity, temperature, etc.

Table 2.6 Comparison of leachate characteristics of landfills surveyed in Asia, Europe and America

Parameter	Thailand[1,2]					Malaysia[3]			Hong Kong[4]		USA[5]			Europe[6]
	Phitsanulok	Pathumthani	Nakhonpathom	Pathumthani	On-Nuch	Air Hitam	Sabak Bernam	Taman Beringin						
Years in operation	1	3	4	9	20	5	7	16	6	10	1	5	16	-
Alkalinity	300-4,700	918-4,250	960-2,740	6,620	-	1,540-9,000	1,200-1,550	3,750-9,375	10,700-11,700	3,230-4,940	800-4,000	5,810	2,250	300-11,500
pH	7.1-8.3	8.2-8.9	8.2-8.5	8.1	7.5	7.6-8.8	8.0-8.1	7.8-8.7	8.1-8.6	7.6-8.1	5.2-6.4	6.3	-	5.3-8.5
Chloride	-	1,220-5,545	655-2,200	2,530	-	1,625-3,200	420-1,820	875-2,875	2,320-2,740	522-853	600-800	1,330	70	-
SS	1,950	29-110	8.4-1.7	12.5	488	410-1,250	111-920	420-1,150	40-53	3-124	-	-	-	-
TS	6,700	350-1,598	274-1,200	848	11,320	13,930-15,380	-	10,300-13,680	-	-	100-700	-	-	-
COD	4,900-11,000	1,488-3,200	800-3,575	3,200	1,200	1,724-7,038	1,250-2,570	1,960-5,500	2,460-2,830	641-873	10,000-40,000	8,000	400	150-10,000
BOD	3,000-7,150	198-260	100-240	280	130	1,120-1,80	726-1,210	562-1,990	-	-	7,500-28,000	4,000	80	100-90,000
TKN	-	240-452	64-1,260	1,256	700	131-930	-	104-1,014	2,219-2,860	889-1,180	-	-	-	50-5,000
NH_3-N	150-1,250	-	-	-	-	2-32	3.8	2-47	1,190-2,700	784-1,156	56-482	-	-	1-1,500
Ni	0.02-1.56	0.01-0.42	0.1	0.25	0.035	0.13-0.95	-	0-0.6	-	-	-	-	-	0.02-2.05
Cd	0.037	0.02	0.001	0.002	-	0-0.23	0-0.001	0-0.15	-	-	-	<0.05	<0.05	0.14
Pb	0.03-0.45	0.07	0.05	-	0.52	0-5.37	0-0.03	0-3.45	-	-	-	0.5	1.0	1.02
Cr	-	0.01-0.52	0.08-2.9	0.07	-	0.24-0.94	-	0.04-0.70	-	-	-	-	-	0.03-1.60
Hg	0.50-1.70	-	-	-	0.684	-	-	-	-	-	-	-	-	0.05

Note: Values are in mg/l with the exception of pH

1. Pollution Control Department, 2000 2. Sivapornpun, 2000 3. Agamuthu, 1999
4. Robinson and Luo, 1991 5. Qasim and Chiang, 1994 6. Andreottola and Cannas, 1992

2.2 Treatment methods for landfill leachate

Normal leachate is harmful to receiving watercourses. Leachate also contains many other substances which, may be toxic to life, or may simply alter the ecology of the stream or watercourse, if not removed by treatment. The treatment of leachate is one of the most important issues in the management of landfill. Leachate that is collected and removed from a landfill must be managed with care. Treated leachate must meet the required regulatory limits for discharge to environment as treated wastewater. The treatment of landfill leachate is often complicated and expensive in order to comply with the effluent standards prior to discharge and is challenge for environmental engineers (Shu et. al, 2005).

Leachate treatment is dependent on the quality and quantity of the leachate influent, discharge limits or removal efficiency requirements, site locations and economic reasons. Leachate can be treated by biological, physical, chemical method or a combination of these methods. Leachate can also be treated by recycling. Each method has inherent advantages and disadvantages with respect to certain problems. However a single method is not sufficient, some combination of the methods might be most effective.

There are many different landfill leachate options. The potential methods for the management of landfill leachate are mainly:

- Physical-chemical treatment
    - Neutralization
    - Precipitation/Flocculation/Sedimentation
    - Chemical oxidation
    - Membrane filtration
    - Adsorption
    - Ion exchange
    - Ozonation
- Biological treatment
    - Aerobic biological treatment
    - Anaerobic biological treatment
    - Constructed wetlands

Selection of a leachate treatment process is highly site specific. It depends on the following factors:

- Effluent discharge alternatives and limitations
- Treatment process
- Permit requirements
- Cost-effectiveness of treatment

2.2.1 Physical-chemical treatment

The physical/chemical method is used mainly to reduce suspended solids, refractory organic, heavy metals and phosphates. Old leachate is characterized by ammonia, refractory substances and low biodegradable organic fraction (BOD/COD in the range of 0.1). Therefore old leachate cannot be treated to required standards by biological processes alone. Effective removal of these substances could be achieved by more sophisticated

methods such as advanced oxidation, adsorption with activated carbon or combination of these with conventional physico-chemical methods (Zamora et. al., 2000).

Firstly, inorganic constituents are removed from the leachate before the organic constituents. This is to protect the biological, adsorption and stripping processes from problems caused by the metal's toxicity, corrosivity and scaling. The treatment process always begins with equalization. Equalization involves mixing incoming leachate in a large tank to create a uniform product. After that many methods can be employed to treat the inorganic portion. These methods include:

a) Neutralization

It involves addition of acid and base to adjust the pH to an acceptable level, usually between 6 and 9. Some common base is lime, calcium hydroxide, caustic, soda ash. Common acid includes sulfuric, hydrochloric and nitric acid to pH adjustment.

b) Precipitation/Flocculation/Sedimentation

This treatment is effective on leachate with high molecular weight organic material such as fulvic and humic acids. In precipitation reactions, chemicals are added to transform dissolved constituents to form insoluble precipitates. Metals are precipitated as hydroxides, sulfides and carbonates by adding appropriate precipitant and adjusting to the favorable pH. Chemical coagulation should bring about removal of particulates and possibly color, depending upon the nature of color and pH and possibly the removal of other contaminants that precipitates with the coagulants. In flocculation reaction, alum, lime, ferric chlorides are added to the inflow to reduce repulsive forces between the precipitated particles. These particles aggregate, forming larger flocs which can be settled out in a sedimentation tank.

A most widely used method for removing heavy metals is lime-addition hydroxide precipitation. Chemical precipitation for leachate treatment mainly makes use of lime because of its low cost, high availability with regard to high dose required and effectiveness in removing arsenic, cadmium, nickel, lead, zinc. Raw leachate from medium aged fills or pretreated biological leachate with BOD/COD ratio of 0.1-0.5 provide the best effectiveness of lime precipitation not only for color and metals removal but also for COD reduction due to a predominant typical molecular weight fraction (Chian et al., 1976). Provided that lime dose (1.4-6.4 g/l) is sufficient to raise the pH value above 10, precipitation combined with aerobic process is able to lower COD value by 30 to 70% (Ho et al., 1974).

In the same way, coagulation-flocculation process with ferric chloride or alum is better applied to remove high molecular weight compounds that represent a large part of the organic matter in raw stabilized leachates or biologically pretreated ones (BOD/COD<0.1) (Diamadopoulos, 1994). From literature results, the optimum coagulation-flocculation is obtained with ferric chloride (1-4.8 g/l) under controlled acid pH (4.3-4.9); in these conditions, the treatment ensures considerable reduction of suspended solids content and gives about 60% COD removal.

Sulfide precipitation was also very effective for removing mercury and silver (Cawley et. al., 1981). However the high concentration of organic and inorganic complexing agent could inhibit, even at high pH, the heavy metals removal efficiency (Sletten et. al., 1995).

Chian and Dewalle (1977) and Ho et. al, (1974) reported that precipitation-using lime could remove organic matter with molecular weight greater than 50,000 Da. This particular fraction is present in low concentrations in young landfills and absent in older landfills. Therefore lime treatment is most effective for medium age landfill leachate. Suspended solids and color in the leachate could be effectively reduced by using coagulation process. However, due to high concentration of soluble organics, the total organic strength can not be reduced by coagulation. This could be reduced by adsorption using activated carbon adsorption.

Hamidi et. al, 2006 investigated that ferric chloride could be a viable coagulant in managing color problems associated with landfill leachate. Among four types of coagulants (Aluminum (III) sulphate or alum, Ferric (III) chloride, ferrous (II) sulphate and ferric (III) sulphate), ferric chloride was superior to the other coagulants and remove color up to 94% at an optimum dose of 800 mg/l at pH 4 and it showed similar trend as for COD, turbidity and suspended solids.

Roger et. al (2005) reported that by using ferric chloride and alum as coagulants, greater than 90% removal of organic material, measured as suspended COD, was obtained at pH 2, which was considered as the optimal value.

Chang (1998) using alum dose of 1,000 mg/l at pH 5.5 obtained 92% removal in color, 72% in COD, 80% of cadmium and 87% of chromium. Ho et. al. (1974) using alum found that 53% of COD removal for a high strength leachate of 9,100 mg/l COD with dose of 1,000 mg/l.

However, the main disadvantages of this technology associated with its high operating costs due to chemical dose, and sludge produced.

c) Chemical oxidation

Chemical oxidation technologies are useful in the oxidative degradation or transformation of a wide range of pollutants present in wastewater treatment (Venkatadri and Peters, 1993). The reaction involves the addition of chemical oxidizing agents under controlled pH. Generally, chemical oxidation processes are incorporated into treatment sequences to treat constituents of wastewaters that are resistant to biodegradation or create toxicity in biological reactors. Chemical oxidation processes are widely used in leachate treatment. A variety of chemical oxidants are used for leachate treatment including hydrogen peroxide, ozone, chlorine, chlorine dioxide, hypochlorite, UV radiation and wet oxidation. Since oxidation processes are energy intensive and expensive, their application is limited. Moreover a large amount of oxygen required for higher organic concentration because it is dependent on the stoichiometry (Webber and Smith, 1986).

Chemical oxidation is a convenient method for the removal of organics but it is not efficient in removal of ammonia nitrogen. Chemical oxidation can make refractory organics capable of undergoing subsequent biological treatment. During chemical oxidation, organic compounds can be converted to simple final products such as water and carbon dioxide. Various oxidants such as hydrogen peroxide, potassium permanganate, oxygen, ozone and chlorine can be used. Chlorine has the drawback of producing toxic

products. Most reactive oxidant is the pH radical on which most of the advanced ozonation processes are based.

Chian and DeWalle (1976) did ozonation of aerated lagoon effluent for three hours using a dose of 400 mg/L/h for a COD of 627 mg/l and achieved about 48% COD removal. But they did not observe any removal of organics by using calcium hypochlorite.

Bjorkman and Malvinic (1977) by using lime and ozone, were able to remove some metallic ions, color and turbidity but main components of COD are not effectively reduced or removed.

d) Membrane filtration

Membrane filtration can be defined as the separation of solid immiscible particles from a liquid or gaseous stream primarily based on size differences. The classification of membrane separation processes are based on particle and molecular size. Membrane processes are very effective, do not require additional chemicals and can be operated in ambient condition, making it both environmentally attractive alternatives to the conventional operating units., but the rate of transfer across the membranes is generally slow and pressures are high; large membrane areas are required. The processes are such as Reverse Osmosis (RO), Nanofiltration (NF), Ultrafiltration (UF) and Microfiltration (MF).

In case of leachate treatment, membrane filtration is less effective in treating young (acidogenic) leachate since membranes cannot retain volatile fatty acids. The reason for the poor removal efficiency is the high percentage of high molecular weight organic material and ammonium concentration to be removed which may be present in the leachate. Although NF and RO are quite effective in leachate treatment in terms of organic, inorganic, nitrogen removal, the major disadvantage is its susceptibility to fouling and short lifetime. In addition, high COD in the leachate is not good for this technology.

RO membranes can remove more than 99% of colloids from feed-water (leachate) and up to 99% of the inorganic ions. RO permeate can be discharged into surface water without any harm to the environment and the recovery rate is about 80% (Steven et. al., 1995). However, one of the major disadvantages of reverse osmosis is bio-fouling and premature clogging. RO is expensive because of pre- and post-treatment costs and high energy consumption (Gierlich and Kollbach, 1998).

When a reverse osmosis in Germany was operated at a capacity of 1.8 m^3/h, a salt rejection of 98 % and COD removal of 99 % could be achieved. The membrane was changed after three years of operation due to the flux reduction. The illustrations indicated that reverse osmosis is effective in landfill leachate treatment provided that the leachate characteristic is considered and the membrane module modified adapted to meet the design criteria (Peters, 1997).

For stabilized leachate, biological treatment combined with reverse osmosis produces permeate conform to regulations together with a residual concentrate representing about 20% of raw leachate.

The coupling of microfiltration membrane bioreactor with reverse osmosis further allows overcoming process limitations due to fouling by residual organic compounds or suspended solids. This combination achieves full purification of aged landfill leachate.

Lerdpornsawan (1999) determined the efficiency of organic compounds removal in landfill leachate using reverse osmosis with spiral wound membrane. The results of the experiment showed that for strong leachate, COD removal was in the range of 82.9% to 91.4%. The removal efficiency of other parameters is

- Dissolved solids (78.6% to 78.7%),
- Suspended solids (87.9% to 99.0%),
- Color (98.6% to 98.8%),
- Total phosphorus (50.0% to 90.6%),
- Ammonia nitrogen (57.4% to 63.5%),
- Organic nitrogen (39.3% to 91.5%),
- Chloride (31.7% to 57.3%),
- Total alkalinity (57.3% to 71.5%),
- Calcium hardness (97.9% to 98.2%) and
- Magnesium hardness (95.3% to 98.0%)

The use of reverse osmosis for raw leachate treatment was quite successful in treating organics. Chian and DeWalle (1976) found COD reductions of between 56 and 89% for a high strength leachate of 53,300 mg/l COD using a cellulose acetate membrane. Removal of total solids was up to 99% when influent pre-filtration was performed. Organic removal was greatly increased when pH of raw leachate was increased. They concluded that reverse osmosis was found to be more effective in removal of organic especially when applied to biologically treated and highly stabilized leachate but special precaution may have to be used for membrane fouling and also the concentrate of RO should be treated properly.

e) Adsorption

The carbon adsorption technique removes dissolved organics from the leachate. The cost of the carbon in the regeneration stage can make the process one of the most expensive treatment options. However, the adsorption techniques, using carbon to remove dissolved organics from the leachate, is useful with some older leachate.

Carbon adsorption achieved 50-70% removal of COD from leachates. Other materials such as zeolites yielded similar treatment performance (Chian and DeWalle, 1976; Holm and Zhu, 1994).

