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CYANIDE DETOXIFICATION BY SOIL MICROORGANISMS

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Surrey or any other University, Institute of Learning

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Sept, 2001

الدعي الاجل فاطمي اقا سيدنا محمد برهان الدين (ط ع)

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Thesis ا في الحضرة الامامية النورانية اشرق الله انوارها شكر ما ادبً

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Respectfully dedicated to His Holiness

Dr Syedna Mohammed Burhanuddin Saheb (TUS)

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ABSTRACT

Cyanides enter the environment through both natural and man-made sources. Natural sources include cyanogenesis by bacteria, fungi and plants. A number of cyanide catabolising microorganisms have also been reported in literature.

This is the first reported instance of cyanide catabolism in *Trichoderma harzianum*. Four strains of *T. harzianum*, one of *T. pseudokoningii* were evaluated. An investigation was made into the occurrence and distribution of the cyanide catabolising enzymes. Three enzymes, cyanide hydratase, β-cyanoalanine synthase and rhodanese, were studied. All the strains showed a high capacity to degrade cyanide via both the cyanide hydratase and rhodanese pathways. β-cyanoalanine synthase activity, however, was not detected in any of the selected strains.

In the studies on the kinetic characterization of the rhodanese enzyme, a broad pH optimum of 8.5 - 10.5 was obtained for all the strains and a broad temperature optimum of 35 - 55 °C was also observed. The Km^{CN} and Vmax values ranged from 7 - 16 mM and from 0.069 - 0.093 μmoles. min^{-1}. mg $protein^{-1}$, respectively, between the selected strains of *Trichoderma*.

Strong evidence of cyanide biodegradation and co-metabolism emerged from studies with flask cultures where glucose was provided as a co-substrate. The rate of degradation of 2000 ppm CN^- was enhanced almost three times in the presence of glucose.

Plant microcosm studies carried out using pea and wheat seeds too gave further corroboration of the cyanide degrading and plant growth promotion capabilities of *Trichoderma*. Microcosms set-up with cyanide at 50 or 100 ppm CN^-, in the presence of *Trichoderma*, showed germination of both pea and wheat seeds. There was no seed germination in any of the controls in the absence of *Trichoderma* inoculation.

ABBREVIATIONS

approx.	approximately
cfu	colony forming units
CHT	cyanide hydratase
conc.	concentration
DDT	Dichloro Diphenyl Trichloroethane
DETR	Department of Environment, Trade and the Region
e.g.	example
FHL	formamide hydrolyase
hrs	hours
ICRCL	Inter-departmental Committee on the Redevelopment of Contaminated Land
MGP	Manufactured Gas Plant
mins	minutes
mls.	millilitres
nm	nanometre
PAH	poly aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCP	penta-chloro phenol
PDA	potato dextrose agar
PDB	potato dextrose broth
ptn.	protein
R.O. water	Reverse osmosis water
rpm	revolutions per minute
RT	room temperature
secs.	seconds
sp. gravity	specific gravity
spp.	species
sup	supernatant
TCAB	3,3,4,4 – tetra-chloro-azo-benzene
TNT	2,4,6-trinitro toluene
UST	underground storage tank
WAD	weak acid dissociable

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CHAPTER 1:

INTRODUCTION

This chapter provides a general introduction into the chemistry and occurrence of cyanide in nature, the traditional as well as the potential treatments of cyanide contamination. The first part of the chapter deals with the physical and chemical characteristics, its prevalence in the environment including its toxicity in biological systems. This is followed by a brief description of the conventional chemical treatment processes used for the detoxification of cyanide. The final part deals with bioremediation and biotechnology specifically on the enzymes evolved in nature to allow biological life to tolerate and utilize cyanide for growth.

1.1 CYANIDE

1.1.1 *Chemistry and forms of cyanide*

Cyanides are defined as organic and inorganic compounds that contain the C-N grouping. Cyanide can be found in the environment in a variety of forms, such as free cyanide (HCN), salts (NaCN or KCN), and complexed forms.

Cyanide, in its free form, is a weak acid, known as hydrogen cyanide or hydrocyanic acid or is sometimes also referred to as prussic acid. It is colourless and exists in either gas or liquid form, with a boiling point of 27.5 °C. Cyanides emit a faint distinguishing bitter almond odour (Chemweb online).

HCN in the pure state is highly volatile and is less dense than air. With the pKa of HCN being 9.3, formation of HCN is favoured as acidity is approached. At pH 8, 95.8 % is in the form of HCN, which increases to 99% at pH 7 (Mc Graw Hill, 1987;

Chatwin *et al*, 1988). Cyanide is, therefore, present largely as HCN at physiological pH values (Knowles and Bunch, 1986).

Simple forms such as compounds of the type A(CN)x, where A may be an alkali, alkaline earth or a metal form the second type of cyanide species. Salts of cyanides such as NaCN and KCN are easily dissociable and tend to form HCN in acidic or mildly alkaline solutions (Padiyar *et al*, 1995).

The complexed forms of cyanide can be split into two categories, viz, decomposable and sparingly decomposable forms. The former include weak complexes such as $Zn(CN)_2$ (Towill *et al*, 1978) or ones with lead, cadmium and nickel that are stable in alkaline conditions but weakly ionise under acidic/neutral conditions to form the toxic free cyanide. Metallo-cyanides such as iron or potassium ferri-cyanides fall in the latter category. These complexes have a wide range of chemical and biological stabilities and so do not dissociate easily into the free forms of cyanide. These forms, therefore, tend to be less toxic (Padiyar *et al*, 1995).

The form in which cyanide is found in the environment is dependant upon pH, metal availability and the redox potential (Meeussen *et al*, 1992). Prussian blue ($Fe_4[Fe(CN)_6]_3$) for example, although highly stable and insoluble under acidic conditions, at pH above 6, it is soluble and gives rise to cyanide concentrations in groundwater or soil solutions (Meeussen *et al*, 1992A). The toxicity of these various cyanide species is dependant upon their chemical form, stability constant and

bioavailability to the exposed flora and fauna including microorganisms (Dubey and Holmes, 1995).

Another species of cyanide are the nitriles that have the form R-CN, where R is an aliphatic or aromatic compound. These compounds are widely used in a range of industries as precursors in the synthesis of dyes, emulsifiers, animal feed, cosmetics and antiseptics. Nitriles are also used as herbicides (3,5-dibromo-4 hydroxybenzonitrile) (Hoyle *et al*, 1998).

1.1.2 *Toxicity of cyanide*

The recognition of cyanide as a poison in bitter almonds and cassava dates as far back as the Egyptian times (Sykes, 1981). Cyanide is believed to have been one of the more frequently employed preparations for suicides and homicides during the nineteenth century. Cyanide was used in both World Wars; in the first, by France with little success and in the second war, various countries including USA and Japan employed cyanide. High concentrations of Zyklon B, a derivative of hydrogen cyanide, was used in Nazi gas chambers (Chemweb online).

Cyanide is a potent inhibitor of growth and cellular metabolism, including respiration and nitrogen and phosphate metabolism. It is toxic to a wide range of microorganisms, including bacteria, fungi and algae (Solomonson, 1981). The cyanide ion inhibits DNA replication in *E.coli* and DNA repair in *Chlamydomonas*

species at concentrations as low as 0.5 to 17 mM KCN (Olivera and Lundquist, 1971). It is shown to alter the motility of *Spirillum volutans* (Bowdre and Kreig, 1974) and causes mutation in *Neurospora crassa* (Bergquist *et al*, 1974). The protozoa, *Microregma heterostoma* and green algae *Scenedesmus quadricauda*, have toxicity thresholds of 0.04 and 0.16ppm respectively (Becker and Thatcher, 1973).

Hydrogen cyanide in aqueous solution (hydrocyanic acid) is readily absorbed from the skin, via the lungs and from all mucous membranes whereas the cyanide ion is readily absorbed after oral administration (Egekeze and Oehme, 1980).

The principal mode of action of cyanide is thought to be by inhibition of respiration. It binds to the metallic co-factors in metallo-enzymes thereby inhibiting their activity. CN^- reacts with the haem [Fe(III)] in the cytochrome oxidase a3, the terminal oxidase of the mitochondria or bacterial respiratory chain, thus preventing further redox reactions. This inhibits electron transfer to oxygen and, in turn, prevents tissues, especially in the central nervous system, from utilising oxygen, resulting in rapid death (Stryer, 1988). Solomonson, 1981, has summarised a range of metabolic effects of cyanide including increase in catabolism of glucose, reduction in thyroid activity by the thiocyanate formed during detoxification, as an indirect effect of cyanide, inhibition of other metallo-enzymes (apart from haem) and cyanide toxicity in other non-metallo interactions like reactions with a Schiff base intermediate.

Inhalation is the main route of cyanide exposure. Inhalation of high concentrations of cyanide affects the central nervous system, respiratory system and the cardiovascular

system and can lead to coma and/or death (Chemweb online).

Padiyar *et al*, 1995, have reported cyanide to begin to have a toxic effect above 0.3ppm and 1mg/kg body weight on inhalations and injection or ingestion, respectively in man and lethal doses in animals have been found to be slightly higher at 9 mg/kg body weight by Brebion *et al*, 1986. Concentrations of 20ppm of cyanide in air are known to cause slight symptoms; worsening at concentrations of 50ppm by causing disturbances within 30-60 minutes and eventually proving to be fatal at concentrations of 300ppm unless prompt first-aid is administered (Padiyar *et al*, 1995).

1.2 Sources of cyanide

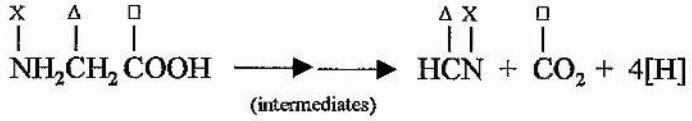
Cyanide and cyanide precursors are ubiquitous in nature (Solomonson, 1981). Cyanide can enter the environment through both natural and artificial sources. Cyanide being a potent inhibitor of many biochemical systems, it is perhaps surprising that cyanogenesis (metabolism or formation of cyanide) has been found in a wide range of different life forms. These include many photosynthetic bacteria and algae, non-photosynthetic bacteria, fungi and plants (approx 2000 species reported) as well as a few insects (Harris *et al*, 1987). The principal role is generally believed to be as a defence mechanism against predation (Conn, 1981). There is documented evidence of cyanogenesis occurring even in the animal kingdom; in arthropods such as centipedes (class Chilopoda); beetles (Insecta) and even in a few species of butterflies (Duffey, 1981).

1.2.1 Natural Sources

1.2.1.1 Cyanogenic bacteria

Cyanide producing bacteria include *Chromobacterium violaceum,* and certain strains of *Pseudomonas aeruginosa, P. fluorescens and P. chloraphis* (Knowles and Bunch, 1986; Castric, 1975). Harris *et al*, 1987, have reported that these organisms exhibit typical characteristics of secondary metabolism. Cyanide is produced from a defined primary metabolic precursor such as glycine at the end of growth, with another primary metabolite, methionine acting as a stimulator. Iron and phosphate content play an important role in the level of cyanogenesis (Harris *et al*, 1987).