Activated carbon adsorption is an effective unit process for removing colored wastewater. It was investigated as an alternative treatment method to chemical precipitation for raw or biologically pretreated young landfill leachate and also as polishing treatment combined with coagulation-flocculation for stabilized leachate. Because the adsorptive capacities increase while the volatile fatty acid fraction decreases, activated carbon adsorption is shown more effective in treating stabilized or biological pretreated young leachate. However, even if substantial reduction of COD are observed for raw or chloride ferric pretreated leachate from old landfill, high carbon loadings (6 and 1.5 g/l, respectively) required make this process implementation prohibitive (Diamadopoulos, 1994).

Cook and Foree (1974) conducted experiments on activated carbon column treatment of lime pre-treated leachate and achieve 81% COD removal for a leachate of 3,290 mg/l COD with 15 minutes HRT. But because of high organic loading, low adsorptive capacities and presence of suspended solids in raw leachate, regular fouling of carbon column has been observed.

Ammoniacal nitrogen often present in leachate in concentrations of up to thousands of mg/l and must be reduced to well below five mg/l before there is no danger to fresh water fish life.

The results of the study from_Hamidi et.al (2004) show that about 40% of ammoniacal nitrogen with concentration of more than 1,000 mg/l could be removed either by activated carbon or a mixture of carbon with limestone at mixture ratio of 5:35. This result shows that limestone is potentially useful as a cost-effective medium to replace activated carbon for ammoniacal nitrogen removal at a considerably lower cost.

The ratio of $SBOD_5$/SCOD increased from 0.1 to 0.17 upon using clinoptilolite, a type of zeolite, which indicates that the process rendered the leachate more amenable to the biological process (Kim et. al, 2003). When using clinoptilolite as a pretreatment step as a sink for ammonia nitrogen and on an average it reduced the levels of ammonia nitrogen, soluble chemical oxygen demand (SCOD) and color by 72, 4.7 and 25%, respectively.

Qasim and Chiang (1994) observed that adsorption by activated carbon was more effective in organic removal from raw leachate than chemical precipitation with COD removal efficiencies of 59-94%.

Dewalle and Chian (1977) also concluded that activated carbon was more effective in organic removal after biological treatment or with leachate collected from stabilized landfill site. Seventy percent of COD in the effluent of aerated lagoon and aerated effluent of anaerobic filter can be removed by carbon column.

f) f) Ion exchange

Ion exchange removes ions from the aqueous phase by the exchange of cations or anions between the contaminants and the exchange medium. Ion exchange materials may consist of resins made from synthetic organic materials that contain ionic functional groups to which exchangeable ions are attached. They also may be inorganic and natural polymeric materials. After the resin capacity has been exhausted, resins can be regenerated for reuse. Ion exchange can remove dissolved metals and radionuclide from aqueous solutions. Ion exchange is suited for diluted solutions. Other compounds that can be treated include nitrate, ammonia nitrogen. There are some drawbacks from using ion exchange; suspended solids content greater than ten ppm may cause resin blocking and pH of the influent may affect the ion exchange resin selection.

Kim et. al. (2002) found that the arsenic speciation using an ion exchange method was effective to separate As(III) and As(V) in leachate of mine tailings. Street et. al. (2002) investigated that the ion exchanger is excellent for removing lead from the synthetic leachate. Fernández et. al, 2005 studied the removal of Cd and Zn present in the leachate from an inorganic industrial waste landfill using cationic exchange resins (Amberlite 200, 252-C, IR-120, Duolite C-464), a chelating resin, Amberlite IRC 718, and an adsorbent

resin, XAD-2. The chelating resin Amberlite IRC 718 presented the higher removal in batch experiments for both metals (93% for Zn and 50% for Cd).

The use of ion exchange process for raw leachate treatment was considered only for the removal of ammonia in low strength leachate. It was found to be very ineffective means of ammonia removal, due to the presence of interfering substances which compete for bounding sites. Organic removal was not examined but was believed to be very low (David, 1981). Chian and Dewalle (1976) did ion exchange column treatment of effluent of aerated lagoon and achieved 50% COD removal with an initial COD of 527 mg/l.

g) Ozonation

Ozone can be used to for disinfection, color and odor removal. Ozone has the ability to convert organic compound into carbon dioxide and water, which reduce the toxicity. Ozone has an oxidizing potential, which in many cases is sufficient to directly convert organic substances. It is effective to be used as a pretreatment to remove refractory compounds or in post-treatment to increase the biodegradability. After the use of ozone, the BOD is increased without a significant reduction in COD. Since ozone has many advantages, it can be hazardous because the third unstable atom has a strong tendency to break away and attach itself to other substances. Therefore, many ozone treatments usually have ozone trapped or ozone destroyer to trap the residual ozone in the exhaust gas from being emitted to the atmosphere.

Oxidation of ozone is accelerated when a radical reaction occurs. By having initiators such as OH^-, H_2O_2, UV rays, the extremely reactive OH radicals, which are the strongest oxidant in water, formed via intermediate reaction (Steensen, 1997). Ozone need to be transformed to OH radicals for oxidation of ozone resistant compounds. The oxidation potential of ozone alone is high enough for direct oxidation of organic materials. Since the landfill leachate has high humic substances content, the oxidation by ozone is advantages since the initiator is presented in wastewater. The ozone reaction is slow down by radical scavenger such as carbonates/hydro carbonates, or alkyl compounds (Staehelin and Hoigne, 1983). This radical scavenger can interrupt the chain reaction. Generally, the consumption of ozone is about 2.3-3 g ozone/g COD removed (Cossu et al., 2003). The solubility of ozone in water at different temperature is given in Table 2.7.

Table 2.7 Solubility of ozone in water

Temperature (°C)	Solubility (kg/m^3)
0	1.09
10	0.78
20	0.57
30	0.40
40	0.27
50	0.19
60	0.14

(Source: Ullmann's, 1991)

Ozone is an irritating pale blue gas, heavier than air, very reactive and unstable. It can not be stored and transported so it has to produce in situ. Ozonation process is generally designed for process time lasting in the range of 10 minutes. Ozone is toxic and explosive

even at low concentrations. Ozone is about 14 times soluble in water. The initiation of ozone can be artificially accelerated by increasing pH or by adding hydrogen peroxide (Gunten, 2003). Table 2.8 shows ozonation results according to leachate type.

Table 2.8 Ozonation Results

Leachate Type	Initial COD (mg/l)	AOP dosage (g/g)	Ozone dosage (g/g COD)	COD removal (%)
Young	881	O_3/H_2O_2 0.7	1.4	40
		O_3	1.4	43
Young MBR	2,500	O_3/H_2O_2 0.4	2.8	94
		O_3	4.3	70
Biological	1,250	O_3/UV	3	90
		O_3	3	75
-	1,700	O_3/UV	1.5	94
		O_3	1.5	36
Biological	750	O_3/UV	0.7	70
		O_3		
Stabilized	2,300	O_3/UV or O_3	1.5	60
		O_3/H_2O_2 0.3	1.5	92
		O_3	1.5	89
Stabilized biological	1,400	O_3/catalyst	1.5	89
		O_3	1.5	63

(Source: Hausler et al., 1995; Wable et al., 1993; Steensen et al., 1993; Weichgrebe et al., 1993; Leitzke et al., 1993; Bigot et al., 1994; Bigot et al., 1995)

Disadvantages of Ozonation process are

- High cost for reagents
- High energy consumption
- Low reduction of inorganic compounds such as ammoniacal nitrogen

Direct Ozonation (0.4-1.3 g ozone/g COD) is shown to provide the most excellent color removal with limited COD reduction (25-44%) when applied to both young and aged leachate (Sandhya et al, 1995). Recent developments and applications involve advanced oxidation processes (AOP) based on ozone activation by hydrogen peroxide, UV-light with the aim to lower the ozone dose and to enhance organic matter degradation (Table 2.8). Ozonation is not so appropriate for young leachate because major part of the COD is made up of the fatty acids refractory to ozone attack (Chian, 1976).

Chang (2001) treated leachate by using ozone at a concentration of 331.2 mg/l and could remove 17% of color, 6% of COD but using ozone at 1342.8 mg/l, the removal efficiency increased to 54% of color and 12% of COD.

The advantages and drawbacks of different non-biological processes applied to textile wastewater decolouration were summarized in Table 2.9.

Table 2.9 Advantages and drawbacks of some non-biological decolouration processes applied to textile wastewater

Physical/chemical methods	**Method description**	**Advantages**	**Disadvantages**
Fenton reagents	Oxidation reaction using mainly H_2O_2–Fe(II)	Effective decolouration of both soluble and insoluble dyes	Sludge generation
Ozonation	Oxidation reaction using ozone gas	Application in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical oxidation	Reaction using mainly H_2O_2–UV	No sludge production	Formation of by-products
NaOCl Oxidation	Reaction using Cl^+ to attack the amino group	Initiation and acceleration of azo-bond cleavage	Release of aromatic amines
Electrochemical destruction	Oxidation reaction using electricity	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Dye removal by adsorption	Good removal of a wide variety of dyes	Very expensive
Membrane filtration	Physical separation	Removal of all dye types	Concentrated sludge production
Ion exchange	Ion exchange resin	Regeneration: no adsorbent loss	Not effective for all dyes
Electrokinetic coagulation	Addition of ferrous sulphate and ferric chloride	Economically feasible	High sludge production

(Source: Robinson et al., 2001)

2.2.2 Biological treatment

Biological treatment methods are the process where microbes are used to destroy or at least reduce the toxicity of waste stream. Biological treatment is the most common practice for leachate treatment, which is relatively economical when comparing with physico-chemical methods. Biological treatment is capable of providing high BOD, COD and SS removal but it is less effective for color removal. A suitable culture of the micro-organism, either aerobic or anaerobic, is chosen. Biological process is commonly used for the removal of the bulk of leachate containing high concentrations of BOD.

It has been reported that the acidic phase is best suited for biological systems whilst physical-chemical systems are better for old leachate (Boyle and Ham, 1974). High ammonia concentrations and phosphorus deficiency in leachate hamper the efficiency of biological treatment. High nitrogen levels are also hazardous to receiving water and need

to be removed prior to discharge. This is generally carried out through physico-chemical processes in the stabilized leachate.

However, biological treatment of leachate is effective in COD removal even in low biodegradable leachate of BOD_5:COD < 0.2 (Hayer and Stegman, 2003). In many cases, stabilized leachate from biological treatment and old landfills still shows high COD values because the presence of high molecular weight compounds. Therefore, an additional treatment step is often required (Trebouet et. al, 2001). In addition, the organic loading and pH are significant in influencing the growth of nitrifying bacteria in nitrification process (Abeling et. al., 1992; Bae et. al., 1997; Kabdasli et. al., 2000).

The advantage of biological treatment is that the organic material in the leachate is degraded mainly to harmless end products such as water, carbon dioxide and/or methane (Kettunen and Keskitalo, 1999). However, the drawbacks in biological leachate treatment originate from operational problems such as foaming, metal toxicity, nutrient deficiency and sludge settling (Qasim and Chiang, 1994).

Only biological treatment of landfill leachate usually results in low treatment efficiencies because of high COD, high ammonium-N content and also presence of toxic compounds such as heavy metals. Relative biodegradability of leachate is shown in Table 2.10.

Table 2.10 Relative biodegradability of leachate

Biodegradability	BOD/COD	COD/TOC
Low	<0.5	<2
Medium	0.5-0.75	2-3
High	>0.75	>3

(Source: http://msw.cecs.ucf.edu/landfillprocesses.ppt)

2.2.2.1 Aerobic biological treatment processes

Conventional aerobic systems consist of either attached or suspended growth systems. The advantages and disadvantages of each system is case specific. Suspended growth systems range from aerated lagoons, activated sludge and Sequencing Batch Reactor (SBR) while attached growth processes include trickling filters and rotating biological contractors. Trickling filter is generally not used for leachate treatment when leachate contains high concentration of organic matter because of the large sludge production resulting in clogging of the filters.

A problem associated with aerobic treatment is that the C:N ratio is expressed as BOD:N ratio in methanogenic leachates may be 100:100 while the optimum suited ratio for bacteria is 100:5. Due to excess nitrogen, the biological process may not run reliably producing unacceptable concentrations of ammonia (Fletcher and Ashbee, 1994).

It was concluded that aerobic biological treatment of leachate would not be successful at high organic loading and low retention time without addition of nutrients (Robinson and Maris, 1979). In addition to organic compound that require biological treatment, leachate contains inorganic dissolved solids (chloride, sodium) which experience limited removal by biological treatment, combination of biological and physico-chemical unit operation are preferred.

a) Activated sludge process

Activated Sludge Process is efficient in leachate treatment. Activated sludge gives greater BOD reduction, but entails large units to provide the long detention times usually needed and also requires highly qualified supervision. Keenan et al., (1984) investigated the combined physico-chemical process with activated sludge process. It is observed that the reduction in ammonia by stripping and neutralization with sulfuric acid and phosphoric acid after that entered to activated sludge process. The organic matter in terms of BOD was reduced 99% and COD removal of 95%. The effluent BOD/COD was 0.16. The ammonia reduction was 90% and heavy metals removal ranged from 27 to 75%.

b) Sequencing batch reactor (SBR)

SBR is commonly used for leachate treatment. A basic sequencing batch reactor cycle comprises of leachate filling during aeration, settlement, decanting the treated effluent. Several studies have been conducted to find out the applicability of SBR in leachate treatment. Among the various biological treatment processes, Sequencing Batch Reactor (SBR) has been proved as a reliable and robust method for leachate treatment to meet specified effluent values. Doyle et al., (2001) conducted a study of high rate nitrification in SBR on a mature leachate obtained from a domestic landfill. The leachate possessed high ammonia content with average concentration of 880 mg/l while the average BOD and COD concentration were 600 and 1,100 mg/l, respectively were achieved in this study.

c) Constructed wetland

Other treatment options include the use of more natural engineered systems. Land application utilizes the natural treatment processes in soil and in plants uptake to remove contaminants. This method can be inexpensive, require simple operation and low maintenance but in many cases, requires pretreatment to reduce high contaminant concentrations before application. Inconsistent results can be attributed to the variable nature of the leachate and the lack of universally accepted design standards for wetland treatment systems. Besides it is clear that before the constructed wetland, a pre-treatment unit should be installed to remove high organic carbon loads.

Arash et al. (2005) observed that reductions in contaminants from diluted leachate were consistently achieved after passing through six pilot-scale wetland cells, four planted with cattail (Typha latifolia) and two unplanted controls., with average removals for BOD, COD, VFAs and tannin and lignin of 60, 50, 69 and 42%, respectively. Tjasa (1997) investigated that constructed wetland was fairly efficient, simple and inexpensive in landfill leachate treatment. They found the reductions after the initial periods of landfill leachate from constructed wetland has been achieved in COD (68%), BOD_5 (46%), NH_3-N (81%), Fe (80%) and bacteria (85%).