Use of radio-labelled cyanide has revealed glycine to be converted to cyanide without the breakage of the carbon-nitrogen bond (Harris *et al*, 1987) as shown below:



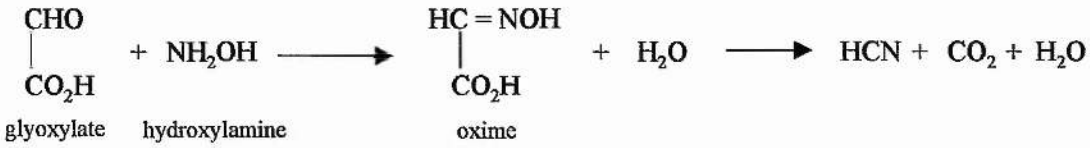
1.2.1.2 ***Cyanogenic algae***

Some photosynthetic bacteria and algae are also found to produce small quantities of cyanide. Two mechanisms are known (Knowles, 1988):

a) The amino acid oxidase/peroxidase system: Illumination of extracts of *C.vulgaris* in the presence of oxygen, manganese, peroxidase and any one of the amino acids (especially histidine), alongwith a soluble flavoprotein amino acid oxidase, results in cyanogenesis (Knowles, 1988). Vennesland *et al*, 1981 have suggested that the action of amino acid oxidase on the amino acid results in the oxidation of the amino acid to an imino acid and hydrogen peroxide. This imino acid in the presence of a peroxidase is further oxidised to give rise to cyanide and imidazole aldehyde. This type of mechanism is particularly seen in *Chlorella vulgaris.*

b) The glyoxylic acid system: Another system for cyanide metabolism in *Chlorella* is from glyoxylate and hydroxylamine involving a non-enzymic synthesis of the oxime of glyoxylate and then followed by enzymic decarboxylation to release of

cyanide:



Cyanogenic algae include the species of *Chlorella vulgaris, Nostoc muscorum, Plectonema boryanum and Anacystis nidulans* (Vennesland *et al*, 1981).

1.2.1.3 ***Cyanogenic or pathogenic fungi generating cyanide***

The first report on microbial cyanogenesis was by von Losecke, 1871, on the cyanide production by the fungus *Marasmius oreades*. Due to the difficulty in growing and handling of fungi, very little is known about the mechanism and physiology of cyanogenesis in fungi compared with bacterial and algal cyanogenesis (Knowles and Bunch, 1986).

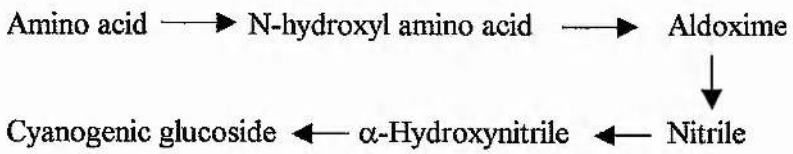
Cyanide production is seen to be stimulated in the presence of glycine but unlike bacterial cyanogenesis, is insensitive to the content of methionine, iron and phosphate in the medium (Bunch and Knowles, 1980).

The link between cyanide and plant diseases has been demonstrated in certain studies where plant diseases involving fungi progress with the liberation of cyanide in host plant tissues (Conn, 1981). These include the pathogenic fungal species like *Stemphylium loti, Gloeocercospora sorghi* and snow moulds (Millar and Higgins, 1970; Knowles and Bunch, 1986; Fry and Munch, 1975).

1.2.1.4 Cyanogenic plants

Cyanophoric plants synthesise cyanogens that liberate hydrogen cyanide on hydrolysis. Amongst the wide variety of cyanogenic plant species, cassava, almonds, sorghum, alfalfa, peaches loquat, apples and beans are some of the agriculturally important species of cyanogenic crops (Knowles, 1976). The widespread neurological disorders reported in West Africa is generally thought to be due to the staple diet of cassava that can contain one-half the lethal dose of cyanide (Knowles, 1976).

The pathway of cyanide production in plants is as follows (Knowles, 1986):



On injury or death, cyanide is released from these cyanogenic glucosides by the action of glucosidase and hydroxynitrilase. These enzymes are normally in different cellular compartments or tissue (Knowles, 1976). For example, in the cyanogenic plant sorghum [*Sorghum bicolor* (L.) Moench], the cyanogenic glucoside, dhurrin, is located in the vacuoles of the leaf epidermal cells whereas the two enzymes, β-glucosidase and α-hydroxynitrilase lyase, are found in the chloroplast and cytosol, respectively (Thayer and Conn, 1981).

1.2.2 *Anthropogenic sources*

Although there are many natural sources of cyanide, including bacteria, fungi and plants that synthesise it, as described in the previous section, industrial wastes contribute significantly to the cyanide in the environment. These include petrochemical emissions, the fumes from burning plastics such as polyurethane, tobacco smoke and the emissions and wastes from industries producing synthetic fibres, pharmaceuticals, manufactured gases, paints and pesticides and from those electroplating metals. The wastes from electro-plating operations contain 0.5 to 20% cyanide by weight (Dubey and Holmes, 1995).

Metal-processing and metal mining industries use cyanide-containing solutions in the extraction of precious metals such as gold and silver. Cyanide, when mixed with the ore from mines, extracts the metal by forming a soluble metal cyanide complex. The metal is then recovered by precipitation. Mine waste-waters have been reported to contain 0.1 to 0.5 μg/ml and the tailings from 7 to 30 μg/ml total cyanide (Mudder and Whitlock, 1983). Other sources include ferricyanides and ferrocyanides used in the dyeing industry which when irradiated with uv light releases HCN and automobile exhausts which contain approximately 1ppm cyanide where it is formed by the reaction of NO with organic free radicals present in the combustion mixture (Towill *et al*, 1978). Cyanide is used in photographic processes and metal polishes (especially silver polish) (Egekeze and Oehme, 1980).

Solutions of cyanide are used in the production of chemicals such as the important nitriles, acrylonitrile and adiponitrile manufactured world-wide, and

methylmethacrylate (Wild *et al*, 1994). The halogenated aromatic nitriles, bromoxynil, ioxynil and dichlorobenil are amongst the most widely used pesticides and herbicides (Knowles and Wyatt, 1992). HCN and its salts like sodium or potassium cyanides are also found in vermicidal fumigants, insecticides and rodenticides (Egekeze and Oehme, 1980).

Cyanide-containing wastes are commonly found in soils at former manufactured gas plant (MGP) sites, also know as town gas sites (Shifrin *et al*, 1996). MGPs produced gas for lighting and heating during the nineteenth and the mid - twentieth centuries for over 150 years. As many as 5000 former MGP sites sites exist in UK (Barclay, 1997) and the U.S. E.P.A. lists 152 waste sites in New York alone (Radian Corporation, 1987). These sites are typically contaminated with a wide range of substances of both an organic and inorganic nature (Fowler *et al*, 1994). The gas generated at these MGPs, before use, was scrubbed with lime and iron oxides in a purifier box in order to remove H_2S and HCN. During this process, cyanide, mainly in the form of the stable iron cyanide was formed in appreciable quantities. Since these wastes or impurities were often dumped on-site, large-scale contamination of the soil and groundwater occurred. Most concentrations of cyanide at MGP sites are below 2000ppm, although isolated cases of concentrations greater than 20,000 ppm have been reported (Shifrin *et al*, 1996). The most prevalent forms of cyanide at these sites are the relatively non-toxic iron cyanides rather than the toxic free forms. Metal complexes of cyanide are readily formed due to the highly reactive nature of the cyanide ions (Patil and Paknikar, 2000). The form of cyanide present is governed by the conditions present at the site. It is the complex forms of cyanide that are

responsible for the blue-stained soils and rocks found at these sites. Ferric ferrocyanide, a major constituent in MGP cyanides, imparts this striking blue colour (Shifrin *et al*, 1996).

Hydrogen cyanide and other volatile, cyanide-containing compounds occasionally occur in the air as a result of emissions from electroplating plants and the fumigation of building and ships. Incomplete combustion of nitrogen-containing substances from chemical processing operations also contributes to cyanide in the air (Katz, 1968).

1.3 Conventional Treatment of cyanide-containing wastes

Cyanide in waste can either exist in the free form or as complexed cyanides. The processes for cyanide removal may be broadly classified into two categories, viz., physico-chemical and biological treatment, the latter is described in detail in Sec. 1.4.

1.3.1 Physico-chemical treatments

Over the years, many chemical treatment processes have been used to degrade cyanide. Since a single process normally does not satisfy all the requirements of control necessary for disposal, in most instances a combination of two or more treatments are used (Padiyar *et al*, 1995). These include alkaline chlorination, electrolytic decomposition, ozonation, incineration, chemical precipitation, coagulation adsorption, stripping, reverse osmosis and ultra filtration, encapsulation, ferrocyanide precipitation and the copper catalysed hydrogen peroxide and Prussian-blue precipitation (Padiyar *et al*, 1995). Of these, only the first three have sufficient versatility for widespread use (Dubey and Holmes, 1995).

Alkaline chlorination

Various oxidants such as chlorine, hypochlorite, ozone, hydrogen peroxide, formaldehyde, chloramine-T, dichromate, permanganate, oxygen and sulfite/air have been used for oxidation of cyanide wastes. Alkaline chlorination is frequently used for both large and small-scale treatments especially of effluents from the

electroplating and steel industries. Poor removal of iron cyanides is the main disadvantage of this method (Padiyar *et al*, 1995).

Ozonation

Ozone, as an oxidising agent in chemical reactions is well documented but its use in cyanide removal is relatively recent. Ozonation has been reported for mining wastes, electroplating wastes from the automobile industry, the galvanising industry and the coke oven effluents (Padiyar *et al*, 1995). Ozonation effectively degrades iron, nickel, zinc and copper cyanide complexes but is ineffective with cobalt cyanide complexes. A considerable amount of energy is consumed with both ozone and the combination ozone - uv treatments and the frequent presence of other oxidizable species in waste-waters can significantly increase the ozone requirements (Dubey and Holmes, 1995).

Electrolysis

Electrolytic treatment has been found to be economically attractive for destroying cyanide in spent liquor baths, cyanide dips and strip baths from the plating industry containing inorganic cyanides (Padiyar *et al*, 1995). This treatment is effective only for wastes containing high concentrations of cyanide (3.5mg/ml) and is not suitable for processing dilute solutions of cyanide (0.1mg/ml) due to the accumulation of cyanate in the electrolyte. A secondary treatment may be employed to satisfactorily remove the residual cyanide (Padiyar *et al*, 1995).

Cyanide is classed as a highly toxic priority pollutant by pollution control authorities in developed and developing countries. The United States Environment Protection Agency has set up a cyanide concentration limit of 0.02mg/ml for effluent disposal, with 0.005mg/l cyanide specified as an ambient standard concentration for freshwater and marine aquatic life and a primary drinking water standard of just 0.2μg cyanide/ml (Padiyar *et al*, 1995). The EC List 2 includes cyanide on the basis of its potential to cause deleterious effects on aquatic environment at elevated concentrations. The Department of Environment, Transport and the Region (DETR), UK, ICRCL's guidelines have proposed threshold trigger values for complexed cyanides as 250mg/kg of soil and 25mg/kg soil for free cyanides (DETR, 1987).

1.4 *Bioremediation and its applications*

1.4.1 *Introduction to bioremediation*

Despite the best efforts of industry and regulators there have been a number of reports on incidents of contaminant spills particularly oil spills. One of the largest tanker spills to date was the Amoco Cadiz spill in 1978 where $67x10^6$ US Gallons (1 US Gallon = 3.8 litres) entered the sea (Prince, 1992). Another example of a massive release of oil in to the environment is the Exxon Valdez spill, described later on in this section.

The Romanian cyanide spill is one of the most recent examples of entry of toxic pollutants into the environment. The spill on 30th Jan 2000, occurred in the Baia Mare region of northwest Romania and flowed into the shared water systems of Hungary, Romania and Yugoslavia (Kovac, 2000). The report compiled by the United Nations Environment Programme and the Office for the Coordination of Humanitarian Affairs (UNEP/OCHA) found: 'acute effects, typical of cyanide, occurred for long stretches of the river system; phyto- and zooplanktons were down to zero and fish were killed in the cyanide plume. After the plume had passed there was rapid recovery of the aquatic microorganisms. Although the spill had produced 'minimal' immediate health risk, the pollution 'might have a longer term impact on human and environmental health' (Sorentino, 2000).

The rapidly increased use of cyanide in many chemical-manufacturing processes has exerted much impact on the thinking of industrial waste treatment and pollution control engineering scientists (Howe, 1965). Waste management strategies

in the past have focussed more on disposal than on treatment of the pollutants or effluents. Conventional treatment systems may simply transfer the pollution, creating new waste such as incineration residues, and not eliminate the problem (Heitzer and Sayler, 1993). Also, the realization of the high cost of cyanide waste reduction or removal has thus opened the door leading to investigations into new technologies other than the classical alkaline-chlorination process.

Bioremediation is the process of encouraging the natural process of biodegradation to clean-up spills and other environmental problems caused by humankind's activities (Prince, 1992). Bioremediation relies upon the enzymatic activities of living microorganisms to catalyse the destruction or transformation of pollutants to less harmful forms. This ranges from simple hydrolytic reactions, catalysed by enzymes such as proteases or cellulases, to more complex multi-step pathways capable of degrading a range of compounds including fatty acids, petroleum compounds (Alexander, 1981).

Microorganisms, including bacteria and fungi are especially useful for bioremediation because of their great metabolic diversity. This relates to their important role in the biochemical cycling of organic and inorganic compounds in the environment, which often include their abilities to metabolise xenobiotic pollutants such as petroleum and chlorinated hydrocarbons including the aliphatic and the aromatics (Atlas, 1998; Muller, 1992).

The end products of effective bioremediation must be non-toxic such as carbon-dioxide and water or alternatively, innocuous metabolites that can be further

biodegraded easily, such that they can be accommodated without harm to the environment and the living microorganisms (Atlas, 1998).

Environmental conditions must permit the *in situ* growth of the living organisms used for the bioremediation of a pollutant or on the other hand, extraction of the pollutant from the environment so that it can be degraded *ex situ* in bioreactors (Atlas, 1998). Microorganisms possess a unique ability to quickly evolve and develop a biochemical trait that confers selective advantage. Thus, microbes at a contaminated site that acquire the capability to use a particular contaminant as its source of food and energy, will eventually out-compete other microflora. As a result new pathways for man-made xenobiotics would eventually evolve, for which a naturally occurring pathway does not exist. An appropriate example would be the class of environmental pollutants, polychlorinated biphenyls (PCBs). These have long been thought to be non-biodegradable, but recent research has indeed reported on biological degradation of PCBs, involving a combination of aerobic and anaerobic processes (Bedard *et al*, 1987; Quensen *et al*, 1988). Other examples include synthetic chlorinated insecticides and pesticides.

The biodegradation of pollutants in the environment is a complex process, whose quantitative and qualitative aspects depend on a variety of biotic and abiotic environmental factors. These include the nature and the amount of pollutant present, the composition of the indigenous microbial community and the extent of its acclimatization. The ambient and seasonal environmental conditions as well as the

horizontal and vertical variability occurring within the soil matrix also play an important role (Atlas, 1998). Some common environmental limitations to biodegradation of pollutants are excessively high waste concentrations, lack of oxygen, unfavourable pH and temperature and lack of mineral nutrients and moisture. Once these limitations are corrected, the ubiquitous distribution of microorganisms allows, in most cases, for a spontaneous enrichment of appropriate microorganisms (Atlas, 1998).

The general consensus amongst the research publications seems to be the following:

a) the rate of biodegradation and the behaviour and fate of the contaminants are site-specific and it may be difficult to generalize from one case to another; bioremediation strategies must be tailored specifically to each polluted site. Each waste site has unique characteristics and thus requires individual attention (Caplan, 1993).
b) the complexity and its age may affect the rate and extent of transformation of the contaminant, and
c) that there are large variations (Fig. 1.1) between small and large-scale studies in the biological removal of pollutants (Blackburn and Hafker, 1993).

Bioremediation has numerous applications that include clean-up of ground water, soils, sludges, lagoons and process waste streams. These systems have even been used on very large-scale applications, as demonstrated by the shore-line clean-up efforts resulting from the Exxon Valdez oil spill in 1989, at Prince William Sound,

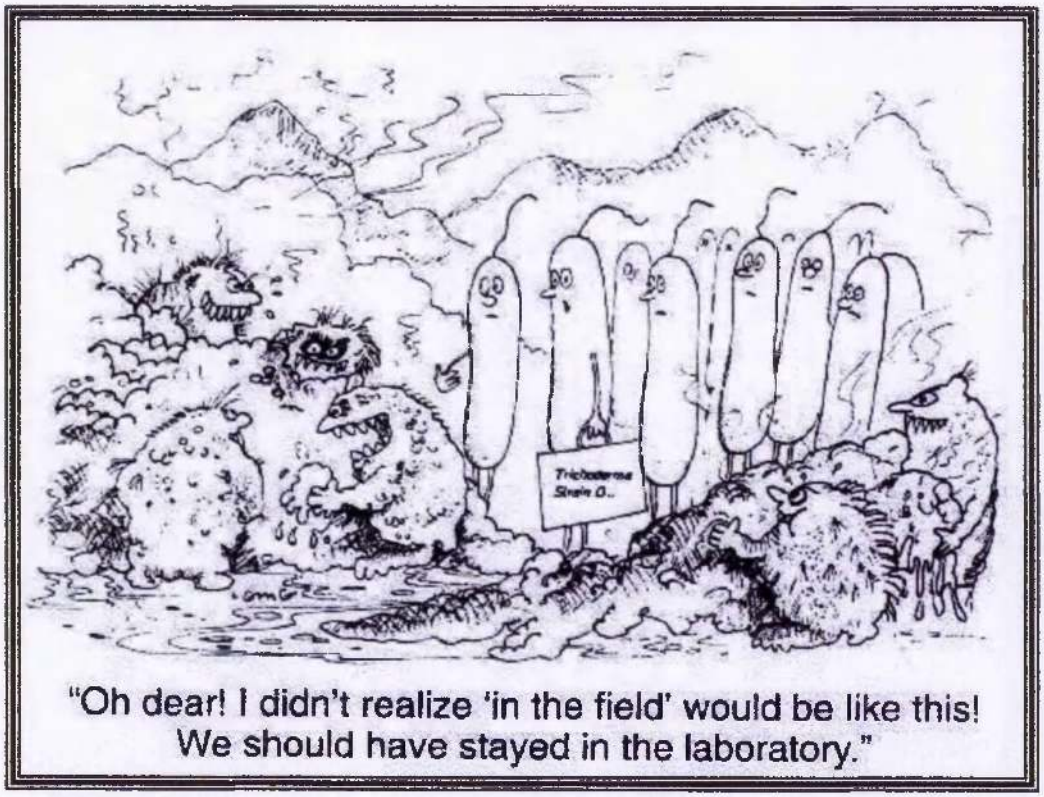


Fig. 1.1 Cartoon illustrating the large variations between lab and field-scale studies - adapted from Liu and Suflita, 1992.

Alaska (Caplan, 1993). A monitoring programme to assess the safety and efficacy of bioremediation in the field set-up came up with the following conclusions (Prince, 1992):

a) that biodegradation was proceeding at a significant rate even in the absence of bioremediation,

b) that fertilizer application stimulated the microbial activity responsible for that process by several fold, and

c) finding no detrimental environmental effects.

Based on the above findings, the joint USEPA/Alaska Department of Environment Conservation/Exxon, concluded that the bioremediation in Prince William Sound was both safe and effective. Bioremediation, thus, remained in use as a major tool in the clean-up and was also used on those isolated areas where surface and sub-surface oil remained (Prince, 1992). Although the Alaskan oil spill clean-up represents the most extensive use of bioremediation on any one site to date, there have been numerous other successful applications including remediation of pollution due to chemical spills, leaking underground storage tanks (USTs) and industrial process wastes.

At present, there are three methods commonly used to carry out bioremediation of contaminated soil or ground water:

Addition of nutrients

This procedure, also called as *in situ* bioremediation or 'biostimulation', is the most common method of bioremediation of soils and aquifers (Sims *et al*, 1989). The rationale behind this approach is that the sub-surface already contains indigenous micoorganisms adapted to the contaminant (s) but due to the deficiency in both nutrients and oxygen, they cannot metabolise the contaminants to the end-products, carbon-dioxide and water.

The supply of these growth-limiting factors by addition of an appropriate nutrient formulation and/or periodic introduction of hydrogen peroxide to the sub-surface environment, stimulates the microorganisms to mineralise the contaminants. The hydrogen peroxide breaks down to create oxygen in water. In a process known as 'bioventing' oxygen in a gaseous form, is injected into the unsaturated zone above a water table, to stimulate the indigenous organisms. On the other hand, in

'biosparging' involves injection of the oxygen below the water table from where bubbles of oxygen then rise into the unsaturated zone where natural biodegradation is stimulated (Lee *et al*, 1988).

Chemical nutrients, for example, similar to fertilizers, such as carbon, nitrogen and phosphorus, surfactants to break up oily wastes, and in certain cases, nitrates to act as an alternative electron acceptor are usually added. These, normally in aqueous solutions, are simply sprinkled onto the soil or, otherwise, through sophisticated plumbing (Sims *et al*, 1989).