The use of constructed wetlands for leachate treatment was reported by Martin and Johnson (1995). The treatment efficiencies of constructed wetlands with respect to organic matter, nitrogen, phosphorus, iron and pathogen removal were between 70-95% (Mahlum, 1995 and Bulc et al., 1997).

2.2.2.2 Anaerobic biological treatment

Anaerobic biological treatment uses microorganisms which grow in the absence of dissolved oxygen and convert organic material to carbon dioxide, methane and other metabolic products. The most common aerobic biological treatment methods are Up-flow Anaerobic Sludge Blanket (UASB) reactors, Up-flow Anaerobic Filter or Anaerobic Digester. The main advantages of anaerobic treatment over aerobic treatment are:

- Lower energy requirement as no oxygen is required and thus reduce the operational cost.
- Low sludge production as only about 10-15% of organics is transformed into biomass.
- Biogas production (85-90%) favors the energy balance with a low nutrient requirement making it appropriate for leachate treatment.
- Anaerobic microorganisms seldom reach endogeneous phase, important for the treatment of leachate with variable volume and strength.
- Elimination of odor problem.
- Anaerobic sludge is highly mineralized than aerobic sludge which increases its value as fertilizer if toxic metals are removed.

Although anaerobic treatments may appear to offer benefit over aerobic treatment, it has disadvantages of long retention time and high capital cost. The result from laboratory scale has been reported that leachate with high BOD and COD would appear to require organic loading of around one kg COD/m^3.day at 30 to 35°C (Crawford and Smith, 1985). For UASB, soluble COD removal efficiency was consistently between 77 and 91% at hydraulic retention time of 24, 18 and 12 h (Kennedy and Lentz, 2000).

Boyle and Ham (1974) showed that greater than 90% removal of organic matter from leachate was possible by storage in anaerobic conditions for 10-12 days at temperatures of between 23 and 30°C with organic loading of 1.05 kg COD/m^3.day. For anaerobic treatment, ammonia removal is low but BOD removal can be significant with the methane collection and used to maintain the temperature in the process.

Simple anaerobic reactors were used to treat metal contaminated water. There was one research reported that 95% of Al, Cd, Fe, Mn, Ni, and Zn were removed through the reactor (Dvorak et al., 1992).

Chian and Dewalle (1976) using anaerobic filter with a detention time of 27 days could achieve 97% removal of COD for an initial COD of 30,000 mg/l. Robinson et al. (1985) could achieve 98% removal of BOD and 92% removal of COD by aerobic biological process at 10°C with retention time greater than 10 days.

There will be five steps involved in anaerobic degradation with the presence of sulfate.

1. Hydrolysis: large molecules are broken down into smaller molecule that can be easily transported into the cell and metabolized.
2. Acedogenesis: the soluble compound such as sugar, amino acid are fermented and changed into volatile fatty acids (VFA), hydrogen, carbon dioxide and small molecules like ethanol and lactate.

3. Oxidation: the oxidation of long chain fatty acid to become acetate and some kinds of volatile fatty acid.
4. Acetogenesis: the oxidation of volatile fatty acid (VFA) from the yielding of acedogenesis to form acetate and hydrogen depending on the types of VFA and carbon dioxide produced from the previous step.
5. Methanogenesis: the methane generation by decarboxylation of acetate by acetotrophic methanogenic bacteria and by hydrogenation of cabon dioxide by hydrogenotrophic methanogenic bacteria.

2.2.3 Treated leachate discharge limit

Leachate treatment regulations vary from country to country. Some countries have strict regulation, some countries require simply collection of leachate and some countries have no definite requirements. Germany is one such country that has a treated leachate requirement. The discharge limits according to German and Indian standard are presented in Table 2.11 and 2.12. According to Pollution Control Department of Thailand, such standard is under preparation.

Table 2.11 Limiting concentrations for the discharge of treated leachate according to German Standards

Parameters	Limiting Concentration (mg/L)	Parameters	Limiting Concentration (mg/L)
COD	200	Chromium	0.5
BOD_5	20	Chromium (VI)	0.1
Total Nitrogen	70	Nickel	1
Total Phosphorus	3	Lead	0.5
Hydrocarbons	10	Copper	0.5
Nitrite nitrogen	2	Zinc	2
AOX (absorbable organic halides)	0.5	Cyanide	0.2
Mercury	0.05	Sulfide	1
Cadmium	0.1		

(Source: Heyer and Stegman, 2003)

Table 2.12 Indian Standards for discharging treated leachate

Parameter	Standard mode of disposal		
	Inland surface water	Public sewers	Land disposal
Suspended solids mg/l, max	100	600	200
Dissolved solids (inorganic) mg/l, max	2,100	2,100	2,100
pH	5.5-9.0	5.5-9.0	5.5-9.0
Ammonical nitrogen (as N) mg/l, max	50	50	-
TKN (as N) mg/l, max	300	350	100
BOD mg/l (3 days at 27°C). max	250	-	-
COD mg/l, max	200	200	200
As mg/l, max	0.01	0.01	-
Hg mg/l, max	0.1	0.1	-
Pb, mg/l, max	2.0	1.0	-
Cd, mg/l, max	2.0	1.0	-
Total Cr mg/l, max	2.0	2.0	-
Cu mg/l, max	3.0	3.0	-
Zn mg/l, max	5.0	15	-
Ni mg/l, max	3.0	3.0	-
CN mg/l, max	0.2	2.0	0.2
Cl mg/l, max	1,000	1,000	600
F mg/l, max	2.0	15	-
Phenolic compounds (as C_6H_5OH) mg/l, max	1.0	5.0	-

(Source: www.cleantechindia.com)

Leachate wastewater is a complex mixture of many polluting substances such as organochlorine-based pesticides, heavy metals and color. The majority of this color is slowly removed by the wastewater treatment plant (WWTP) because of their toxicities to indigenous microorganisms. Color removal from WWTP processes are expensive and need careful application (Vandervivere et al., 1998; Robinson et al., 2001). Furthermore, following anaerobic digestion, nitrogen containing color is transformed into aromatic amines that are more toxic and mutagenic than the parent molecules (Shaul et al., 1985; Chung and Stevens, 1993; Ganesh et al., 1994). To overcome these difficulties, fungi are being investigated for their potential to decolorize. Only few researches have been studied about the color removal from the leachate by the fungi. Utilization of fungi for biological treatment of wastewater will be reviewed in the next chapter.

2.3 Fungi

2.3.1 Fungal growth characteristics

Growth of fungi is readily recognized by its fuzzy or cottony appearance. The main part of the growth commonly appears whitish but may be colored or be dark or smoky. Colored spores are typical in some of mature species and some give color during whole life cycle (Frazier and Westhoff, 1978). Growth of mycelial fungi is accomplished by the extension

of the hyphal tip. Although the older portions of the hypha are not capable of growth, they have an important role in supporting growth of the tip as new protoplasm is formed throughout the hypha and forms branches in acropetal succession behind the hyphal tip (Moore-Landecker, 1972).

The typical pattern involved in the growth rate of fungi was shown in Figure 2.2.

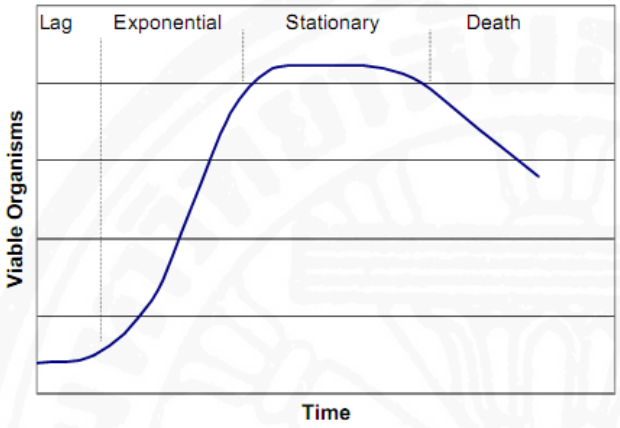

Figure 2.2 Typical growth curve for a fungal population

Stage I: Lag phase

The lag phase represents a period during which the fungal cells or spores adapt to a new environment. It is therefore the period between inoculation and the beginning of growth. Adaptation includes formation of enzymes and intermediates to support resumption of growth. The length of this phase is dependent not only on the physiological state of the fungus, but also on the morphology and level of inoculum. Spore inocula require a germination period (Smith and Calam, 1980), while pelleted inocula may require a certain degree of mechanical disruption prior to inoculation (Greasham, 1991). Physiological adaptation of the organism includes synthesis of enzyme systems required for substrate utilization, or removal of inhibitory compounds carried over with the inoculum (Prosser, 1995).

Stage II: Log phase

The exponential phase is characterized by a significant increase in cell mass. The rate of hyphal growth depends not only on the strain of the fungus, but also on the physicochemical environmental conditions. As on solid media, exponential growth results from autocatalysis through exponential production of branches, each of which extends at a linear rate. A reduction in the specific growth rate occurs when the fungus begins to experience an unfavorable growth environment such as the limitation of a required nutrient, the development of an adverse pH value or the accumulation of end products of metabolism that are inhibitory. This stage is the correct time for the harvest of fungi when the greatest increase in cell numbers and the highest content of protein production occur. However the time of the harvest depends on the environment and on the type of fungus.

During the deceleration phase, growth kinetics during this phase is largely uncharacterized, despite the importance of this phase for biotechnological processes, as the period when secondary metabolite production begins. Deceleration in growth rate due to oxygen limitation is of particular importance for cultures of filamentous fungi because of the influence of their morphology on rheological properties. Dispersed growth leads to non-Newtonian rheological behavior. The apparent viscosity increases with growth, reducing the transport of nutrients, oxygen and heat. Nutrient limitations can accelerate entry into the deceleration phase, in comparison with unicellular cultures of equivalent biomass and activity.

Stage III: Stationary phase

The stationary phase may be defined simplistically as the balance between hyphal mass increase and decrease. However, if the hyphal mass accumulates intracellular storage material during the reduced growth phase, a slight increase in hyphal mass may be observed during endogenous metabolism of these storage materials. Also, if the hyphae begin to autolyse, new growth could be expected from the products of autolysis, e.g., release of a limited nutrient.

Stage IV: Death phase

Death phase is the period preceding the stationary phase where the fungus cell dies. In some cases, death leads to loss of cellular integrity with the release of cytoplasmic constituents or lysis. However incubation may continue after the stationary phase and cells may remain alive and continue to metabolize.

2.3.2 Fungal growth condition

Fungi are morphologically complex organisms differing in structure at different times in their life cycle, differing in form between surface and submerged growth, differing also with the nature of the growth medium and physical environment. Factors affecting morphology include the type and concentration of carbon substrate, levels of nitrogen and phosphate, trace minerals, dissolved oxygen and carbon dioxide, pH and temperature, salts, inhibitory molecules (sulfur compounds, surfactants and heavy metals), fermenter geometry, agitation systems, rheology and the culture modes, whether batch, fed-batch or continuous. The change in morphology during growth affects nutrient consumption and oxygen uptake rate in submerged culture (Schügerl et al., 1983).

White rot fungus works best in a nitrogen-limited environment. A high C/N ratio and SBOD/SCOD in the leachate was found preferable for the fungal treatment (Kim et. al, 2003). It requires nutrient limitation to trigger ligninolytic activity, it is very sensitive to agitation and it only reaches full degradative potential under an oxygen enriched atmosphere. Low ambient temperatures can decrease biodegradation rates. High contaminant concentrations and heavy metals may be toxic and inhibit the growth of the fungus as it affects the reproduction of *Basidiomycetes.* The culture conditions of *P.chrysosporium* required for the expression and activity of its ligninolytic enzymes (low nitrogen, high temperature (37ºC), and static cultures) (Kirk et al., 1978) have limited the practical application of this organism in bioremediation processes.

The degradation of contaminants may not be sufficient to meet cleanup levels. The weak decolorization of these effluents by complete cultures could be explained by the influences of temperature, pH, carbon and nutrients etc. (Swamy and Ramsay, 1999; Mester and Tien, 2000).

2.3.2.1 Physical requirements of microbial growth

a) Temperature

Temperature not only affects the growth of the microorganism and survival, also has an effect on cell size, metabolic products such as pigments and toxins, nutritional requirements, enzymatic reactions and the chemical composition of cells. The optimum growth and highest color removal occurred at 35ºC for C.versicolor and 40ºC for *P.chrysosporium* (Kumar et al., 1998). These temperatures are also optimum for the enzymes responsible for decolorization.

b) pH Effects

Most fungi grow within an optimal pH range of 4-8. In general, the change in pH of the medium affects two types of metabolic activity:

1) The production of the organic acids, or the release of the ammonia.

2) The unbalanced utilization or excretion of cations and anions.
   In solid substrate fermentation of cassava, Senez (1979) suggested an initial pH of 3.5 to avoid ba cterial contamination. But mycelial growth promoted a rapid acidification that slowed down enrichment and even stopped it when an ammonium salt was used alone as the nitrogen source.

C.versicolor and *P.chrysosporium* promote the highest color removal, 52% and 33.5% at p 5.0, respectively (Kumar et al., 1998). The best decolorization (80%) and highest COD removal (75%) were shown to occur at an initial pH of 5 (Benito et al., 1997). Total color elimation was 69% at an initial pH of 5 (Miranda et al., 1996).

c) Moisture

Water is essential for the growth of all viable cells. Fungi require relatively high moisture levels, although many of the higher fungi are able to grow in the absence of free water. Water is used to bring nutrients into the cells and is involved in the chemical reactions which break down substrates to usable molecules. Chemical reactions include the hydrolysis of the peptide bonds in protein, the ester bonds in fats and in the conversion of polysaccharides to monosaccharide (Banwart, 1981).

Raimbault et al., (1977) studied the influence of initial moisture content on the enriched cassava protein yield and pointed out an optimum range of moisture content is 50-60%. If the moisture content is too high, the inter particle spaces become filled with water and the air needed for fungi growth is forced out, here the growth of the micro organism is inhibited (Moo-Young et al., 1971).

d) Agitation and aeration

Fungal growth is an aerobic process and the growth majority of fungal species are inhibited by high concentrations of carbon dioxide. Oxygen is vital to cellular respiration in which an energy source is oxidized to carbon dioxide and water, with the evolution of a considerable amount of heat which needs to be removed for proper monitoring of the temperature. High partial pressures of carbon dioxide depressed amylase production in solid – substrate fermentation of rice with *Aspergillus oryzae* (Bajracharya et al., 1980) increased partial pressures of oxygen stimulated amylase production. Effective utilization of carbon and nitrogen compounds as sources of nutrients may by affected by the amount of oxygen available in the atmosphere.