Augmentation of selected microbial cultures

The second alternative is to inoculate cultured microorganisms to the site of contamination. This method is often used when there is not enough effective micro flora. One way is to introduce non-indigenous, contaminant-degrading bacteria with a balanced nutrient formulation into the sub surface environment. Another more costly and generally less practised method is to select the best waste-degrading microorganisms from the site to be remediated, scaling up their numbers in the laboratory and then returning these enriched cultures to the site (Skladany and Metting, 1993). Another strategy, called as 'bioaugmentation', is the introduction of non-native cultures selected from other sites for their ability to degrade specific wastes (Skaldany and Metting, 1993).

***Ex-situ* methods of bioremediation**

The third method is bio-reactor based (Caplan, 1993). Here the contaminants are flushed from the soil and then treated in an above-ground bioreactor into which a continuous supply of nutrients, oxygen and microorganisms is introduced. In addition

to the above bioreactor technology, *ex situ* soil bioremediation is also been carried out using the conventional land-farming techniques (Caplan, 1993).

In summary, the most appropriate strategy to treat a specific site can be guided by considering three basic principles: the amenability of the pollutant to biological transformation to less toxic products (biochemistry); the accessibility of the contaminant to microorganisms (bioavailability); and the opportunity for optimization of biological activity (bioactivity) (Blackburn and Hafker, 1993). A general knowledge of the ecology and evolution of degradative microbial poulations is essential for the design and assessment of any cost-effective, ecologically safe and environmentally sound bioremediation plan (Liu and Suflita, 1992) (Fig. 1.2).

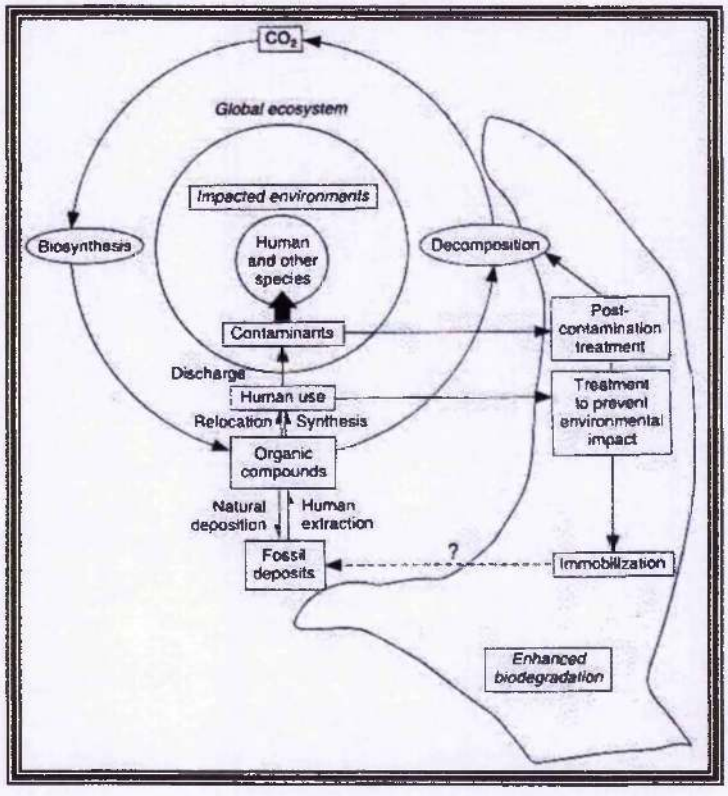


Fig. 1.2 Lending a helping hand to nature – adapted from Liu and Suflita, 1993

Phytoremediation

Phytoremediation is an emerging method for decontaminating metal-contaminated soils and waters. Various plants have the ability to concentrate heavy metals, including lead and radionuclides in their plant biomass. Plants such as Indian mustard (*Brassica juncea*) and ragweed (various *Ambrosia* spp.) are being field-tested. These have been reported to accumulate up to 40% of their biomass as heavy metals. The plants can then be harvested, incinerated and the heavy metals thus recovered can be reused (Atlas, 1998).

Applications of molecular biology are fast gaining popularity in this field. Tools now available can be used in the detection of specific genes responsible for degradation. Specific DNA gene probes can be used for hybridisation with extracted DNA from contaminated sites to give an indication of potential for bioremediation by indigenous microorganims at a given site. The main drawback of this method is in the measurement of potential enzyme activity, i.e. the gene may be present though not necessarily be active (Barclay, 1997). Conversely, gene expression can also directly be measured using mRNA probes.

The bioremediation industry

Bioremediation currently comprises only a small fraction of the very large hazardous-waste treatment market but is one of the fastest growing sectors in environmental management. Within Europe, a thriving bioremediation industry exists in the

Netherlands. They have made the most progress among the European nations, having decontaminated over 6000 sites since 1982 (Caplan, 1993). In the UK although R&D efforts are underway in several small companies, use of waste-treatment processes by industry is minimal, due to a less stringent regulatory climate and weak incentives for efficient industrial clean-up. The US bioremediation market was estimated at US$60 million in 1990, which grew to around US$250million in 1996. The international market in 2000 was estimated to be around US$1 billion with roughly half in the US and the most of the other half in Europe (Caplan, 1993; Glass, 1993).

A multitude of abiotic (physical and chemical) and biotic factors can affect the rate and extent of biodegradation of environmental pollutants. These include temperature, light, pH, volatilisation, water activity and moisture content, and the presence of other toxicants (Johnston and Robinson, 1984). A particularly important factor affecting the biotransformation of an environmental compound is its concentration. In soils, the most important constraint is the inaccessibility of the pollutant to the microbe as well as the horizontal and vertical variations within the soil matrix. A number of factors have to be considered during the scale-up from the laboratory to field conditions including the adaptability of introduced organisms, if any, temperature and aeration. The use of an organism (s) tolerant of the prevailing physical and biological conditions seems to be a critical aspect of successful bioremediation (Johnston and Robinson, 1984).

In summary, although bioremediation is considered an innovative waste treatment

method, it has gained acceptance over the recent years, and is now generally regarded as a viable technology in certain defined uses.

1.5 *Bioremediation by fungi*

Fungi, with the possible exception of mushrooms, are generally associated with unpleasant moulds on foods or wet/dry rots in homes. These are surprisingly minor attributes when compared to the tremendous potential of fungi for cleaning-up in processes as diverse as detoxifying noxious xenobiotics, oil spill clean-up and reducing animal waste (Evans and Bucke, 1998).

Composting from garden and agricultural wastes is the oldest known use of fungi in bioremediation where different species of fungi, alongwith a variety of species of micoorganisms including bacteria, crustacea and insects, degrade plant refuse into organic manure. Mushroom production is an ideal bioremediation process since it not only destroys a waste product, but also generates a useful saleable commodity (Wood and Smith, 1987).

Phanerochaete chrysosporium (within the group basidiomycetes) causing white-rot in wood (whitening of the wood) is one of the main foci of current bioremediation. They produce peroxidase and polyphenyl oxidase - enzymes that attack lignin, one of the strengthening polymers of wood. The non-specificity of these lignolytic enzymes can be potentially exploited to break down a range of compounds structurally similar to lignin. These types of compounds include the highly recalcitrant PCBs, DDT, dioxins and polyaromatic hydrocarbons (PAHs), all of which can otherwise remain in the environment for infinite periods of time. They have all however, been found to be degraded by the white rot fungi (Field *et al*, 1993).

The first successful field trial, using fungi in soil bioremediation, was carried out by the USDA Forest Product Laboratory in Wisconsin, in 1993, on a heavily pentachloro phenol (PCP) contaminated soil. The study which used *Phanerochaete chrysosporium* and another strain of *Phanerochaete*, grown on wood chips, found the disappearance of 80% of the PCP after 30 days (Lamar *et al*, 1993).

Another white-rot fungi, *Phlebia radiata* has been used in trials to degrade wastes arising from military use of explosives, for example, TNT (2,4,6-trinitrotoluene). These trials, although in preliminary stages, have shown transformation of TNT into the less explosive aminodinitrotoluenes. Due to the formation of toxic intermediates under certain conditions, more research is needed. Large-scale processes for TNT removal from soil, though, are still some time away (van Aken *et al*, 1999).

Enzyme systems from the white-rot fungus, *P. chrysosporium* and *Pleurotus ostreatus* have also been shown to degrade the PAH, phenanthrene. Results from a field study on an oil-contaminated soil with *P. chrysosporium* concluded that with further refinement, this system would be effective for soil bioremediation (Brodkorb and Legge, 1992).

The *Trichoderma* and *Gliocladium* sp., like many other fungi, trap metal ions in their cell walls, a finding that has been employed for industrial removal of metal ions from waste streams (Kubicek-Pranz, 1998).

Cyanide, one of the most toxic substances known to man, can be used by microbes like fungi as their carbon and nitrogen source for growth. Barclay *et al*, 1998, have

shown that fungi such as *Fusarium* are effective cyanide and metallo-cyanide degraders at both alkaline and acidic pHs. There are also a number of other fungal species, like for e.g. *Gloeocercospora sorghi* and *Stemphilium loti*, which attack plants that produce cyanide in defence and the fungi, in turn, have enzyme systems which can detoxify the cyanide. Commercial preparations of cyanide-degrading enzymes, such as CYANIDASE ® sold by Novo Nordisk A/S (using cyanide di-hydratase from *Alcaligenes xylosoxidans* subsp. *denitrificans*) (Ingvorsen *et al*, 1991) and CYCLEAR ® from ICI (utilizing cyanide hydratase from *Fusarium lateritium*) are also available.

The breakdown products of a pollutant in bioremediation could sometimes be more toxic and resistant to the original compound, for e.g., with the herbicide propanil, the aliphatic portion of the compound is degraded by the bacteria leaving behind the aromatic part which gives rise to persistent residues such as TCAB (3,3,4,4 – tetrachloroazobenzene) and other azo compounds (Evans and Bucke, 1998). No field studies have been reported on degradation of pesticides although laboratory experiments have proved that fungi degrade pesticides without generating undesirable products.

1.6 *Microbial degradation of cyanide*

The cyanogenic (cyanide-producing) microorganisms mentioned earlier, also have been reported to possess the ability to assimilate cyanide. A wide variety of products are formed including alanine, glutamic acid, α-aminobutyric acid, β-cyanoalanine and γ–cyano-α-aminobutyric acid (Harris *et al*, 1987).

The degradative capability of microorganisms is not restricted to the ones producing cyanide. Many non-cyanogenic microorganisms also degrade cyanide (Castric, 1981). Water hyacinths (*Eichornia crassipes* (mart.) solms.) too, have been reported to degrade cyanide (Granato, 1993). Bendall and Bonner, Jr., 1971 found the existence of a cyanide-insensitive respiratory pathway consisting of an alternative terminal oxidase instead of cytochrome c, in certain higher plants that are found to tolerate cyanide. There are specific enzyme systems and pathways involved in the degradation of cyanide, as will be discussed below.