Senez (1979) maintained aeration by a forced air flow that was saturated with moisture by bubbling it through water in the lower part of a fermenter. In this way, a high relative humidity can be attained. Maximum growth of most fungi occurs at a relative humidity of 95-100%, and growth declines in humidity of 80-85% (Moore-Landecker, 1972)

2.3.2.2 Nutritional requirements of fungi

Some mineral elements are important in the growth of fungi and if an essential element like N, P or K is lacking in the medium, fungi will not survive no matter how abundant are other elements. Table 2.13 summarized the elemental requirement of fungal cells.

Nitrogen is a component of cell wall polymers and is important for protein synthesis. A low-nitrogen medium favors the highest decolorization of MSW by *F.flavus* (Raghukumar and Rivonkar, 2001). Shojaosadati et al. (1999) reported a COD reduction of 35.7% by the supplementation of 4g/dm^3 ammonium sulfate and 0.4 g/dm3 KH_2PO_4 to sugar beet stiallage in a continuous culture of Hansenula sp. The highest productivity is achieved in a dilution rate of 0.12 per hour. Supplementation of these nutrients increases the levels in COD reduction and biomass production, but its use depends on sources, yeast extract and peptone were shown to support the highest growth and decolorization by *C.versicolor* and *P.chrysosporium*, respectively (Kumar et al., 1998). The highest color removal occurred in the absence of the addition of ammonium nitrate by *T.versicolor* (Benito et al., 1997) The COD removal rate was 65% to 70%. The color elimination and COD reduction were similar and more or less unaffected by the various manganese and magnesium concentrations. However, white rot fungus require trace amount of essential heavy metals such as Cd, Mn or Zn for their growth, but these metals are toxic when present in excess. The toxicity of some heavy metals such as Hg, Cu or Ni has been used for the development of antifungal wood preservatives. Fungi are susceptible to water stress.

Table 2.13 Elemental requirements of fungal cells

Element	Common sources	Cellular functions
Carbon	Sugars	Structural element of fungal cells in combination with hydrogen, oxygen and nitrogen. Energy source
Hydrogen	Protons from acidic environments	Transmembrane proton motive force vital for fungal nutrition. Intracellular acidic pH (around 5-6) necessary for fungal metabolism
Oxygen	Air, O_2	Substrate for respiratory and other mixed-function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4^+ salts, urea, amino acids	Structurally and functionally as organic amino nitrogen in proteins and enzymes
Phosphorus	Phosphates	Energy transduction, nucleic acid and membrane structure
Potassium	K^+ salts	Ionic balance, enzyme activity
Magnesium	Mg^{2+} salts	Enzyme activity, cell and organelle structure
Sulphur	Sulphates, methionine	Sulphydryl amino acids and vitamins
Calcium	Ca^{2+} salts	Possible second messenger in signal transduction
Copper	Cupric salts	Redox pigments
Iron	Ferric salts: Fe^{3+} is chelated bysiderophores and released as Fe^{2+} within the cell	Haem proteins, cytochromes
Manganese	Mn^{2+} salts	Enzymes activity
Zinc	Zn^{2+} salts	Enzymes activity
Nickel	Ni^{2+} salts	Urease activity
Molybdenum	$Na_2 MoO_4$	Nitrate metabolism, vitamin B_{12}

(Source: Graeme, M. W.; Nia, A. W., 2005)

Little is known of the ability of the white rot fungus to compete with other forms of fungi. Concerning enzymatic (Laccase, LiP, MnP) degradations, these reactions are quite complicated, involving numerous low molecular weight cofactors that serve as redox mediators (Reyes et al., 1999; Wasenberg et al., 2003). These cofactors, in addition to enzymes themselves, influence fungal color removal rates.

2.3.2.3 Effect of co-substrates for fungal growth

Effect of difference co-substrates on decolorization by *T.versicolor* is shown in Table 2.13. A separate carbon source (co-substrate) is required for growth and metabolic activity. Ashish (1995) reported that it was possible to decolorize the effluent from first alkali extraction stage with *T.versicolor* by 92% in 7 days at pH 4.5, temperature 30ºC, inoculums concentration 5.0 g/l, sucrose concentration 7.5 g/l; NH_4NO_3 1.75 g/l, $MgSO_4$ $7H_2O$, 0.5 g/l; KCL, 0.5 g/l; $FeSO_4$ $7H_20$, 0.01 g/l; and KH_2PO_4, 1.0 g/l. In addition to color reduction, COD and BOD_5 were also reduced to 68.5% and 67.7%, respectively. The fungus was grown in the form of pellets thus eliminating the problem with the recycling of biomass and making it possible to use large amounts of fungus. Table 2.14 show the effect of difference co-substrates on decolorization by *T.versicolor*

Table 2.14 Effect of difference co-substrates on decolorization by *T. versicolor*

Co-substrate	Color reduction (%)
Control	5
Sucrose	82
Glucose	80
Corn starch	65
Carboxymethylcellulose	70
Ethylalcohol	72
Pulp	65
Bagasse pith	45
Primary sludge	26

(Source: Ashish et al., 1995)

Of several carbon sources, glucose or sucrose proves best for decolorization of raw MSW at 10% concentration in LN medium using *F.flavus* (Raghukumar and Rivonkar, 2001). Optimum growth and decolorization of digested MSW occur at up to 3% glucose (w/v) for *P.chrysosporium* and up to 5% (w/v) for *C.versicolor* (Kumar et al., 1998). The decolorization activity correlates very well with the growth of mycelia of *A.niger* (Miranda et al., 1996).

Several factors that regulate growth and cell production of micro-organisms, such as source of carbon, nitrogen, temperature, pH, moisture, aeration, inoculum dose and toxicity are known to influence the rate of decolorization and COD removal of distillery and brewery effluents by fungi (Table 2.15). These factors are described below.

Table 2.15 Influence of supplements on fungal fermentation and decolorization of distillery and brewery waste

Fungus	Stillage origin/ dilution	Supplements	COD reduction (%)	Decolorization (%)	Duration (days)	Reference
Flavodon flavus	Cane, MSW/10%	Glucose or sucrose		63-64	6	Raghukumar and Rivonkar, 2001
Phanerochaete chrysosporium ATCC 24725	MSW/6.25%	glucose		85	10	Fahy et al., 1997
Coriolus versicolor	digested	glucose	90	71.5	12	Kumar et al., 1998
P.chrysosporium	Molasses spent wash/6.25%	Yeast extract	73	53.5		Benito et al., 1997
Trametes versicolor	Beet molasses/undiluted	Sucrose $MnSO_4$	77 70	84 76	4	Miranda et al., 1996
Aspergillus niger	Beet molasses/ undiluted	Sucrose $MnSO_4$ KH_2PO_4 NH_4NO_3	75	69	4	Shojaosadati et al., 1999
Hansenula sp.	Beet stillage/ undiluted	NH_4SO_4 KH_2PO_4	36		2	

Bajpai et al. (1993) and Mehna et al. (1995) also found glucose to be the most effective decolorization substrate for *Trametes versicolor*.

Archibald et al. (1990) have reported that *T. versicolor* is able to remove color efficiently in the presence of inexpensive sugar refinery or brewery wastes. About 92% color removal was achieved at a glucose concentration of 1 g/liter in 24 h. Even without any glucose, the fungus was able to remove up to 78% of the color in the same period. Archibald et al. (1990) reported a continuous increase in decolorization with an increase in glucose concentration from 1 to 3.5 g/liter. In the absence of glucose, no decolorization took place. In contrast, *R. oryzae* showed good decolorization with less glucose, 1 g/liter, or no glucose.

Esposito et al. (1991) and Lee et al. (1994) have reported that the fungus *Lentinus edodes* and the fungus KS-62 also removed 70 to 80% of the color without any glucose.

2.3.3 Modes of nutrients transport in fungi

The nutrients can be transported to fungi by ways such as free diffusion, facilitated diffusion, diffusion channels, and active transport. Table 2.16 summarizes the modes of nutrient transport in fungi.

Table 2.16 Modes of nutrient transport in fungi

Mode of nutrient transport	**Description**	**Examples of nutrients transported**
Free diffusion	Passive penetration of lipid-soluble solutes through the plasma membrane following the law of mass action from a high extracellular concentration to a lower intracellular concentration	Organic acids, short-chain alkanes and long-chain fatty acids by fungi and the export of lipophilic gaseous compounds
Facilitated diffusion	Translocates solutes down a transmembrane concentration gradient in an enzyme- (permease-) mediated manner. As with passive diffusion, nutrient translocation continues until the intracellular concentrations equal to that of the extracellular medium	In the yeast *S. cerevisiae*, glucose is transported in this manner
Diffusion channels	These operate as voltage-dependent 'gates' to transiently move certain nutrient ions down concentration gradients. They are normally closed at the negative membrane potential of resting yeast cells but open when the membrane potential becomes positive	Ions such as potassium may be transported in this fusion
Active transport	The driving force is the membrane potential and the transmembrane electrochemical proton gradient generated by the plasma membrane H^+-ATOase the latter extrudes protons using the free energy of ATP hydrolysis that enables nutrients to either enter with influxed protons, as in 'symport' mechanisms, or against effluxed protons, as in 'antiport' mechanisms	Many nutrients (sugars, amino acids, ions)

(Source: Graeme, M. W.; Nia, A. W., 2005)

2.4 White rot fungi

White rot fungi are the most effective lignin (and lignin-like substances) degrading microorganisms in natural environment. These fungi have recently become the object of worldwide attention because of their industrial use in biodecolorization and bioremediation. White rot fungi have a great possibility to be applied to landfill leachate. Wood-rotting enzymes in white rot fungus degrade a variety of pollutants including the predominant conventional explosives, DDT (dichlorodiphenyltrichloroethane), polynuclear aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs), other halogenated aromatics (including dioxins), some dyes, TNT and other nitro explosives, and other toxic chemicals such as cyanides, azide, carbon tetrachloride, and pentachlorophenol.

At present, a number of studies have focused on microorganisms, which are able to decolorize and biodegrade dyes (1, 2). To date, the majority of studies on biological decolorization have focused on fungal strains. Phanerochaete chrysosporium (8), Trametes versicolor (9), Cunninghamella polymorpha (12), F.trogii ATCC 200800 (13) abd Rhizopus arrhizus (14) are the major fungal strains used for decolorization purposes.

Phanerochaete chrysosporium

Phanerochaete chrysosporium (the scientific name means visible hair, golden spore) is a fungus with unusual degradative capabilities and is the most extensively studied white rot fungi. Its lignin-degradaing system has been implicated in the degradation of several xenobiotic compounds including benzene, toluene, ethylbenzenes and xylenes (BTEX), polyaromatic hydrocarbons (anthracene, benzo [a] pyrene), chlorophenols (PCP, dioxins), chlorinated and halogenated polycyclic aromatics (DDT), chloroalkanes, bleach plant effluents, nitrotoluenes, polychlonnated bipheny ls, atrazine, and insecticides such as chlorpyrifos, fonofos, and terbufos (Bumpus et al., 1985; Bumpus and Aust, 1987; Field et al., 1993; Reddy, 1995; Yadav and Reddy, 1993).

Trametes versicolor

Trametes versicolor is commonly known as the Turkey Tail. This shelf-fungus has small, thin, and leathery overlapping stalkless caps with multicolored concentric zones that alternate between smooth and hairy. It has a white pore surface. The tough basidiocarps will persist overwinter and can be found year-round on dead hardwood logs and stumps. It was also known as *Polyporus versicolor* and *Coriolus versicolor.*

Ligninolytic cultures of *Trametes versicolor* have been shown to degrade phenanthrene, anthracene, benzo[a]pyrene, anthroquinone, pentachlorophenol, 3,4-dichloroaniline, polychlorinated biphenyls (Aroclor 1254) and dieldrin (Addleman et ai.1995; Vyas et al., 1994). The ligninolytic enzymes of *T.versico1or* include laccase in addition to LiP and MnP. This organism has been studied extensively in the decoloration of bleach plant effluents (BPE) fiom pulp mills and the biobleaching of pulp (Addleman et al., 1995; Addleman and Archibald, 1993; Archibald, 1992b; Archibald et al., 1990; Mehna et al., 1995; Paice et al., 1989; Reid et al., 1990).

Flavodon flavus

The fungus *Flavodon flavus* belonging to the class Basidiomycetes produces fertile basidiomata in medium containing alpha-cellulose and sometimes in malt extract agar medium on prolonged incubation. Most of the times the fungus is in non-sprouting form and can be recognized by crystals deposited around fungal hyphae.

Although presences of lignin-modifying enzymes are reported in several fungal taxa belonging to the class Basidiomycetes, the applicants reported their presence in Flavodon flavus for the first time (Raghukumar et al., 1999). Lignin-modifying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment.

2.4.1 Degradation mechanisms

White rot fungi use a variety of mechanisms to accomplish the complete degradation of lignin and a wide variety of environmental pollutants which are resistant to biodegradation. Both oxidative and reductive reactions are required for the metabolism of both lignin and environmental pollutants. The decolorization can be achieved either by adsorption or oxidative degradation by the enzymes (Fu and Viraraghavan, 2001). The fungi secrete unique extracellular enzymes including a family of peroxidases; lignin peroxidase, manganese peroxidase, and laccase to catalyse both direct and indirect oxidation of chemicals. White-rot fungi contain all three enzymes and are therefore able to breakdown and mineralize several environmental pollutants into nontoxic forms. Some white-rot fungi produce all three enzymes whereas others produce only one or two of them (Hatakka, 1994).

Fountoulakis et. al (2002) reported that lignin can be degraded only by a few microorganisms such as white rot fungi basidiomycete, which produce manganese (MnPs) and lignin peroxidases (LiPs) and laccases that are responsible for the oxidation of lignin compounds. Color is removed by fungi by biosorption (Contato and Corso, 1996; Tatarko and Bumpus, 1998; Payman et al., 1998; Zheng et al., 1999; Fu and Viraraghavan, 2000), biodegradation (Nigam et al., 1995; Conneely et al., 1999) and enzymatic mineralization (LiP, MnP, Manganese independent peroxidase (MIP), Laccase) (Young and Yu, 1997; Ferreira et al., 2000; Ollikka et al., 1998; Podgornik et al., 1999; Wong and Yu, 1999; Zheng et al., 1999; Pointing and Vrijmoed, 2000, Wesenberg et al., 2003). However, one or more of these mechanisms could be involved in color removal depending on the fungus used.