P. fluorescens, Citrobacter freundii, Escherichia coli, Bacillus subtilis and certain other *Bacillus* strains, *Fusarium* spp., snow moulds and a variety of other species of fungi, all possess the ability to assimilate cyanide and use it for growth as both carbon and/or nitrogen sources (Knowles, 1976). The pathway involved is generally via NH_3. Several possible pathways have been elucidated by Knowles, 1976 (Fig. 1.3). The simplest method of detoxifying cyanide is to convert it to formate and then to carbon dioxide by formate dehydrogenase. The formamide is formed due to the action of cyanide hydratase, which is then converted to formate in the presence of formamidase.

There are other pathways for detoxifying cyanide, which are discussed later in this chapter.

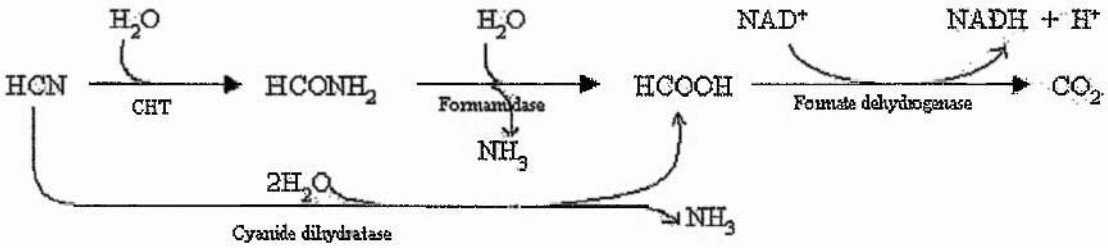


Fig. 1.3 Different possible pathways of cyanide degradation as elucidated by Knowles, 1976

1.6.1 ***Cyanide hydratase*** (EC 4.2.1.66 formamide hydrolyase)

A number of microorganisms have been reported to possess the enzyme cyanide hydratase. The enzyme is found to be mainly restricted to fungal species, with *P. fluorescens* NCIMB 111764, reported by Kunz *et al*, 1992, being the only exception. Snow moulds, one of the most frequently encountered fungi utilize cyanide hydratase to degrade cyanide (Fry and Myers, 1981). *Stemphilium loti* (Fry and Millar, 1972), *Gloeocercospora sorghi* (Wang *et al*, 1992) also degrade cyanide using the enzyme cyanide hydratase. There appears to be some correlation between cyanide hydratase activity and the ability of certain fungi to infect cyanogenic plants (Cluness *et al*, 1993).

The cyanide hydratase pathway results in the conversion of cyanide to formamide, which eventually gets converted into CO_2 and NH_3 in the presence of

another enzyme, amidase, discussed later in this chapter.

$$HCN + H_2O \xrightarrow{\text{Cyanide hydratase}} HCONH_2$$

S. loti, from which this enzyme was first found by Fry and Millar in 1972, is the principal pathogen of the cyanogenic plant, birdsfoot trefoil (*Lotus corniculatus L*). The partially purified enzyme from *S. loti* was shown to be fairly stable, with about half the activity retained after 2 days at room temperature (RT) (25 °C) and more than 6 weeks at 4 °C. The enzyme was found to be inducible with a wide pH optimum from pH 7 - 9 (Nazly and Knowles, 1981).

Another phytopathogenic fungus, *Gloeocercospora sorghi* also produces cyanide hydratase (Fry and Munch, 1975 and Wang *et al*, 1992). *G. sorghi* is a pathogen of the cyanogenic plant sorghum (*Sorghum bicolor (L)* Moench). The enzyme was again found to be inducible with pH optimum between pH 7 - 8. It was reported to be stable for more than a week at 4 °C and more than 2 years at – 80 °C (Wang, *et al*, 1992).

Dumestre *et al*, 1997, have reported the presence of cyanide hydratase in *Fusarium solani* IHEM 8026. The fungus was reported to utilize cyanide as its sole source of nitrogen and grew under alkaline conditions.

The cyanide hydratase enzyme has been purified, cloned and sequenced in the species, *Fusarium lateritium* Nees. Similar properties to that described above for *S.loti* and *G.sorghi* were observed with the pH optimum being pH 8.5. The enzyme

was also readily induced in the presence of cyanide (Cluness *et al*, 1993).

Cultures of *Fusarium oxysporum* have been reported to convert cyanide to formamide in the presence of cyanide hydratase (Pereira *et al*, 1996). Only induced cells were capable of significant cyanide degradation and the authors have proposed that the synthesis of the hydratase enzyme was dependant on the energy provided by an alternative cyanide-resistant respiration pathway (Pereira *et al*, 1999).

CYCLEAR, the commercial immobilized enzyme product from ICI Biological Products, uses the cyanide hydratase enzyme from *F. lateritium* for degrading liquid cyanide wastes. Operating at a pH of 8 - 8.5, it can reduce 5000ppm of cyanide to less than 10 ppm in 6 hours (Evans and Bucke, 1998).

1.6.2 ***β-cyanoalanine synthase*** [EC 4.4.1.9, L-cysteine hydrogen sulphide-lyase (adding HCN)]

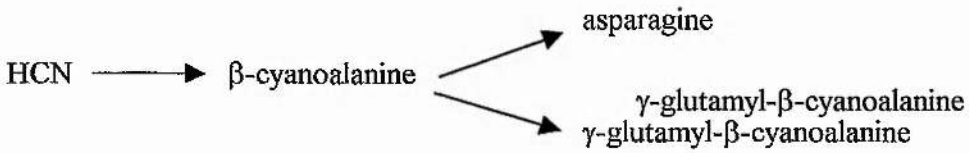
An alternative route of cyanide detoxification is through conversion of cyanide to β-cyanoalanine or an α-aminonitrile catalysed by the enzyme β-cyanoalanine synthase (Bluementhal *et al*, 1968).

$$\underset{\text{Cysteine}}{HSCH_2CHNH_2CO_2H} + HCN \longrightarrow \underset{\text{β-cyanoalanine}}{NCCH_2CHNH_2CO_2H} + H_2S$$

CO_2 is not released in this process and also O_2 or NAD(P)H are not required.

The enzyme β-cyanoalanine synthase has been shown to be present in a variety of plant species and in some bacteria (Miller and Conn, 1980). Bluementhal *et al*, 1968 found that seedlings of blue lupine (*Lipinus angustifolia*), sorghum and common vetch could extensively convert $H^{14}CN$ into the amide carbon of asparagine and also utilize L-cysteine - 3 -^{14}C as the source of the other three carbon atoms of asparagine. They also found that the reaction between cyanide and cysteine in the presence of β-cyanoalanine synthase yielded β-cyanoalanine; the nitrile group of which is then hydrolysed to give asparagine.

Assimilation of cyanide in many plants is found to occur via two pathways, viz. formation of asparagine or the dipeptide, γ-glutamyl-β-cyanoalanine (Knowles, 1976). Formation of both, the dipeptide (common in *Vicia* species) and asparagine occurs via β-cyanoalanine:



The peptide, though being a normal constituent of *Vicia*, can only be found in plants that are fed cyanide (Knowles, 1976).

Blue lupine also contains an enzyme, O-acetyl-L-serine sulfhydrase that catalyses the formation of cysteine from O-acetyl-L-serine and sulphide and also the reaction between O-acetyl-L-serine and cyanide to form β-cyanoalanine, albeit at a low rate (Knowles, 1976).

Chlorella pyrenoidosa has been observed to form β-cyanoalanine and γ-glutamyl-β-cyanoalanine from cyanide (Bluementhal *et al*, 1968; Knowles, 1976). Further experiments showed that in *Vicia sativa* and several other vetches, the formation of the γ-glutamyl peptide was catalysed by an active γ-glutamyl transferase (Bluementhal *et al*, 1968). *E. coli* has been shown to form β-cyanoalanine from cyanide and its been suggested that the *E. coli* β-cyanoalanine synthase activity represents a function of a non-specific enzyme, possibly serine sulfhydrase (Knowles, 1976) whose primary function is the formation of cysteine from serine and sulphide. The other non-specific reactions were:

$$\text{Serine} + \text{HCN} \longrightarrow \beta\text{-cyanoalanine}$$

$$\text{Cysteine} + \text{HCN} \longrightarrow \beta\text{-cyanoalanine} + H_2S$$

Chromobacterium violaceum (strain 9) can assimilate the cyanide it produces into β-cyanoalanine and then into the amide carbon of asparagine (Knowles, 1976).

A cyanide-metabolizing strain of *Bacillus megaterium* was isolated (Castric and Conn, 1969), and the studies concluded that β-cyanoalanine was synthesised from cyanide and serine, which was converted to asparagine and then aspartate. They later reported that cysteine and O-acetyl-L-serine were significantly more effective substrates than serine and showed that one of the enzymes in cysteine biosynthesis was indeed able to catalyse β-cyanoalanine formation (Knowles, 1976).

The enzyme from *Enterobacter* strain 10-1, isolated later, was found to resemble the

B. megaterium enzyme in that it was a cysteine synthase rather than a true β-cyanoalanine synthase and also inclusion of cyanide did not induce the enzyme (Knowles, 1976).

1.6.3 ***Rhodanese*** [EC 2.8.1.1, thiosulfate:cyanide sulfurtransferase]

Rhodanese, first reported by Konrad Lang in 1933, catalyses the conversion of cyanide to a less toxic form thiocyanate (Lang, 1933):

$$HCN + S_2O_3^- \longrightarrow HSCN + SO_3^-$$

Rhodanese has since been extensively studied and catalyses a number of reactions involving transformations of sulfur-containing molecules.

$$\{RSO_xS\}^- + \text{rhodanese} \rightleftharpoons \{RSO_x\}^- + \text{rhodanese} - S$$

$$\text{rhodanese} - S + X^- \rightleftharpoons \text{rhodanese} + SX^-$$

R can be an aryl or alkyl residue or O^- and x is an integral value in the range 0-2. The sulfur donor $\{RSO_xS\}^-$ can be practically any sulfane-containing anion including thiosulfate, thiosulfonate, persulfide and polysulfide. The acceptor molecule can be of a number of thiophiles including cyanide, thiols, dihydrolipoate, sulfite or organic sulfinates (Knowles, 1976; Westley, 1973).

Although sulfurtransferases have been found in almost all living forms of life, their physiological roles still remain in question. Proposed roles include sulfur

metabolism, cyanide catabolism/detoxification and mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Ray *et al*, 2000).

Indeed, the primary role of this enzyme is thought to be of sulfur metabolism (Harris *et al*, 1987) and has been implicated in forming iron-sulfur centres important for electron transport (Horowitz and Bowman, 1987). Cyanide detoxification by rhodanese is thus an accessory function of the rhodanese (Volini and Alexander, 1981). The cyanide to thiocyanate reaction is atypical amongst the rhodanese catalysed reactions in that it is irreversible (Knowles, 1976).

Rhodanese is found to be widely distributed in bacteria, fungi, few higher plants (Miller, 1983) and all phyla of the animal kingdom (Westley, 1973), including humans.

Lang's original report of the discovery of rhodanese contained a very substantial survey of mammalian tissues as well as work with frogs, birds and *E. coli*. Rhodanese activity was found in all mammalian tissues, except blood and muscle, being the highest in the liver and adrenals. Frogs, followed by rabbits and cattle had the highest activity. Humans, fowl, cats and dogs had lower levels, so did *E. coli* (Westley, 1973). This was followed by work by Saunders and Himwich, 1950, on the studies on the distribution of rhodanese activity in mammalian tissues. They found liver tissue to be the most active source, although in dogs it was the adrenals. Mammalian kidneys were reported to be less rich in rhodanese and about 10% activity of that of the liver was found in the brain.

Later studies, however, indicated Lang's earlier report on the utilization of colloidal sulfur to be incorrect in that crystalline rhodanese does not use elemental sulfur as substrate (Westley, 1973). Rhodanese activity has also been reported in blowfly larvae, pupae and adults, however, only in the order of 1-2% of that of bovine liver (Westley, 1973).

Whereas most land animals tended to have activity primarily in the liver and kidneys, marine animals had large amounts in the gills (Westley, 1973). The highest levels of rhodanese, in mammals, is found to occur within the mitochondria in the liver where detoxification normally takes place. In rodent tissues, particularly the liver and the nasal epithelium, the enzyme is found to be synthesised constitutively at high concentrations and acts as a primary detoxification mechanism against inhaled HCN (Dubey and Holmes, 1995).

In general, rhodanese activities differ greatly from class to class but are relatively constant in species of the same class (Westley, 1973).

Westley, 1973, in studies on distribution of the rhodanese enzyme did not find any appreciable amounts of rhodanese activity in different strains of *E. coli* and *N. crassa*. However, considerable quantities of the enzyme was detected in *B. subtilis, B. coagulans* and *B. stearothermophilus* (Westley, 1973).

One of the first reports on rhodanese activity seems to be by Mc Chesney who found its presence in *Thiobacillus thiocyanoxidans* (Mc Chesney, 1958). The rhodanese enzyme from *T. denitrificans* was isolated by Bowen *et al* in 1965. In their studies on its mechanism and molecular properties they found that the presence of cyanide in the growth medium increased the yield of rhodanese enzyme several-fold (Bowen *et al*, 1965). Subsequently, there have been a number of reports on the presence of rhodanese in *Thiobacillus* species. LeJohn *et al* in 1967 reported an interesting observation in *T. novellus*, where they found that alternative energy substrates like glucose, lactose, glycerol or pyruvate could markedly repress all enzymes involved in thiosulfate oxidation in this organism, including rhodanese. Extracts of sulfur-growing and iron-oxidising *Ferrobacillus ferrooxidans* (*Acidithiobacillus ferrooxidans*) were reported to contain rhodanese enzyme activity. *F. ferrooxidans* was found to be similar to other thiobacilli in terms of its rhodanese having broad pH optimum and inhibition of rhodanese activity by iodoacetamide and N-ethylmaleimide (Westley, 1973).

Both, *E. coli* and *P. aeruginosa* were found to exhibit maximal rhodanese activity during the transition from exponential growth to early stationary phase when the latter strain reaches peak cyanogenic capacity. Since the levels of enzyme activity remained stable during the mid-stationary phase when cyanogenesis decreased, it suggested rhodanese served another function in addition to cyanide detoxification (Westley, 1973). In *E. coli*, studies indicated the enzyme was largely involved in a physiological role in aerobic energy metabolism, in the biosynthesis of iron-sulfur proteins (Alexander and Volini, 1987).

Extracts of *Pseudomonas aeruginosa* have been shown to contain rhodanese. These extracts showed a marked enhancement of activity when grown on mercaptosuccinate (Westley, 1973). Ryan and Tilton, 1977, also have reported on the isolation of rhodanese from *P. aeruginosa*.

Rhodanese activity has been reported within three families of photosynthetic bacteria, viz. Thiorhodaceae, Athiorhodaceae and Chlorobacteriaceae (Westley, 1973). Smith and Lascelles, 1966, have reported rhodanese activity in another photosynthetic bacterium, the *Chromatium* sp strain D. A partial purification of rhodanese has been reported from the sulfate-reducing bacterium *Desulfotomaculum nigrificans* by Burton and Akagi, 1971.

Knowles, 1976, has observed that the frequent appearance in literature of rhodanese in *Thiobacillus* species, in photosynthetic bacteria and in *Desulfotomaculum* suggest that in these organisms at least, it has a role of sulfur metabolism rather than cyanide detoxification. There have also been conflicting reports on the presence of rhodanese in certain microorganisms. Westley, 1973, has attributed that, partly to real biological variability, and much of it to the ubiquitous occurrence of non-specific catalysts being mistaken for rhodanese activity.

There have been few reports on fungal and plant rhodanese. Chew and Boey, 1972, found the leaf of the tapioca plant, *Manihot utilissima* to contain rhodanese in significant amounts that could allow assimilation of any cyanide released *in vivo* by the cyanogenic glycoside, linamarin, that occurs in that plant.

The presence of an extracellular, constitutive rhodanese in the cyanide-degrading fungus, *Rhizopus oryzae* has also been reported (Ray, *et al*, 1991). The fungus was isolated from rotten cassava tubers.

Kakes & Hakvoort, 1992, however, in their comparative studies on various plants, already reported in literature to have rhodanese activity, disagree with the high activities reported. No correlation between cyanogenesis and rhodanese activity was found in their study. They found very low enzyme activities in those same plants and in certain plant species none at all. They suggest that the values were possibly inflated due to spontaneous non-enzymatic reactions not accounted for by the respective authors.

The thiocyanate formed during the detoxification of cyanide in the presence of rhodanese, although relatively non - toxic compared to cyanide, has been shown to be toxic in certain organisms (Wood *et al*, 1998). Chemolithotrophic and heterotrophic bacteria have, however, been demonstrated to possess the capability of its biodegradation (Wood *et al*, 1998). Thiocyanate metabolism has been observed to occur in *T. thiocyanoxidans*, by the hydrolysis of thiocyanate to sulphide, ammonia and carbon-dioxide (Happold *et al*, 1954). They proposed that the carbon requirements of the organism is satisfied by the hydrolysis of thiocyanate and the subsequent absorption of carbon-dioxide.

Stafford and Callely, 1969, have reported on a '*Pseudomonas stutzeri* - like

pseudomonad' to utilize thiocyanate as its sole source of nitrogen and sulfur for growth.

Shivaraman *et al*, 1985, found thiocyanate removal to be dependant on the concentrations of cyanide and phenol present in a mixed effluent from coal carbonisation. At concentrations of above 22 and 1251ppm cyanide and phenol respectively, rate of thiocyanate removal was observed to decrease in an activated sludge process.

Wood *et al*, 1998, have reported utilization of thiocyanate or cyanate by *Methylobacterium thiocyanatum* sp.nov. as the sole source of nitrogen and as a sole source of sulfur in the former, in the absence of any sulfur compounds.

Recently, Sorokin *et al*, 2001, have for the first time reported thiocyanate degradation under highly alkaline conditions (pH 10). The study demonstrated the utilization of thiocyanate as the nitrogen source and as an energy source in alkaliphillic obligately organoheterotrophic and obligately lithoautotrophic sulfur-oxidising bacteria, respectively under alkaline conditions.

1.6.4 Cyanide oxygenase

Harris *et al*, (1987) reported *P. fluorescens* NCIMB 11764 utilizes cyanide as a nitrogen source for growth by converting cyanide to CO_2 and NH_3 under aerobic conditions, with NH_3 being assimilated. . The presence of NADH or NADPH was found to be necessary as also the utilization of oxygen which led the authors to suggest the presence of a cyanide oxygenase system. (Harris *et al*, 1987). Two possible pathways were proposed:

A dioxygenase where there is a direct conversion of cyanide into CO_2 and NH_3

$$NAD(P)H + 2H^+ + HCN + O_2 \longrightarrow CO_2 + NH_4^+ + NAD(P)^+$$

If the cyanide degrading system is a monooxygenase, then an intermediate, cyanate could result:

$$HCN + O_2 + H^+ + NADPH \longrightarrow HOCN + NADP^+ + H_2O$$

The cyanate formed then in the presence of another enzyme, Cyanase [EC 3.5.5.3 cyanate aminohydrolase], undergoes catalytic decomposition to form CO_2 and NH_3.

$$HOCN + H_2O \longrightarrow CO_2 + NH_3$$

Cyanate is found to be degraded by cell free extracts of both ammonium and cyanide-grown *P. fluorescens* (Harris *et al*, 1987).

Dorr and Knowles, 1989, however, noted that although the bacterium *P. fluorescens* NCIMB 11764 was able to degrade cyanate via a cyanase system, it was not co-

induced with the cyanide oxygenase system. Kunz *et al,* 1994 later showed that *P. fluorescens* had concentration-dependant routes of cyanide degradation in that at low concentrations the dominant pathway was via cyanide oxygenase. At higher concentrations, two mechanisms were involved, viz. formation of formamide via cyanide hydratase and the other involving cyanide dihydratase (cyanidase) resulting in the production of formate.

Apart from the *Pseudomonas* species, cyanase has also been identified in some plants. Cyanase has been sequenced and has been found to contain 156 amino acid residues (Dubey and Holmes, 1995).

1.6.5 Cyanide dihydratase (Cyanidase)

The enzyme cyanide dihydratase catalyses the conversion of cyanide into formate and ammonia:

$$HCN + 2H_2O \longrightarrow HCOOH + NH_3$$

The difference between this enzyme, and the cyanide hydratase and other enzymes is that formamide is not formed in the degradation process.

Ingvorsen *et al,* 1991, have reported the pathway in *Alcaligenes xylosoxidans* subsp. *denitrificans* DF3. They found conversion of cyanide into formate without any formation of intermediates such as formamide. Novo Nordisk A/S have patented the immobilized enzyme product sold as CYANIDASE ® (Novo Industri A/S European

patent, 88), which utilizes cyanide dihydratase from this *Alcaligenes* species. Cyanidase shows resistance to metal-cyanide complexes, organic nitriles and aromatic and aliphatic alcohols.

Another cyanide dihydratase enzyme has been isolated from *Bacillus pumilus C1* (Meyers *et al*, 1991). The divalent ion, Mn^{+2} at a certain concentration was found to be essential in producing cyanide-degrading activity and the activity was found to be intracellular. The authors suggested the requirement of manganese to be linked to the induction of the gene(s) involved in cyanide degradation or manganese could serve as an important component of the cyanide degrading activity. Activity was also found to be enhanced by other metals such as Cr^{+3}, Fe^{+3}, Tb^{+3} and Sc^{+3} (Meyers *et al*, 1993). This enzyme has also been observed in *P. fluorescens* NCIMB 11764 by Kunz *et al*, (1992).