The main reactions that are catalyzed by the ligninolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening (Reddy and Mathew, 2001). The process of chemical conversion by white rot fungi occurs via a free radical process. The ligninolytic enzymes in white rot fungi catalyze the degradation of pollutants by using a non-specific free radical mechanism (Pointing, 2001 and Law et al, 2003). Once the peroxidases have opened the aromatic ring structures by way of introducing oxygen, other more common species of fungi and bacteria can mineralize the products intracellularly into products such as CO_2 and other benign compounds (Pointing, 2001). The ability of white rot fungi to adsorb and accumulate metals together with the excellent mechanical properties of fungal mycelial pellets provide an opportunity for application of fungal mycelia in selective sorption of individual heavy metal ions from polluted water (Baldrian and Garbriel, 2003).

Fungi remove metals essentially by adsorption, chemisorptions (ion exchange), complexation, coordination, chelation, physical adsorption and microprecipitation (Guibal et al., 1995); Huang and Huang, 1996; Kapoor and Viraraghavan, 1997; Sarret et al., 1998). Because biomasses walls are composed of macromolecules (shitin, chitosan, glucan, lipid, phospholipids), which contain carboxyl groups, amino groups, phosphates, lipids, melanin sulfate and hydroxides (Caesartonthat et al., 1995; Kapoor and Viraraghavan, 1998 a, b; Fogarty and Tobin, 1996; Kapoor et al, 1999). Those functional groups are metals sorption sites (Tsezos and Volesky, 1982; Mullen et al., 1992; Guibal et al., 1995; Gardea Torresdey et al., 1996; Kapoor and Viraraghavan, 1997; Matheickal and Yu, 1997, Zhang et al., 1998; Sarret et al., 1999; McHale and McHale, 1994; Mashitah et al., 1999; Tereshina et al., 1999., Zhou, 1999).

2.4.2 The lignin-degrading system

A great number of white rot fungi have been reported to produce the lignin-degrading enzymes lignin-peroxidase, manganese peroxidase, laccase, or at least one of these enzymes (Elias et al., 2000). Lignin degradation occurs during the secondary metabolic phase (idiophase) when carbon, nitrogen, phosphorous or sulfur sources are limiting. The fungi produce and secrete several extracellular hydrolytic and non-hydrolytic digestive enzymes under these "ligninolytic" conditions.

Three lignin-modifying enzymes (LMEs) are thought to catalyse lignin degradation: lignin peroxidase (LiP), manganese (II)-dependent peroxidase (MnP) and laccase (phenol oxidase) (de Jong, 1993; de Jong et al. 1994). These LMEs have the ability to catalyze both the cleavage of carbon-carbon or carbon-oxygen bonds in complex lignin polymer or lignin model compounds (Kirk and Hammel, 1992; Hammel et al., 1993; Marzullo et al., 1996) and also one-electron oxidations resulting in the formation of radicals which undergo numerous spontaneous reactions. These, in turn, lead to various bond cleavages including aromatic ring fission (Shoemaker and Leisola, 1990; Tuor et al, 1992; Akthar et al., 1997; Zapanta and Tien, 1997). Several isoforms of each enzyme are secreted simultaneously by the fungus at different stages during lignin degradation.

a) Lignin peroxidase

LiP, originally designated as ligninase, was discovered for the first time in cultures of Phanerochaete chrysosporium (Tien and Kirks, 1983). Lignin peroxidase (LiP) is a monomeric N- and probably O-glycosylated protein expressed in several iso-forms. LiPs are extracellular glycosylated heme proteins. LiP requires H_2O_2 to be active. The enzyme is acidic having an iso-electric point between 3 and 5 and its optimum pH which is unusually low is between 2.5 and 3(Cho et al., 1999b).

In H_2O_2-dependent oxidations, aryl cation radicals are generated which are then further degraded non-enzymatically (Reddy, 1995). LiPs are of particular interest in pollutant degradation due to their very high oxidation-reduction potential that permits the oxidation of xenobiotics not attacked by other enzymes (Lamar et al., 1993). Several isoenzymes of LiP are produced, each distinct in terms of pH sensitivity and substrate specificity (Ollika et al., 1993; Tien and Kirk, 1988). Veratryl alcohol (3.3-dimethoxybenzyl alcohol) is a fungal metabolite which is necessary for LiP activity. Its exact role is unclear, but it is believed to play a role in preventing H_2O_2 inactivation of the enzyme (Harvey et al., 1 986; Wariishi and Gold, 1990). A schematic of the LiP reaction mechanism is depicted in

Figure 2.3. H_2O_2 oxidizes LiP to the LiP(I) state. LiP (I) oxidizes the substrate and is simultaneously reduced to LiP(II). It is believed that LiP(II) then oxidizes veratryl alcohol to veratraldehyde and is reduced back to its native state in the process (Barr and Aust, 1994; Kersten et al., 1985).

Strong evidence for the involvement of LiP in degradation of humic substances was found during the incubation of *P. chrysosporium* with brown coal using two different media, one which suppressed LiP and MnP (high N) and one which induced both of these enzymes (low N). No decrease in absorbance (400 nm) was found using the high-N medium, while a strong decrease occurred using the low-N medium (Ralph and Catcheside, 1999). The crude extract which exhibited high LiP activity was found to depolymerize humic substances (Catcheside and Ralph, 1999). In addition, polymerization of humic substances from coal was detected by using a mutant that was unable to produce LiP but synthesized MnP, suggesting that LiP is the key enzyme in degradation. A mutant that does not secrete either of the enzymes failed to depolymerize brown coal (Catcheside and Ralph, 1999).

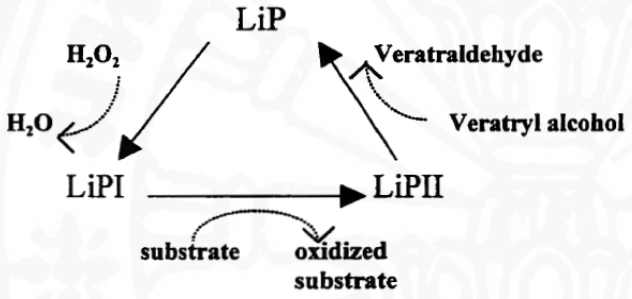

Figure 2.3 Catalytic cycle of substrate oxidation by LiP (Swamy, 1998)

b) Manganese peroxidase

MnPs are extracellular glycosylated heme proteins that catalyze the H_2O_2-dependent oxidation of Mn(II) to Mn(II), which then further mediates the oxidation of phenolic substrates. The structure of MnP is quite similar to that of LiP except that it has five rather than four disulfide bonds (Reddy, 1995). Several isoenzymes of MnP have been identified (Leisola et al., 1987; Paszczynski et al., 1986; Ruttimann-Johnson et al., 1994). MnP optimum pH varies from 3.5 to 5 for *T. versicolor*, (Nyman and Johansson, 1997).

The catalytic cycle of substrate oxidation by MnP is depicted in Figure 2.4. MnP is oxidized fiom its native state to MnP(I) by H_2O_2. MnP(I) oxidizes Mn^{2+} to Mn^{3+}, and is simultaneously reduced to MnP(II). Simple organic acids chelate and form organic complexes that oxidize the substrate, regenerating Mn^{2+} and the organic acid in the process. By oxidizing another Mn^{2+} ion to the Mn^{3+} state, MnP(II) is reduced back to native-state MnP.

Moreover, MnP has been suggested as a key enzyme in the degradation and mineralization of HAs (Hofrichter and Fritsche, 1997b, Hofrichter et al., 1998, Hofrichter et al., 1999). Ralph and Catcheside (1999) found that solubilized macromolecules from Morwell brown coal were depolymerized by MnP when incubated under hyperbaric O_2. However, under N_2 or air, they were polymerized, suggesting that a net depolymerization by Mn^{3+} requires molecular oxygen to inhibit the coupling of coal radicals. In contrast, they found that

solubilized brown coal inhibited the activity of both MnP and LiP (Ralph & Catcheside, 1994). Mn^{3+} acetate alone was also shown to bleach HA extracted from forest soil (Gramss *et al.,* 1999). Hofrichter *et al.* 1999 detected that synthetic ^{14}C labeled HA mineralization by MnP was considerably enhanced in the presence of the thiol mediator glutathione.

Recently investigations has revealed that the enzyme is capable of completing its catalytic cycle effciently in the absence of external H_2O_2 by oxidizing organic acids such as oxalate and malonate in an "oxidase-like" auto-catalytic reactions involving the transient formation of several radical species (Hofrichter et al., 1998).

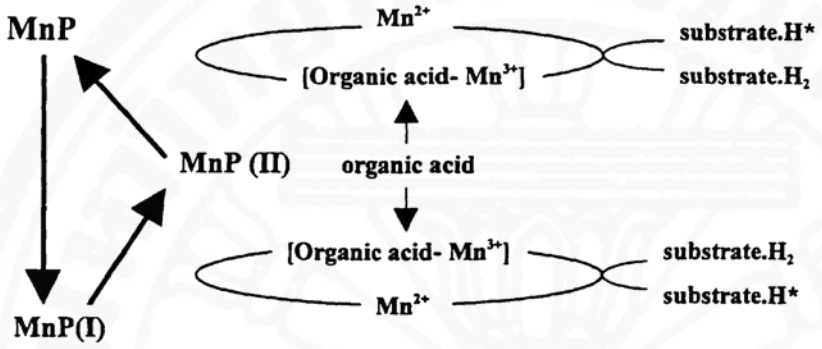

Figure 2.4 Catalytic cycle of substrate oxidation by MnP (Picard, 1995)

c) Laccases

The major enzyme laccase is a multicopper enzyme, and is the primary enzyme involved in the degradation process, which catalyses the oxidation of phenolic and non-phenolic compounds. It was first described in 1883, making it one of the oldest enzymes ever described (Mayer and Staples, 2002). It uses dioxygen as an oxidant, reducing it to water and it has the ability to catalyze the oxidation of a wide-range of dihydroxy and diamino aromatic compounds (Mester and Tien, 2000). Laccase usually contains four copper ions (Cu^{2+}) per monomer (Solomon et al., 1996). Laccases (benzenedioVoxygen oxidoreductase, phenol oxidase) are coppercontaining oxidases that oxidize polyphenols, methoxy-substituted phenols, diamines and other aromatic substrates (Thurston, 1994). In the presence of certain mediators such as 2, 2'-azlIio-bis (3-ethyIbe11~-thiazoline-6-sulfonic acid) the substrate range can be expanded to include non-aromatic substrates (Bourbonnais and Paice, 1990).

Laccase activity is highly dependent on Cu^{+2} which are distributed to 3 red-ox sites and defined according to their spectroscopic properties. The type 1 site contains the type blue copper (Cu1) whose 50tight coordination to a cysteine is responsible for an intense absorption band around 600 nm, giving its blue color to the enzyme. The type 2 site contains a type 2 copper (Cu2) with a characteristic electron paramagnetic resonance (EPR). In the T3 site, the pair of strongly coupled type 3 coppers (Cu3a and Cu3b) are EPR-silent in the presence of oxygen, indicative of a strongly antiferromagnetically coupled Cu pair bridged by a hydroxide.

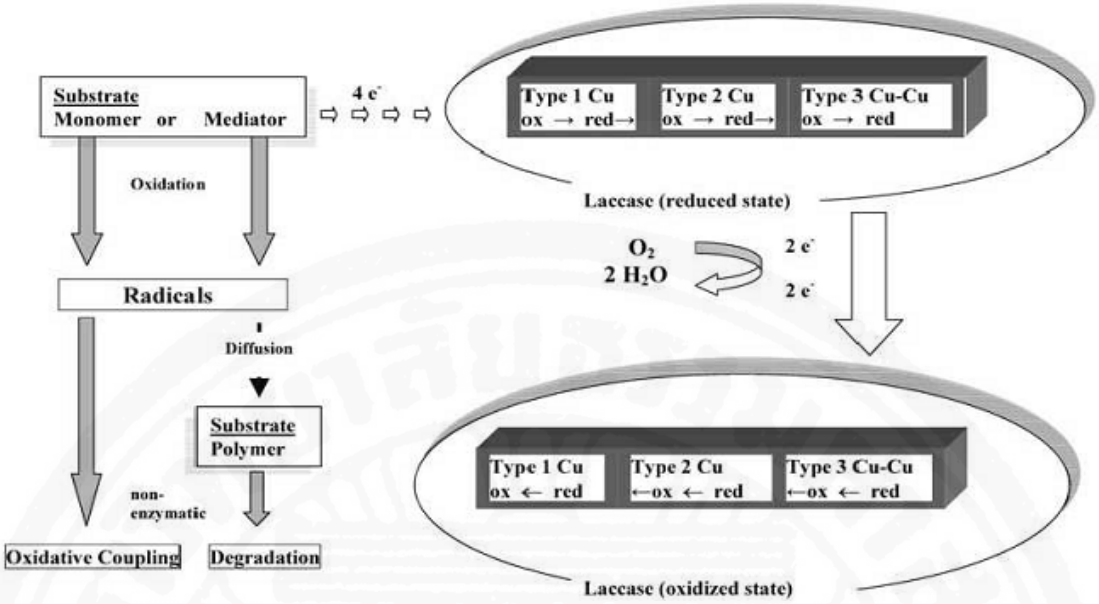

Figure 2.5 Catalytic cycle of laccase (Claus, 2004)

The mononuclear type 1 extracts electrons from the reducing substrate and mediates their transfer to the tri-nuclear type1/type3 cluster center where molecular oxygen is reduced as shown in Figure 2.5 (Claus, 2004). Laccase oxidizes preferentially phenolic lignin structures to phenoxy radicals which subsequently form quinones. This spontaneous rearrangement can also lead to the fission of carbon-carbon or carbon-oxygen bonds inside the lignin phenyl-propane subunits resulting either in the degradation of both side chains and aromatic rings (Kawai et al., 1988b), or in demethylation processes (Leonowicz et al., 1984). In this respect, laccase can co-operate with various FAD containing oxidases like glucose oxidase (Szklarz and Leonowicz 1986), veratryl alcohol oxidase (Marzullo et al., 1996), cellobiose:quinone oxidoreductase (Westermark and Eriksson, 1975) and cellobiose dehydrogenase (Ayers et al., 1978). It removes a hydrogen atom by a one-electron abstraction from hydroxyl or amino groups of ortho- and para-substituted phenolic substrates to form free radicals capable of undergoing further de-polymerization, re-polymerization, de-methylation, or quinone formation (Abadulla et al., 2000)

Although the actual role of laccase in lignin biodegradation is still under discussion and not completely understood, several authors have reported that laccase acts on the lignin polymer in ways different from ligninolytic peroxidases. In order to present the role of laccase more clearly several conceptions of biochemical routes in lignin biodegradation have been reviewed, especially with respect to the co-operation of laccase with other biocatalysts. The laccases as well as laccase mediator systems may be used in biopulping, biobleaching, removal of toxic phenolic compounds from aquatic and terrestrial systems, synthesis of useful polymers by reticulation of phenolic compounds, degradation of lignite and hard coal structures, as analytical tools and as biosensors to estimate the quantity of phenols in natural juices or the presence of other enzymes (Ahn et al., 1999).

However, not all white rot fungi produce all LMEs. It is accepted that the ligninolytic systems are not fully characterized and that there may be other unidentified enzymes that contribute to ligninolytic activity. Apart from these three enzyme families, other enzymes are known to be involved in lignin degradation, including glyoxal oxidase (GLX), cellobiose dehydrogenase (CDH), aryl alcohol oxidase (AAO), versatile peroxidase (VP) and cytochrome P450 and may also be involved in turnover of humic substances.

Table 2.17 Ligninolytic enzymes and their main reactions

Enzyme and abbreviation	Cofactor	Substrate, mediator	Reaction
Lignin peroxidase, LiP	H_2O_2	Veratryl alcohol	Aromatic ring oxidised to cation radical
Manganese peroxidase, MnP	H_2O_2	Mn, organic acids as chelators, thiols, unsaturated fatty acids	Mn(II) oxidised to Mn(III); chelated Mn(III) oxidises phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Versatile peroxidase, VP	H_2O_2	Mn, veratryl alcohol, compounds similar to LiP and MnP	Mn(II) oxidised to Mn(III), oxidation of phenolic and non-phenolic compounds, and dyes
Laccase	O_2	Phenols, mediators, e.g., hydroxybenzotriazole or ABTS	Phenols are oxidised to phenoxyl radicals; other reactions in the presence of mediators
Glyoxal oxidase, GLOX		Glyoxal, methyl glyoxal	Glyoxal oxidised to glyoxal acid; H_2O_2 production
Aryl alcohol oxidase, AAO		Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohols oxidised to aldehydes; H_2O_2 production
Other H_2O_2-producing enzymes		Many organic compounds	O_2 reduced to H_2O_2

(Source: Hatakka, 2001)

Table 2.17 summarises the ligninolytic enzymes and their substrates and reactions. The main extracellular enzymes participating in lignin degradation are lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and Cu-containing laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). A new group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the versatile peroxidases (VPs). In addition, enzymes involved in hydrogen peroxide production such as glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (EC 1.1.3.7) are considered to belong to the ligninolytic system.

2.4.3 Immobilisation of fungi

Immobilised fungal cells have several advantages over dispersed cells such as simple reuse of the biomass, easier liquid–solid separation and minimal clogging in continuous-flow systems (Arica et al., 1993 and Tieng and Sun, 2000). In addition, immobilised cultures tend to have a higher level of activity and are more resilient to environmental perturbations such as pH, or exposure to toxic chemical concentrations than suspension cultures (Shin et al., 2002) and immobilisation protects the cells from shear damage (Abraham et al., 1991,

Fiedurek and Ilczuk, 1991 and Vassilev and Vassileva, 1992). Moreover, cell immobilisation lowers the apparent broth viscosity and makes the rheological features more favourable for oxygen supply and mass transfer (Thongchul and Yang, 2003). When applied to recombinant strains, cell immobilisation can also alleviate strain genetic stability problems (Caunt et al., 1988 and Dincbas et al., 1993). Another advantage of cell immobilisation is a reduction in the protease activity and contamination risk.

Basically, there are two types of cell immobilisation: entrapment and attachment. In the former, the micro-organisms are entrapped in the interstices of fibrous or porous materials or are physically restrained within or by a solid or porous matrix such as a stabilised gel or a membrane. In the latter, the micro-organisms adhere to surfaces or other organisms by self-adhesion or chemical bonding. Self-adhesion is not considered in the present review.

2.4.3.1 Entrapment

A variety of matrices have been used for cell immobilisation *via* the entrapment technique, such as natural polymeric gels (agar, carrageenan, alginate, chitosan and cellulose derivatives) and synthetic polymers (polyacrylamide, polyurethane, polyvinyl) (Katzbauer et al., 1995). Entrapment in natural polymeric gels has become a preferred technique for cell immobilisation because of the toxicity problems associated with synthetic polymeric materials (Lusta et al., 1990). The use of natural gels is, however, limited by their mechanical strength and the lack of open spaces to accommodate active cell growth resulting in their rupture and cell release into the growth medium (Barbotin and Nava Saucedo, 1998). Their efficiency is also limited by diffusion restrictions and alginate gels are unstable in contact with various quelating agents such as phosphate and citrate which are frequently used in culture media (Birnbaum et al., 1981). These drawbacks have impelled the search for other types of biomaterials such as those from plant sources. The advantages accruable from such bio-structures are reusability, freedom from toxicity problems, mechanical strength for necessary support and open spaces within the matrix for growing cells, thus, avoiding rupture and diffusion problems (Abdullah et al., 1995 and Akhtar et al., 2004). In this regard, Iqbal and Saeed (2006) developed a novel immobilisation technique by using a structural fibrous network of papaya wood as an immobilising matrix for the entrapment of *Aspergillus terreus* cells.

2.4.3.2 Attachment

The materials commonly used for the attachment procedure are synthetic foams like polyurethane foam (Nakamura et al., 1997) and nylon sponge (Haapala and Linko, 1993). Recently, stainless steel sponge has also been described as a very suitable synthetic material for fungi immobilisation (Rodríguez Couto et al., 2004a). One of the advantages found when using stainless steel sponges was that dyes did not adsorb onto it. Also, natural supports (organic materials) can be used to immobilise fungi by the attachment technique. In addition, it would be an environmentally friendly way for waste management. The use of a natural support instead of an artificial inert one to immobilise fungi is advantageous because an immobilisation material that mimics the natural habitat of the fungi can provide them with additional nutrients and stimulate the production of ligninolytic enzymes (Rodriguez et al., 1999, Rodriguez Couto et al., 2003 and Rodriguez Couto et al., 2004b). Nevertheless, the use of natural supports can cause problems in a bioreactor; for example, degradation and loss of the supporting material may lead to blockages in the waste stream and a constant flow of wastewater may lead to permanent losses of enzymes from the

fungal extracellular matrix. Recently, Böhmer et al. (2006) ameliorated some of these problems by the use of a temporary immersion system.

In order to select both the immobilisation method and the immobilisation material Biria et al. (2008) have proposed the utilisation of a novel model called corrugated parallel bundle model (CPBM). This model showed the possibility of evaluating the lifetime cycle of immobilised cells in order to replace them on time and prevent any reduction in the process efficiency. At the same time, their findings indicated the impact of various kinetic conditions on issues such as effectiveness factor and plugging. In this way it is possible to detect and investigate the defects, which may arise during the operational process.

2.4.4 Bioreactor configurations for white rot fungi cultures

Possible bioreactor configurations for the culture of white rot fungi may be divided into agitated and non-agitated growth processes. Examples of bioreactor processes with white rot fungi are discussed in the following section.

2.4.4.1 Agitated growth processes

The ability of white rot fungi to degrade lignin and other compounds has been shown to decrease drastically in mechanically aerated cultures (Alleman et al., 1991; Faison and Kirk, 1985; Jager et al., 1985; Kirk et al., 1978). The main concern with agitated cultures is decreased or lost production and/or activity of the ligninolytic enzymes, especially LiP, due to shear from mechanical agitation. Although detergents such as Tween and polyethylene glycol prevent some inactivation, relatively low agitation speeds are still required (Asther et al., 1987; Jager et al., 1985).

2.4.4.2 Agitated processes – suspended growth

When agitated, freely suspended fungal biomass grows as mycelial pellets. Pellet size is determined by the speed of agitation and the amount of biomass in the inoculum (Jimenez-Tobon et al., 1997). Typical reactor configurations for suspended growth processes are conventional stirred tank reactors with mechanical mixing and air-lift fermentors, Bonnarme et al. (1993) compared growth and ligninolytic enzyme production by *P.chrysosporium* in a stirred tank reactor, airlift fermentor and a bubble column reactor. Aeration in the pneumatically agitated reactors (airlift and bubble column) was achieved by sparging and in the stirred tank reactor, with marine impellers operated between 150 to 300 rpm. More biomass and significantly higher levels of ligninolytic enzymes (e.g. 6 to 9-fold more LiP) were produced in the pneurnatic reaction and were attributed to improved oxygen transfer.

LiP production by *P.chrysosporium* was demonstrated in a 42L submerged stirred tank reactor, but scale-up to a 300 L stirred tank reactor was unsuccessful (Jansheka. and Fiechter, 1988). While investigating growth and LiP production by *P.chrysosporium* in a stirred tank reactor, Venkatadri and lrvine (1990) found that the mycelia clumped and adhered to the walls and impellers, resulting in relatively low yields of LiP.

2.4.4.3 Agitated processes - immobilized growth

Fungal mycelium adheres to hydrophobic supports and is readily immobilized on porous particles such as agar, agarose, alginate or silicon beads, polyurethane foam or nylon web cubes, and macroporous glass (Kirkpatrick and Palmer, 1987; Linko et al., 1986; Rogalski et al., 1992). These processes are often described as "immobilized biocatalyst fermentations". The "biocatalysts" (support + fungus) can be agitated to improve mass and oxygen transfer without the problems encountered with freely suspended fungal pellets. Advantages include improved oxygen and mass transfer and the recycling and repeated use of the "biocatalysts". However, such methods, in general, are expensive at an industrial scale. LiP production by agitated cultures of P. *chrysosporiurn* immobilized on agar, agarose and k-carrageenan gel beads, nylon web and polyurethane cubes was several fold higher than by freely suspended mycelial pellets (Linko et al., 1986). The nylon and polyurethane-based biocatalysts were active for at least 38 days after the addition of veratryl alcohol, which triggered LiP expression. In a subsequent study, *P.chrysosporiurn* immobilized on 0.5 cm nylon web cubes or polyurethane foarn cubes in an 8 L bioreactor produced high maximum yields of LiP continuously for up to a week (Linko, 1988). Agitation was either with air and/or oxygen 80w only or with mechanical stimng at 220 rpm. *T* versicolor, immobilized in calcium alginate beads, achieved 80% decoloration of kraft wastewater compared to 60% by suspended pellets (Livernoche et al., 1983).

2.4.4.4 Non-agitated immobilized growth processes

Several studies have examined fixed bed reactors, rotating biological contactors, rotating tube reactors and other configurations for the culture of white rot fungi. In fixed growth processes, the fungus forms a biofilm on the given surface. Hollow fiber reactors typically consist of hollow fibers mounted inside a cylindrical cartridge. The microporous structure and hydrophilic surfaces of the hollow fibers permitted the exchange of nutrients and metabolic products between the immobilized biofilm on the outside and the medium flowing within (Venkatadri and Irvine, 1993). The silicone membrane reactor used by Venkatadri and Irvine (1993) consisted of 15 feet of silicone tubing wrapped around five stainless steel rods in a stirred tank reactor and submerged in 1.5 L of growth medium. Pressurizing air through one end of the tubing, with the other end sealed, transferred oxygen across the walls to the cells immobilized on the outside wall of the tubing and the bulk liquid phase. The inherent advantages of such processes include the preference of the fungi to grow attached to surfaces, lack of mechanical shear and increased mass and oxygen transfer.

2.4.5 Treatment of wastewater by immobilized white rot fungi

The removal of several synthetic dyes by fungi immobilised on different systems was summarized in Table 2.18.

Table 2.18 Dye decolouration by fungi immobilised on different supports

Immobilisation support	Microorganism	Dye	Reference
Nylon sponge	*Pycnoporus cinnabarinus*	Remazol Brilliant Blue R	Schliephake and Lonergan (1996)
PVAL hydrogel beads	*Trametes versicolor*	Poly R-478	Leidig et al. (1999)
Alginate beads	Unidentified basidiomycetous	Orange II	Zhang et al. (1999)
Plastic discs	*Coriolus versicolor*[a]	Everzol Turquoise Blue G	Kapdan et al. (2000)
Corncob, Polyurethane foam PUF, nylon sponge	*Phanerochaete chrysosporium*	Poly R-478	Rodríguez Couto et al. (2000)
Grape seeds, wheat straw, wood shavings	*P. crysosporium*	Poly R-478	Rodríguez Couto et al. (2001)
PUF	*P. chrysosporium*	Poly R-478	Mielgo et al. (2002)
Barley bran	*T. versicolor*	Acid Fuchsine, Congo Red, Indigo Carmine	Rodríguez Couto et al. (2002)
Wheat straw, hemp fiber, hemp mat, hemp core, jute twine, polyethylene teraphthalate fiber, hemp-polypropylene fiber	*T. versicolor*	Amaranth	Shin et al. (2002)
PUF, pine wood	*Irpex lacteus*	Remazol Brilliant Blue R	Kasinath et al. (2003)
Nylon sponge, grape cluster stems	*P. chrysosporium*	Poly R-478	Rodríguez Couto et al. (2003)
Plastic discs	*Phanerochaete sordida*	Basic Blue 22	Ge et al. (2004)
Luffa cylindrica sponge	*Funalia trogii*	Reactive Black 5	Mazmanci and Ünyayar (2005)
Plastic net	*Bjerkandera adusta*	Reactive Black 5	Mohorcic et al. (2004)
Jute twine	*T. versicolor*	Carpet dye effluent	Ramsay and Goode (2004)
Stainless steel sponge	*T. hirsuta*	Indigo Carmine, Lanaset Marine	(Rodríguez Couto et al., 2004a) and (Rodriguez Couto et al., 2004b)
Stainless steel sponge	*T. hirsuta*	Sella Solid Blue	Rodríguez Couto and Sanromán (2004)
Corn cob	*Pleurotus pulmonarius*	Remazol Brilliant Blur,	Tychanowicz et

Immobilisation support	**Microorganism**	**Dye**	**Reference**
		Ethyl Violet, Methyl Violet, Methyl Green, Brilliant Cresyl Blue, Methylene Blue, Poly R-478, Congo Red, Trypan Blue, Amido Black	al. (2004)
Alginate beads	*T. hirsuta*	Indigo Carmine, Phenol Red	Domínguez et al. (2005)
Sugarcane bagasse	*Pleurotus sajor-caju*	real textile effluent	Kamida et al. (2005)
$ZrOCl_2$-activated pumice	*P. chrysosporium*	Direct Black 38, Direct Brown 2, Direct Red 23, Direct Blue 15, Direct Orange 26, Direct Green 6, Tartrazine, Chrysophenin and Congo Red	Pazarlioglu et al. (2005)
Alginate beads	*P. chrysosporium*	Methyl violet, Congo Red, Acid Orange, Acid Red 114, Vat Magenta, Methylene Blue and Acid Green	Radha et al. (2005)
Alginate beads	*T. versicolor*	Amaranth	Ramsay et al. (2005)
Coconut flesh	*T. hirsuta*	Lissamine Green B	Rodríguez Couto and Sanromán (2005)
Pine wood chips, palm oil fiber	*T. versicolor*, *P. chrysosporium*	Levafix Blue, Remazol Brilliant Red.	Böhmer et al. (2006)
Kissiris (a mineral)	*P. chrysosporium*	Methylene Blue	Karimi et al. (2006)
Luffa sponge, birch wood	*T. versicolor, Pleurotus ostreatus*, *P. sajor-caju*, *P. chrysosporium*, *Pleurotus flabellatus*	Reactive Blue 4, Reactive Red 2	Nilsson et al. (2006)
Dyed wheat straw	*Fomes sclerodermeus*	Malachite Green	Papinutti et al. (2006)
Na-alginate beads	*F. trogii*	Acid Black 52	Park et al. (2006)
Orange peelings	*T. hirsuta*	Indigo Carmine, Bromophenol Blue, Methyl Orange, Poly R-478	Rodríguez Couto et al. (2006)
PUF	*I. lactus*	Reactive Orange 16	Tavcar et al. (2006)
PUF, pine wood	*Dichomitus squalens*	Remazol Brilliant Blue R, Reactive Orange 16,	Susla et al. (2007)