Cyanidase, as an enzyme system has been used under experimental field conditions to degrade metallo-cyanides (Dubey and Holmes, 1995). Positive results were obtained which could be attributed to its resistance to metallic toxicants and the non-requirement of any co-factors/coenzymes for its catalytic activity. Dubey and Holmes, 1995, have suggested that cyanidase and rhodanese could be used in the treatment of waste streams containing metallic cyanides and other forms of cyanide by using protective devices to keep the enzymes stable and reactive.

1.6.6 Other degradation pathways

Biological removal of metal ions from aqueous solutions can involve different mechanisms. The key mechanisms proposed are volatilisation, extra-cellular precipitation or complexation and intracellular or cell surface accumulation (Goncalves *et al*, 1998). Biological degradation of metallo-cyanide complexes has also been reported. The white rot fungus, *Phanerochaete chrysosporium* has been reported to mineralise cyanide and cyanide complexes of Fe, Cu, Cd, Zn, and Cr (IV) (Shah and Aust, 1993). Tetracyano-nickel (II) has been reported by Rollinson *et al*, 1987, to be utilized by isolates of *Pseudomonas fluorescens* biotype II NCIMB 11764. Isolates of both *Pseudomonas* and *Klebsiella* sp. and have also been reported by other authors, as the sole source of nitrogen (Silva-Avalos *et al*, 1990). Barclay *et al*, 1998, have reported on strains of *Fusarium solani* that degrade Ni and Fe cyanides. Recently, Patil and Paknikar, 2000, reported on more than 99.9 % removal of 0.5mM metal cyanide (ca. 52mg l^{-1} cyanide and 30-40mg l^{-1} copper/zinc) in 15 hours with sugarcane molasses as the carbon source. A rotating biological contractor process, in a continuous mode, was used.

Porter and Knowles, 1979, have observed the role of cysteine in certain strains of *Citrobacter freundii* and *Enterobacter aerogenes* in the detoxification of cyanide. Knowles, 1976; Macadam and Knowles, 1984, have also suggested the role of cysteine as a co-substrate in the catalysis of the cyanide degradation reaction by the enzyme β-cyanoalanine synthase. Another possible role suggested for cysteine is as a chelator of metal cyanides, therefore reducing the toxicity of cyanide (Knowles, 1976).

In certain bacterial species, cystine (and homocystine) produced from the oxidation of cysteine reacts with the cyanide to form β-cyanoalanine, which further gets hydrolysed to CO_2 and NH_3 (Ressler *et al*, 1973).

Dumestre *et al*, 1997, have also reported the presence of an amidase enzyme along with the cyanide hydratase in *Fusarium solani* IHEM 8026. The amidase converts formamide to formate which can be further metabolized to CO_2. It thus helps the microorganism to utilize cyanide as a source of nitrogen for growth.

Babu *et al*, 1995, found cell-free extracts of *Pseudomonas putida* to degrade cyanide, cyanate, formamide and thiocyanate into ammonia. They proposed that the conversion of cyanide to ammonia was via formamide, but due to the high rate of amidase activity, formamide was not detected.

An enzyme for organic cyanide degradation, nitrilase, has been reported in *Klebsiella ozaenae*. It transforms the herbicide bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid. This enzyme has been also reported in *Nocardia* spp., *Arthrobacter* spp. and *P. aeruginosa* and exhibits a broad specificity for a variety of aromatic nitriles converting them to their corresponding acids (Nawaz *et al*, 1991).

A report by Chen and Kunz, 1997, describes a non-enzymatic degradation of cyanide in *Pseudomonas fluorescens* NCIMB 11764, in that an iron-chelating species, identified as a putative siderophore has been used for removing cyanide by oxidation

to ammonia and carbon-dioxide. The authors suggest that both cyanide oxygenase as well as a putative siderophore component play a role in cyanide utilization.

Silva-Avalos *et al*, 1990 have reported a complexed-cyanide degrading bacterium, *Pseudomonas putida* BCN3 and another strain, *P. paucimobilis mudlock* ATCC 39204 has been used in the biological treatment of cyanide containing waste water (Mudder and Whitlock, US patent, 1984).

Finnegan *et al*, 1991, have isolated and characterised an *Acinetobacter* spp. RFB1, capable of degrading and assimilating cyanide containing compounds like the cyano-metal complexes. A constitutive enzyme was reported to be involved.

Fallon, 1992, demonstrated an anaerobic route of cyanide degradation that follows a hydrolytic route similar to that observed in aerobic systems. However, the pathway was not distinguished as to whether it was a direct hydrolysis to formate or involved a two-step hydrolysis involving formamide as an intermediate. The formate was then generally converted to bicarbonate.

1.7 *Biotechnology of cyanide destruction*

Bioremediation offers new alternatives for cyanide degradation, with some cost-effective advantages over the processes involving physico - chemical treatments. Advantages include treatment efficiencies being equal or exceeding those of chemical treatment; significantly lower plant construction and operating costs; costs are relatively fixed since no oxidising chemicals are needed and so greater volumes of wastes do not necessarily increase the costs proportionately; and generally a lower production of total dissolved solids and sludges (Howe, 1965).

Case studies

Although there are a number of reports of bench-scale demonstrations of cyanide biodegradation, the only commercial scale cyanide biodegradation plant perhaps in the whole world, exists at the Homestake Mine, South Dakota (Mudder and Whitlock, 1984). The plant, in operation for more than two decades has proved to be less expensive, in both capital and operating costs (60 and 29% less than the alternative, respectively) than the best available alternative process – the copper catalysed H_2O_2 treatment. Removal rates of the contaminants varied with plant operation but were 91% - 99.5% for total cyanide and 98 - 100% for weak acid dissociable (WAD) cyanide. Biological systems are complex systems and have the key advantage of tending towards self-regulation, rapidly developing their own self-regulatory mechanisms. The Homestake biological system is beginning to approach such a self-regulating passive system that result in mitigating cyanide pollution problems almost indefinitely at low or no cost (Dubey and Holmes, 1995).

A study in the UK has also been carried out at the 10-hectare site of the Green Bank Gas Works, east of Blackburn. This site was contaminated with coal tar, phenols, heavy metals and spent oxides containing complex cyanides. A scheme devised by Biotreatment Ltd of Cardiff, UK, used a combination of conventional engineering and microbiological techniques to reduce the cyanide to pre-determined levels. Part of the site was encapsulated in clay and the remaining treated biologically. The latter treatment represented a saving of US$70,000 over conventional methods (Bewley and Theile, 1988).

The objective of the study at USMX Green Springs Gold operation site was to assess the feasibility of using bacterial oxidation to reduce the levels of cyanide. The site had process liquid from the plant containing 20ppm of WAD cyanide, principally in the form of copper and nickel cyanides. Cultures of immobilized *Pseudomonas pseudoalcaligenes*, used in the treatment reduced the cyanide concentration to 8.5 ppm, with a flow rate of cyanide at 190 litres/min, at the termination of the field trial 15 weeks later (Lien and Altringer, 1993).

Acinetoobacter RFB1, in the separation of gold from other metal-cyanide complexes in mining eluents and use of *Pseudomonas aeruginosa* to upgrade shale oils by the selective removal of nitriles are two other examples of practical application of cyanide-degrading microorganisms (Dubey and Holmes, 1995).

Successful bioremediation in association with H_2O_2 treatment, mediated by indigenous microorganisms has been reported in cyanide-contaminated soil and water in Lake Wood, Colorado (Rouse and Gochnour, 1992).

Fluidized bed reactors with immobilized cultures have also been tested (Babu *et al*, 1992). The main advantages over free cells is there is no cell loss during washing and also provide high population densities at any flow rate.

A microbial cyanide sensor for monitoring river water was reported by Ikebukuro *et al*, 1996). The sensor was based on the inhibition of respiration of *Saccharomyces cerevisiae*. The sensor is composed of two oxygen electrodes that measure the respiration of the *S. cerevisiae* and a reactor containing *S. cerevisiae* immobilized on beads.

A two-step anaerobic process involving an equilibration/pre-acidification reactor followed by a methane reactor has been proposed by Siller and Winter, 1998. These systems have been applied for pre-acidification of highly concentrated, insoluble biopolymers containing wastewater for the food industry. A pre-treatment is recommended to protect the methanogenic reactor from toxic substances and clogging (Siller and Winter, 1998).

Knowles and Bunch, 1986, have reported that although both aerobic and anaerobic processes have been studied, aerobic systems have been the preferred systems, probably since the cyanide degrading ability by methanogens is inhibited in anaerobic conditions.

1.8 *The fungal species, Trichoderma*

The genus *Trichoderma* is cosmopolitan in soils and on decaying wood and vegetable matter. Due to the diverse metabolic capability of these species, their aggressively competitive nature, rapid growth and resistance to noxious chemicals, species of *Trichoderma* are frequently found to be the dominant components of the soil microflora in widely ranging habitats (Gams and Bissett, 1998; Klein and Eveleigh, 1998).

Colonies of *Trichoderma harzianum* grow rapidly with predominantly effuse conidiation appearing granular or powdery that rapidly turn yellowish-green to dark green. The conidia are subglobose to ovoid, smooth walled and pale green in colour (Gams and Bissett, 1998).

Trichoderma species, being well known hyper-producers of degradative enzymes including chitinases and cellulases, possess the ability to transform an extraordinarily wide variety of organic materials of both natural and xenobiotic origin (Klein and Eveleigh, 1998).

Trichoderma spp. are known to be plant-growth promoters and have been reported to increase plant growth in a range of bedding and crop plants, including carnation, chrysanthemum, petunia, pea, radish and tobacco (Ousley *et al*, 1994). They have reported identification of six strains that gave stable growth promoting activity over a period of 4 years. Growth promotion by *Trichoderma* spp. has been shown to be

dependant on the crop, the strain used, the concentrations of inocula applied, and also the form of inocula (Lynch *et al*, 1991; Lynch *et al*, 1991A).

Ousley *et al*, 1993A, have reported that the mechanism of growth promotion by *Trichoderma* was unknown. Various suggestions have been documented such as *Trichoderma* spp. increase the efficiency of nutrient transfer from compost to the roots since *Trichoderma* has been found to penetrate and colonise the interior of the root systems. Another mechanism proposed is that the *Trichoderma* inocula remove toxic material from the soil or alternatively the growth response is due to the production of a heat-stable metabolite promoting growth. The observation of dead autoclaved biomass stimulating growth has given credence to this idea. In some instances this may involve control of minor pathogens. Although direct stimulation by *Trichoderma* could not be ruled out, some soils suppressive to plant pathogens such as *Pythium* and *Rhizoctonia* have been found to contain high levels of *Trichoderma* (Ousley *et al*, 1993).

On the other hand, Ousley *et al*, 1993 have also reported on studies on biocontrol which have shown plant inhibition by *Trichoderma* in lettuce seedlings and radish. This variation in effects on plant growth has been attributed by them, to the differences in age and form of inocula. Biocontrol, in simple terms can be defined as the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook, 1993).