Immobilisation support	Microorganism	Dye	Reference
		Copper(II) phthalocyanine	
PUF	*I. lacteus*	Reactive Orange 16	Svobodová et al. (2007)
Wheat bran	*C. versicolor*[a] f. *antarcticus*	Malachite Green	Diorio et al. (2008)
Stainless steel sponges	*Trametes pubescens*	Reactive Black 5	Enayatzamir et al. (2008)
Rice hull	*Schizophyllum* sp.	Congo Red	Li and Jia (2008)
Reticular carrier, PUF	*P. chrysosporium*	Reactive Brilliant Red K-2BP	Gao et al. (2008)
Sunflower seed shells	*T. pubescens*	Reactive Black 5	Rodríguez Couto et al. (2008)
Carboxymethylcellulose beads	*Aspergillus fumigatus*	Reactive Blue 19	Wang and Hu (2008)

(Source: Rodríguez Couto, 2009)

Schliephake and Lonergan (1996) studied the decolouration of the synthetic dye Remazol Brilliant Blue R (RBBR) by the white-rot fungus *Pycnoporus cinnabarinus* grown on nylon web cubes in a 200 L packed-bed bioreactor. They found that the dye was rapidly decolourised due to the action of the laccases produced by the fungus.

Leidig et al. (1999) showed that encapsulation of *Trametes versicolor* in polyvinylalcohol (PVAL)-hydrogel beads protected the mycelia from mechanical damage and the ligninases from biochemical degradation by presumably proteases of bacterial contaminants. In addition, the polymeric dye Poly R-478 was removed by the encapsulated *T. versicolor* in a stirred tank reactor (working volume 1 L) with an efficiency of 89%, which was due partially to biotransformation (65%) and partially to adsorption onto biomass (24%).

Also, Zhang et al. (1999) studied the decolouration of the azo dye Orange II by free and alginate-immobilised cells of an unidentified white-rot fungus in continuous packed-bed, fed-batch fluidised-bed and continuous fluidised-bed bioreactors. They found that the immobilised fungus performed better than the free one and could continuously be reused for more than two months with high efficiency (97% in 24 h).

Kapdan et al. (2000) studied the decolouration of the textile dye Everzol Turquoise Blue G by the white-rot fungus *Coriolus versicolor* (now classified as *T. versicolor*) immobilised on discs in a rotating biological contactor (RBC). The system was operated in repeated-batch mode with 48 h hydraulic retention time (HRT). They found that control of biofilm thickness, glucose concentration, rotational speed and dye concentration had an acute effect on dye decolouration. Three different disc types: plastic, metal mesh covered plastic and metal mesh discs were used and the plastic ones were found to be the most suitable. The highest decolouration efficiency (80%) was obtained with a rotational speed of 30 rpm and an initial dye concentration of 50–200 mg/L. Also, minimum glucose concentration for 77% decolouration efficiency was 5 g/L.

Rodríguez Couto et al. (2000) reported that *Phanerochaete chrysosporium* immobilised on cubes of polyurethane foam (PUF) decolourised the polymeric dye Poly R-478 (0.03% w/v) in 6 days. Also, Rodríguez Couto et al. (2001) studied the decolouration of the polymeric dye Poly R-478 by cultures of *P. chrysosporium* grown on grape seeds, wheat straw and wood shavings under solid-state conditions. The percentage of biological decolourisation attained by grape seed, wheat straw and wood shaving cultures was around 74%, 40% and 63%, respectively. Later, Mielgo et al. (2002) proposed a pulsed packed-bed bioreactor with *P. chrysosporium* immobilised on PUF cubes for the decolouration of the polymeric dye Poly R-478. They reported a maximum decolouration rate around 7.9 mg/L h. In addition, excessive growth of mycelia was avoided by the limitation of nutrient supply and gas pulsation which made possible a long-term operation.

Shin et al. (2002) assessed different natural and synthetic support materials such as wheat straw, jute, hemp, maple wood chips, and nylon and polyethylene teraphthalate fibers for the surface immobilisation and decolouration of Amaranth by *T. versicolor*. They found that jute was the best material for both fungus growth and dye decolouration. The fungus immobilised on jute, straw and hemp decolourised Amaranth at a rate of about 5 mg/L h without glucose being added. However, the decolouration rate increased when 1 g/L glucose was added (about 8 mg/L h).

Kasinath et al. (2003) studied the effect of growth conditions of the white-rot fungus *Irpex lacteus* on the degradation of different commercial dyes. They found that *I. lacteus* immobilised on PUF and pine wood cubes was capable of efficient decolouration of chemically complex wastewater from the textile industry. In addition, due to their robustness and regenerating ability these cultures were reusable, which was essential for their long-term applicability to bioremediation of dye-polluted wastewater.

Rodríguez Couto et al. (2003) investigated the decolouration of the polymeric dye Poly R-478 by the white-rot fungus *P. chrysosporium* immobilised on nylon sponge and on grape cluster stems. The former led to a decolouration higher than 90% after four days of dye addition, whereas the latter led to a decolouration of around 70% after eight days of dye incubation. The lower percentage of dye decolouration achieved by the cultures grown on grape cluster stems was due to the enzymes produced were not only used in the decolouration of the dye but also in the degradation of the grape cluster stems, since it was found that about 20% of klason lignin, 48% of hemicellulose and 5% of cellulose contained in the above-mentioned support were degraded along cultivation.

Ge et al. (2004) studied the decolouration of the textile dye Basic Blue 22 by the white-rot fungus *Phanerochaete sordida* immobilised on a disc in a RBC operated in repeated-batch mode with 48 h HRT. The effects of different operating parameters (disc type, rotational speed, glucose and dye concentration) on dye decolouration were investigated. The highest decolouration efficiency (78%) was obtained with a rotational speed of 40 rpm, a glucose concentration of 5 g/L and an initial dye concentration lower than 200 mg/L.

Mazmanci and Ünyayar (2005) reported that cultures of *Funalia trogii* immobilised on *Luffa cylindrica* sponge efficiently decolourised the recalcitrant diazo dye Reactive Black 5 (6.86–8.22 mg dye/g dmw day). They found that dye decolouration was only due to *F. trogii* enzymes.

Also, Mohorcic et al. (2004) studied the decolouration of Reactive Black 5 by the white-rot fungus *Bjerkandera adusta* immobilised on a plastic net in a 5-L stirred tank bioreactor. They observed a decolouration of the dye (0.2 g/L) from black-blue to intense yellow in 20 days.

Ramsay and Goode (2004) found that *T. versicolor*, immobilised on jute twine in a RBC was able to decolourise a carpet dye effluent containing two anthraquinonic dyes in four consecutive batches without increasing toxicity.

Rodríguez Couto et al., 2004a and Rodriguez Couto et al., 2004b studied the decolouration of the dyes Indigo Carmine and Lanaset Marine by *T. hirsuta* immobilised on stainless steel sponges in a 1-L fixed-bed bioreactor. Indigo Carmine was almost totally degraded in 3 days, while Lanaset Marine was degraded in two successive batches, reaching in the first batch a decolouration percentage of about 82% in 15 h and in the second one of about 71% in 28 h. In addition, they found that a much higher decolouration percentage was achieved in actively growing cultures of *T. hirsuta* than with the culture filtrate. Moreover, results obtained after inhibition of *T. hirsuta* growth by antibiotics indicated that dye decolouration could not exclusively be attributed to the laccase produced but also other growth associated mechanisms seemed to contribute to dye decolouration by *T. hirsuta.*

Rodríguez Couto and Sanromán (2004) reported the decolouration of the leather azo dye Sella Solid Blue (Direct Blue 78) in an immersion bioreactor operated in continuous with the white-rot fungus *T. hirsuta* immobilised on stainless steel sponges. The dye was almost totally decolourised operating at an HRT of 24 h. In addition, no operational problems were found making possible a long-term operation.

Tychanowicz et al. (2004) studied the decolouration of several structurally different synthetic dyes by the white-rot fungus *Pleurotus pulmonaris* grown on corncob under solid-state conditions. Thus, Amido Black, Congo Red, Trypan Blue, Methyl Green, RBBR, Methyl Violet, Ethyl Violet and Brilliant Cresyl Blue were completely decolourised after 6 days of cultivation, while Methylene Blue and Poly R-478 were partially decolourised. The decolourising capability of the fungal cultures appeared to be correlated with the high titres of laccase (480 U/mL) produced in response to the presence of soluble phenolics.

Domínguez et al. (2005) studied the decolouration of the dyes Indigo Carmine and Phenol Red by *T. hirsuta* immobilised in alginate beads in a 2-L airlift bioreactor. They obtained high decolouration percentages in a short time (96% for Indigo Carmine and 69% for Phenol Red in 24 h).

Kamida et al. (2005) investigated the decolouration of a textile effluent by *Pleurotus sajor-caju* grown on sugarcane bagasse. The effluent was totally decolourised after 14 incubation days.

Pazarlioglu et al. (2005) studied the decolouration of the azo dye Direct Blue 15 (20 mg/L) by *P. chrysosporium* immobilised on $ZrOCl_2$-activated pumice in a small-scale packed-bed reactor. The colour removal efficiency in repeated batches was found to be 95–100%. It was observed that MnP played an important role in dye decolouration, while there was no obvious role for LiP and adsorption was determined as a minor mechanism.

Also, Radha et al. (2005) found that *P. chrysosporium* was able to biodegrade several synthetic dyes of different structures such as azo, anthraquinone, thiazine and vat dyes in both free and alginate-immobilised cultures with a decolouration percentage higher than 75%.

Ramsay et al. (2005) reported that *T. versicolor* immobilised in alginate beads was able to decolourise the synthetic dyes Amaranth, Reactive Black 5, Reactive Blue 19 and Direct Black 22 and mixtures of these dyes in the presence of glucose alone.

Rodríguez Couto and Sanromán (2005) studied the decolouration of the industrial dye Lissamine Green B (0.03% w/v) by cultures of *T. hirsuta* immobilised on coconut flesh. They found that the dye was decolourised higher than 96% in 2.5 h. Decolouration was mainly due to the extracellular laccase secreted by the fungus, since the dye adsorbed onto both biomass and support was less than 2%.

Böhmer et al. (2006) immobilised the white-rot fungi *T. versicolor* and *P. chrysosporium*, separately, on both pine wood chips and palm oil fiber and cultivated them in the temporary immersion RITA® (R*écipient à* I*mmersion* T*emporaire* A*utomatique*) System. The textile dyes Levafix Blue and Remazol Brilliant Red were successfully decolourised in a series of four batches.

Karimi et al. (2006) studied the decolouration of the synthetic dye Methylene Blue (60 mg/L) by *P. chrysosporium* immobilised on the mineral Kissiris. The dye was almost totally decolourised in 8 days due to the action of the MnPs produced by the fungus.

Nilsson et al. (2006) investigated the decolouration of synthetic wastewater containing either Reactive Blue 4 or Reactive Red 2 by *T. versicolor* immobilised on natural luffa sponge at flask scale. The former was also decolourised (70%, initial dye concentration 200 mg/L) by *T. versicolor* immobilised on birch wood discs in a continuous RBC operated in continuous at an HRT of 3 days. In addition, they studied the decolouration of real textile wastewater by *Pleurotus flabellatus* growing on luffa sponge in a packed-bed reactor operated in continuous at an HRT of 25 h and found that about 66% was decolourised.

Papinutti et al. (2006) used wheat bran dyed with the fungicide dye Malachite Green as a substrate for the growth of the white rot fungi *Fomes sclerodermeus* and *P. chrysosporium*. Both fungi were able to grow on the dyed wheat bran and to degrade the dye adsorbed.

Recently, Rodríguez Couto et al. (2008) have used dyed sunflower seed shells as a support-substrate for the production of laccase by the white-rot fungus *Trametes pubescens*. The fungus was not only able to grow on the dyed sunflower seed shells but also to produce high laccase activities and to degrade the dye adsorbed onto the shells.

Park et al. (2006) found that the immobilisation of the white-rot fungus *F. trogii* in alginate beads allowed the decolouration of the dye Acid Black 52 (100 mg/L) in a stirred tank reactor operated in batch. The decolouration rate obtained by the immobilised cultures was higher than that attained by the free ones (93.8% and 88%, respectively). In addition, the decolouration was maintained with repeated batch experiments for a long period (at least for 300 h).

Rodríguez Couto et al. (2006) studied the decolouration of different synthetic dyes by the white-rot fungus *T. hirsuta* immobilised on ground orange peelings in fixed-bed bioreactors in both batch and continuous mode. The batch operation led to high decolouration percentages in a short time (100% for Indigo Carmine in 3 h and 85% for Bromophenol Blue in 7 h). For the continuous process the best results were found operating at an HRT of 3 days (94% for Indigo Carmine, 81.4% for Methyl Orange and 46.9% for Poly R-478).

Tavcar et al. (2006) studied the decolouration of the azo dye Reactive Orange 16 by *I. lacteus* immobilised on PUF in three different reactor systems of laboratory size: small and large trickle-bed reactors and a rotating-disc reactor. They observed the highest dye decolouration efficiency (90% in 3 days) in the small trickle bed reactor.

Šušla et al. (2007) found that the white-rot fungus *Dichomitus squalens* immobilised on PUF and pine wood cubes in a fixed-bed reactor operated in batch was able to decolourise the synthetic dyes RBBR and Reactive Orange 16 (93% and 73% and 94% and 42%, respectively, in 1 day).

Also, Svodobodá et al. (2007) investigated the decolouration of the synthetic dye Reactive Orange 16 by *I. lacteus* cultures immobilised on PUF. They found that 80% of dye decolouration was achieved within 24 h. In addition, they identified three degradation products by using LC-MS analysis. Despite significant laccase activities detected in the fungal cultures, no backward polymerisation of the reaction products resulting in recurrent colouration was observed after fungal treatment of the dye solution as suggested by Zille et al. (2005).

Diorio et al. (2008) studied the decolouration of the dye Malachite Green by *C. versicolor* (now classified as *T. versicolor*) f. *antarcticus* grown on wheat bran using a two-phase bioreactor. In the first phase the dye was decolourised in the presence of the fungus and when the dye solution was decolourised about 50%, it was discharged to a stainless steel coil at 50 °C initiating the second phase of decolouration. The total decolouration attained by this system was 82% after five consecutive cycles. In addition, the dye was also detoxified.

Enayatzamir et al. (2008) studied the decolouration of the diazo dye Reacive Black 5 by *T. pubescens* immobilised on stainless steel sponges in a fixed-bed reactor. They found that laccase production was increased by 10-fold in the presence of the aforementioned dye, reaching a maximum value of 1025 U/L. In addition, Reactive Black 5 was decolourised during four successive batches with high decolouration percentages without addition of redox mediators.

Gao et al. (2008) developed a treatment approach to degrade the dye Reactive Brilliant Red K-2 BP under non-sterile conditions by using the white-rot fungus *P. chrysosporium* immobilised on different inert carriers (reticular carrier and PUF). They found that under non-sterile conditions, higher MnP activity (690 U/L *vs* 125 U/L), higher decolouration efficiency (93.5% *vs* 15%) and shorter reaction period (3 days *vs* 6 days) were achieved in the immobilised cultures in comparison with the suspension ones. In addition, with the immobilised fungal cultures no difference was observed under non-sterile and sterile conditions for the degradation of reactive dye K-2BP.

Li and Jia (2008) developed an efficient and relatively simple continuous decolouration system utilising the combination rice hull-*Schizophyllum* sp. in a packed-bed bioreactor (working volume 1000 mL) for decolourising the azo dye Congo Red. A maximum total decolouration of 89.7% was achieved at a dye concentration of 142.6 mg/L and an HRT of 41 h. However, several aspects related to improving the bioreactor design, optimising more process parameters, enhancing decolouration and greater automation are needed for increasing the industrial exploitation of the continuous decolouration processes similar to the system rice hull-*Schizophyllum* sp.

Wang and Hu (in press) investigated the removal of Reactive Brilliant Blue KN-R using growing *Aspergillus fumigatus* immobilised on carboxymethylcellulose beads. They found that bioaccumulation was the dominant mechanism of dye removal and believed that this dye bioaccumulation biotechnology had a good perspective in the future application due to the efficient dye removal and no toxic by-products produced in the whole process. It overcame the problem of decolouration by biodegradation, which may produce new toxic by-products due to incomplete mineralisation.

Debendra et. al. (2004) reported that *Aspergillus Fumigatus* was an efficient strain for decolorization of effluents of pulp and paper industry based on agricresidues and extracellular enzymes such as laccase, manganese peroxidase and xylanases might be responsible for its effluent decolorization potential.

Levin et. al. (2003) tested the biodegradation of two PAHs, nitrobenzene and anthracene, by a white rot basidiomycete, *Trametes Trogii*. They found that the fungus was tolerant to extremely high levels of these compounds (250-500ppm) and it was able to metabolize 90-97% of highly concentrated anthracene and nitrobenzene, respectively, during the primary phase of fungal growth (trophophasic phase) and 100% of the compounds during the secondary phase of growth (idiophasic phase).

Yateem et. al (1998) found that white rot fungi are considered potentially useful microorganisms for bioremediation applications. *Coriolus Versicolor* showed the highest degradation rate, as the total petroleum hydrocarbon (TPH) concentration decreased from 32 g/kg to reach 7g/kg within 12 months.

Rashmi et. al (2005) reviewed that in the dye processing industry, white rot fungus, namely *Coriolus Versicolor* can remove color as well as COD up to 95% and 75%, respectively in a batch reactor. Decolorizing was observed during the repeated reuse of the fungal pellets for eight cycles during the long term operation, where medium and dye was replenished at the end of each cycle and the fungus was recycled.

Brian et. al (2001) found that decolorization of 53 to 73% could be attained using a hydraulic retention time of 23 hour. With *Rhizomucor Pusillus* strain RM7, a mucoralean fungus, 55% of AOX (Adsorbable Organic Halogen) were removed compared to 40% by *Coriolus versicolor*. Fungal treatment with both *R. Pusillus and C. versicolor* rendered the effluent essentially non-toxic. Additional of glucose to decolorization media stimulated color removal by *C. versicolor* but not with *R. Pusillus*. Ligninolytic enzymes (manganese peroxidase and laccase) were only detected in effluent treated by *C. versicolor*. It seems that there are definite differences in the mucoralean fungus (adsorption+biodegradation) and the mucoralean fungus (adsorption).

Selvam et. al (2003) suggested that the batch mode of treatment using *Thelephora sp.* may be more effective than the continuous mode for color removal. A maximum decolorization of 61% was achieved on the third day in batch mode and 50% obtained by the seventh day in continuous mode. *Thelephora sp.* was used for decolorization of azo dyes such as orange G (50 μm), congo red (50 μm) and amido black 10B (25 μm). Decolorization using the fungus was 33.3%, 97.1% and 98.8% for orange G, congo red and amido black 10B, respectively. An enzymatic dye decolorization study showed that a maximum of 19% orange G was removed by laccase at 15 U/ml whereas lignin peroxidase (LiP) and manganese peroxidase (MnP) at the same concentration decolorized 13.5% and 10.8%, orange G, respectively. A maximum decolorization of 12% and 15% for congo red and amido black 10B, respectively was recorded by laccase.

T.versicolor, in the fom of mycelium, mycelial pellets or mycelial fragments imrnobilized in alginate beads can rapidly decolorize BPE (Archibald et al., 1990; Livernoche et al., 1983; Mehna et al., 1995). Decoloration requires a separate carbon source (Archibald et al., 1990; Livernoche et al., 1983; Mehna et al., 1995) and several organic substrates have been shown to be suitable (Archibald et al., 1990). Although the mechanism of BPE decoloration is not fully understood MnP and laccases appear to be central to the process.

Hestbjerg et. al. (2003**)** found that *Phanerochaete Chrysosporium*, a major bioremediator, has very high temperature requirements (30-37ºC) for growth and ligninolytic enzyme production.

Kim, et. al. (2003) found that the treatment of landfill leachate in a combined with process using the white rot fungus *Phanerochaete Chrysosporium* and the natural zeolite Clinoptilolite which was used in a pretreatment step as a sink for ammonia nitrogen and on average it reduced the levels of ammonia nitrogen, SCOD and color by 72, 4.7 and 25%, respectively. The reduction by fungal alone were 16.6, 21.5 and 31.2% respectively. With the synergy created by pretreatment and fungal growth that was stimulated by the addition of a growth medium, the process could remove ammonia nitrogen, SCOD and color at 81.5, 65 and 59% respectively. The ratio of $SBOD_5$/SCOD increased from 0.1 to 0.17 upon treatment, indicating that the process rendered the leachate more amenable to the biological process. This suggested that the preliminary reduction of ammonia nitrogen was essential in making the fungus process practicable for landfill leachate treatment.

Ulku et. al. (1999) found that both *Polyporous Versicolor and Phanarochaete Chrysosporium* were the most effective in removing Pb(II) from aqueous solutions with maximum biosorption capacities of 57.5 and 110 mg Pb(II)/g dry biomass, respectively. With P. versicolor, the adsorptive capacity order was determined to be Pb(II)>Ni(II)>Cr(III)>Cd(II)>Cu(II) while *P. Chrysosporium* was Pb(II)>Cr(III)>Cu(II)>Cd(II)>Ni(II). As the initial concentration increased, the trend of removal efficiency also decreased.

Prasad and Gupta (1997) reported on a substantial reduction of color and COD by the use of white rot fungi*, T. Versicolor and P. Chrysosporium*.

Saxena and Gupta (1998) showed that white rot fungi *P. Chrysosporium* in combination with the use of surfactants were able to remove color, COD and lignin content.

Choudhury et.al. (1998) found that lignin, BOD, COD and color removal were achieved to the extent of 77%, 76.8%, 60% and 80% respectively by the fungal species, *Pleurotus Ostreatus*.

Manzanares et.al. (1995) observed that the utilization of *Trametes Versicolor* is suitable for the decolorization and phenolic groups removal of straw soda pulping effluents in the presence of carbon source.

Ryan et al. (2007) reviewed that the removal of the cresylic effluent components by *T.versicolor* from the solution with up to 20% v/v (14 mM) concentration of effluent was successfully completed after 12 hour contact time.

Mtui and Nakamura (2008) found that *Flavodon flavus* could decolorize 94% of raw textile wastewater and almost completely decolorize Rhemazol brillian blue-R (RBB-R) dye, congo red, brillian green, reactive black and reactive yellow at low carbon culture medium.

Kapdan et al. (2000) studied the decolorization of the textile dye verzol Turquoise Blue G by the white rot fungus *T.versicolor* immobilized on discs in a rotating biological contactor (RBC). The system was operated in repeated-batch mode with 48 h hydraulic retention time (HRT). The highest decolorization effieciency (80%) was obtained with a rotational speed of 30 rpm and initial dye concentration of 50-200 mg/L. Also minimum glucose concentration for 77% decoloration efficiency was 5 g/L.

Rodriguez Couto et al., (2000) reported that *Phanerochaete chrysosporium* immobilized on PUF decolorized the polymeric dye poly R-478 (0.03% w/v) in 6 days.

Also, Rodriguez Couto et al. (2001) studied the decolorization of the polymeric dye poly R-478 by *P.chrysosporium* grown on grape seed, wheat straw and wood shaving under solid state condition. The percentage of biological decolorization attained by grape seed, wheat straw and wood shaving cultures was around 74%, 40% and 63, respectively.

Diorio et al. (2008) studied the decoloration of the dye Malachite Green by *T.versicolor f.antarcticus* grown on wheat bran using a two-phase bioreactor. In the first phase the dye was decolorized in the presence of the fungus and when the dye solution was decolorized about 50%, it was discharged to a stainless steel coil at 50ºC initiating the second phase of decoloration. The total decolorization attained by this system was 82% after five consecutive cycles. In addition, the dye was detoxified.

Radha et al. (2005) found that *P.chrysosporium* was able to biodegrade several synthetic dyes of different structures such as azo, anthraquinone, thiazine and vat syes in both free and alginate-immobilized cultures with a decoloration percentage higher than 75%.

Leidig et al. (1999) showed that encapsulation of *Trametes versicolor* in polyvinylalcohol (PVAL)-hydrogel beads protected the mycelia from mechanical damage and the ligninases from biochemical degradation by presumably preteases of bacterial contaminants. In addition, the polymeric dye Poly R-478 was removed by the encapsulated *T.versicolor* in a stirred tank reactor (working volume 1L) with an efficiency of 89% which was due partially to biotransformation (65%) and partially to adsorption onto biomass (24%).

Kirby (1999) showed that *P.chrysosporium* degraded 70% of a different artificial effluent over a 7-day fermentation period in N-limited conditions.

It was reported that 14.90, 37.80, 13.45, 5.65, 27.8 and 49.0 mg/L of 50 mg/L Indigo Carmin, Reactive Blue 15, Acid Violet, Reactive Black 5 Acid Blue 25 and Acid Black 45 were decolorized respectively by C.versicolor at the end of 9 day incubation period (Fu and Viraraghavan, 2001).

Amaral et al. (2004) found that by using the real textile wastewater, decolorization reached efficiencies of about 92% in a diluted system (approximately 50 mg dye/L) by *Trametes versicolor*.

Table 2.19 show the examples of fungi used to treat domestic sewage, starch processing and metal bleaching effluents, optimal culture condition and the effect of fungal pre-treatment.

Table 2.19 Examples of fungi used to treat domestic sewage, starch processing and metal bleaching effluents. Optimal culture condition and the effect of fungal pretreatment

Effluent	Fungi	Treatment		Refernces
		Reactor and medium handling	Parameters	
Domestic Sewage	Penicillium citricum, Steganosporium piriforme, Arthrinium arundis, Fusarium oxysporum, Cladosporium herbarum, Cladosporium cladosporioides, Scopulariopsis brevicaulis, Mucor hiemalis, Trichothecium roseum, Epicoccum nigrum, Helminthosporium sativum, Ulocladium atrum, Geotricum, Trichocladium asperum, Paecilomyces cameus, Trichoderma sp., Chrysosporium pannorum	Shake-flask	COD (72.3%); Phosphates (97.5%); N-Total (86.8%); Dry Matter (684 mg/l); Protein content (205 mg/l)	Thanh and Simard (1973)
	Aspergillus niger	Stirred tanks reactor in series	COD (72%); N-Total (65.4%)	Coulibaly (2002)
	Coriolus hirsutus	Continuous immobilized bioreactor; addition (nutrient NH_4(100mg/l), NO_3 (100 mg/l); $MnSO_4$); Co-substrate (Glucose, 0.5%)	Decolorization (80%, 2d); MnP (60 U/l); MIP (40 U/l)	Miyata et al. (2000)
Starch Processing Effluent	A. oryzae; Rhizopus arrhizus; Trichoderma viride; T. reesei; G. candidum; A. terreus; R. oligosporus	Shake-flask, air-lift bioreactor (45 l); addition of nutrient $(NH_4)_2SO_4$; Urea; NH_4NO_3; $NaNo_3$; K_2HPO_4; KH_2PO_4)	TOC (44-48%); SS (95%); starch hydrolysis (53-100%); biomass (2-5.6 g/l); protein (48.8% of biomass weight); COD (97.8%); glucoamylase (3.94 U/ml)	Jin et al. (1999abc; 2001)
	A. niger, A. oryzae	Shake-flask	COD 90%; biomass and amylase production	Fujita et al. (1993); Murado et al. (1993)
Metal Bleaching Effluent	A niger, P. simplicissimum, Geotrichum sp., Fusarium verticillioides, Rhizoctonia solani, Aquathanatephorus pendulus;	Shake-flask, presence of co-ions biomass (produced)	A. niger (Cu 91%; Zn 70%)	Price et al. (2001); Gormes et al. (1998, 1999); Gormes and Linardi (1996); Karavaiko et al. (1996)
	A. niger, A. flavus, A. fumigatus; R. Arrhizus; A. terrus	Shake-flask, presence of co-ions, biomass (industrial waste, produced)	Metal removal (82-100%)	Balakrishnan et al. (1994); Niyogi et al. (1998)
	Mucor meihi	Shake-flask; biomass (industrial waste), dilution (1-20)	Sorption (0.7-1.15 mmol/g)	Tobin and Roux (1998)
	A. niger	Shake-flask; presence of co-ions, biomass (produced)	Metal removal (75%)	Akthar and Mohan (1995)