Isolates of *Trichoderma* sp. have been used against diseases in many different crops, e.g., cotton, grapes, sweet corn, lettuce, onions, peas, plum, apples and carrots, caused by pathogens such as *Pythium, Rhizoctonia, Botrytis* and *Fusarium* (Hjeljord and Tronsmo, 1998).

Trichoderma strains are good biocontrol agents and good plant growth promoters (Lynch et al, 1991). They are adept colonisers and have been promoted as biocontrol agents (Klein and Eveleigh, 1998).

The biochemical capabilities of the *Trichoderma* is manifested in the ability of various species to break down or transform a wide range of organic molecules including several hazardous xenobiotics such as Arachlor, Malathion, DDT, endosulfan, pentachloronitro benzene and pentachloro phenol as well as hydroxylation reactions of aflatoxins (Kubicek-Pranz, 1998).

Lynch, 1990 reported that low concentrations of toxins formed in the rhizosphere could possibly retard plant growth. It was thought that *Trichoderma* added to the compost may detoxify these toxins and hence promote plant growth. Toxins like cyanide may be introduced into the rhizosphere by plants or by microbial activity.

Timonim, 1941 has reported the isolation of *Trichoderma viride* that was resistant to cyanide and also its growth was enhanced by 1.3 mM cyanide.

There has been another instance of a *Trichoderma* spp. being involved in cyanide

metabolism. *Trichoderma* sp. MB 519 was isolated, intact cells of which, were found to metaboilize cyanide and ammonia from diaminonitrile. In the partially purified form, however, ammonia formation was very low (Kuwahara and Yanase, 1985).

Trichoderma koningii was shown to grow at cyanide concentrations of higher than 10mM (260ppm) and also utilize cyanide as the sole nitrogen source. However, growth was not detected above concentrations of 20mM (520ppm) cyanide (Pereira *et al*, 1999).

Immobilized *Trichoderma harzianum* has been reported to breakdown polycyclic aromatic hydrocarbons in soil. Anthracene was found to be completely metabolised to carbon-dioxide (Ermisch and Rehm, 1989).

Lynch (unpublished) demonstrated the capacity of certain strains of *Trichoderma* to catabolize cyanide. Varying rates of cyanide breakdown were observed between the different strains experimented with in that study. Plant growth studies were also carried out with lettuce plant (*Lactuca sativa L*). The present PhD was a follow-up of this work.

1.9 *Aims of the project:*

There has been no reported evidence of studies on the cyanide-catabolizing properties of *Trichoderma harzianum*. The project was initially aimed at endorsing Prof. Lynch's work (unpublished) to be followed by studies on evaluating the possibility of cyanide-degrading enzymes in *Trichoderma* and eventually observing degradation of cyanide in liquid cultures and soil.

The principal objectives of the PhD work were:

a) Investigation of three cyanide-catabolizing enzymes in five selected strains of *Trichoderma* including studies on induction of the enzymes
b) Characterization/biochemical studies on the rhodanese enzyme
c) Flask culture studies to show degradation of cyanide, and
d) Microcosm studies to evaluate effectiveness of the strains in degrading cyanide present in soil.

CHAPTER 2:

OCCURRENCE AND DISTRIBUTION OF THE CYANIDE-DEGRADING ENZYMES IN *TRICHODERMA*

2.1 INTRODUCTION

This Chapter reports the investigation into the occurrence and distribution of cyanide-catabolizing enzymes in a variety of strains of *Trichoderma harzianum*. The enzymes chosen for investigation were cyanide hydratase, rhodanese and β-cyanoalanine synthase. The choice of enzymes was based on past literature. Whereas cyanide hydratase has been reported in a variety of fungal species, rhodanese has been found in almost the whole spectrum of living forms, from bacteria to animals. β-cyanoalanine synthase, on the other hand has been reported only in a few plants and bacteria. In the selected fungal species of *Trichoderma*, we focussed on the enzymes most likely to be found (cyanide hydratase and rhodanese), as well as an unexpected one (β-cyanoalanine synthase). It was thought that by such a choice of enzymes a broad idea of the distribution of the enzymes in the fungal species of *Trichoderma* would be obtained.

The *T. harzianum* strains were chosen based on the previous work done on lettuce where a number of different strains of *Trichoderma* were tested for resistance to and degradation of cyanide (Lynch, unpublished). Of these, the three fastest degraders from that study were chosen along with another one that was not as good but has been found to act as a good plant growth promoter.

Two strains from Professor Chris Knowles in Oxford, viz. *Fusarium oxysporum* and *F. solani* were used for comparisons in the degradation studies that follow later on in this thesis. They were also analysed in a few of the rhodanese studies in this chapter.

2.2 MATERIALS & METHODS

2.2.1 Chemicals and reagents

All the chemicals and reagents used were of the highest grade purity and quality. Most of the chemicals were supplied by Sigma Chemical Company or in other cases, as stated.

2.2.2 Sources of the isolates

2.2.2.1 Sources of the Trichoderma isolates

Isolate name and No.		Species	Source
TH1/ TO90	IMI 275950	*T.harzianum*	J.M. Lynch, University of Surrey.
WT/ TO77	Wild type	*T.harzianum*	R. Baker, Colorado State University, U.S
T12/ TO82	Wild type	*T.harzianum*	G. Harman, Cornell University U.S
10/ TO80	IMI 351107	*T.harzianum*	J.M. Lynch, University of Surrey
TO10	Wild type	*T.pseudokoningi*	M. Welland, Abies seeds

2.2.2.2 *Sources of the Fusarium isolates*

Isolate name	Source
Fusarim oxysporum	C. Knowles, Oxford University
F. solani	C. Knowles, Oxford University

2.2.3 *Preparation of media and growth of fungi*

2.2.3.1 *Autoclaving*

Aqueous media were sterilized by autoclaving at 121° C, at a pressure of 15 lb/in^2 for 20 mins.

2.2.3.2 *Preparation of media*

Potato Dextrose Agar (PDA) and Broth (PDB) were used as media for growing the fungi. PDA (39 g l^{-1}) was sterilized by autoclaving and then plates were poured under sterile conditions. 24g of the PDB was mixed in a litre of reverse osmosis (R.O.) water. PDB (volumes of 25ml each) was sterilized in 250 ml conical flasks.

2.2.3.3 *Plating onto Potato Dextrose Agar (PDA) plates and inoculation into Potato Dextrose Broth (PDB)*

Freeze-dried selected strains of fungi were first resuscitated using sterile R.O. water. Two to three drops of each were then spread onto sterile PDA plates and incubated at 25°C for 5 days. Duplicate plates for each strain were made. Culture flasks were

prepared after the above incubation by inoculating 3-4 blocks (approx. $2mm^2$ each) of the growing fungus along with the agar, into each flask containing sterile 25ml PDB. Five replicate flasks for each of the seven different isolates were incubated at 25°C on a MKV Orbital Shaker (L.H. Engineering Company Limited) set at approx. 220 rpm for 3 days. For experiments in which cyanide was added to the medium, to look for induction of the enzymes due to cyanide, 1ml of KCN prepared in R.O. was added, aseptically, 18 hours before assaying, to make a final concentration of 0.1mM KCN in the medium.

2.2.4 *Preparation of the crude fungal enzyme extract*

On the completion of the above incubation period, the fungal cultures were observed to grow as pellets in the culture flasks. Therefore, on the day of the assay, the fungal cultures were homogenised. Contents of each culture flask was homogenised for 30 secs using a Colworth Stomacher 80, using stomacher bags. The homogenised cultures were then filtered through a Whatman No. I filter paper. The filtrate was used for all the enzyme assays and protein estimations.

2.2.5 *Enzyme assays*

2.2.5.1 *Rhodanese assay*

The assay was based, according to the method of Sorbo (1953), on the formation of a coloured complex between thiocyanate and ferric ions.

Rhodanese catalyses the following reaction:

$$S_2O_3^{-2} + CN^- \longrightarrow SO_3^{-2} + SCN^-$$

Substrate solutions were prepared immediately prior to use. KCN (BDH Chemicals Limited) and $Na_2S_2O_3$ (BDH Chemicals Limited) were prepared at concentrations of 0.125M each by dissolving them separately in 0.1M Tris-HC1 buffer (pH 8.5), and then individually adjusting their pH to 8.5 using a Griffin pH meter model 80.

After individually equilibrating the substrate solutions for 5 minutes at 30 °C, prior to the start of the assay, 1.0 ml of buffered KCN was mixed with 1.0 ml of the buffered $Na_2S_2O_3$ solution and then 0.5 ml of the sample (crude fungal extract) was added. The reaction mixtures were then incubated at 30 °C in a water-bath (Grants Instruments) at different time intervals of 0, 30, 60, 90 minutes.

At the end of the appropriate incubation times, the reaction was stopped by the addition of 0.5ml of formaldehyde (40% w/v) (BDH Chemicals Limited) and the colour developed by adding 2.5 ml of ferric nitrate reagent which consisted of $Fe(NO_3)_3.9H_2O$, 25g; HNO_3 (sp.gravity, 1.4), 262.5 mls (Fisons Scientific

Equipment); R.O. water to make up to 1 litre. The samples were then centrifuged, if necessary, in a bench-top Fisons Centaur 2 centrifuge for 5 minutes at 4000 rpm. The absorbance was recorded at 460 nm.

For the boiled controls, the fungal extract was boiled in a boiling water-bath for exactly 10 mins and on cooling to room temperature it was treated in exactly the same way as the assay samples. The rhodanese activity for each fungal strain was determined after subtracting from the absorbance reading given by the corresponding boiled controls.

Substrate blanks and enzyme blanks were also set-up. This involved the omission of the substrates (R.O. water instead) from the assay reaction in the former and in the latter, replacement of the enzyme with R.O. water to account for colour contributed by the crude extracts. The rhodanese activity for each fungal strain was determined after subtracting the readings obtained from the corresponding substrate and enzyme blanks.

A standard, sodium thiocyanate (NaSCN), ranging from 0-3 μmoles/ml, which gave a linear calibration, was set up and the experimental sample concentrations were determined from the standard graph.

There are a number of reasons for the use of formaldehyde in terminating the rhodanese reaction (Miller, 83):

(a) Formaldehyde inactivates the enzyme.

(b) It prevents the precipitation of sulphur from excess thiosulphate decomposition by the acid in the ferric nitrate reagent.

(c) Formaldehyde prevents the formation of an unstable blue ferric-excess thiosulphate complex.

(d) Formaldehyde increases the stability of the iron-thiocyanate complex.

(e) Formaldehyde removes excess cyanide.

2.2.5.2 Cyanide hydratase (CHT) / Formamide Hydrolyase (FHL) assay

The CHT/FHL assay was based on the reaction between formamide and hydroxylamine, according to the method described by Fry and Millar, (1972). The reaction of amides can be schematically represented as shown in the equation below (Fig. 2.1):

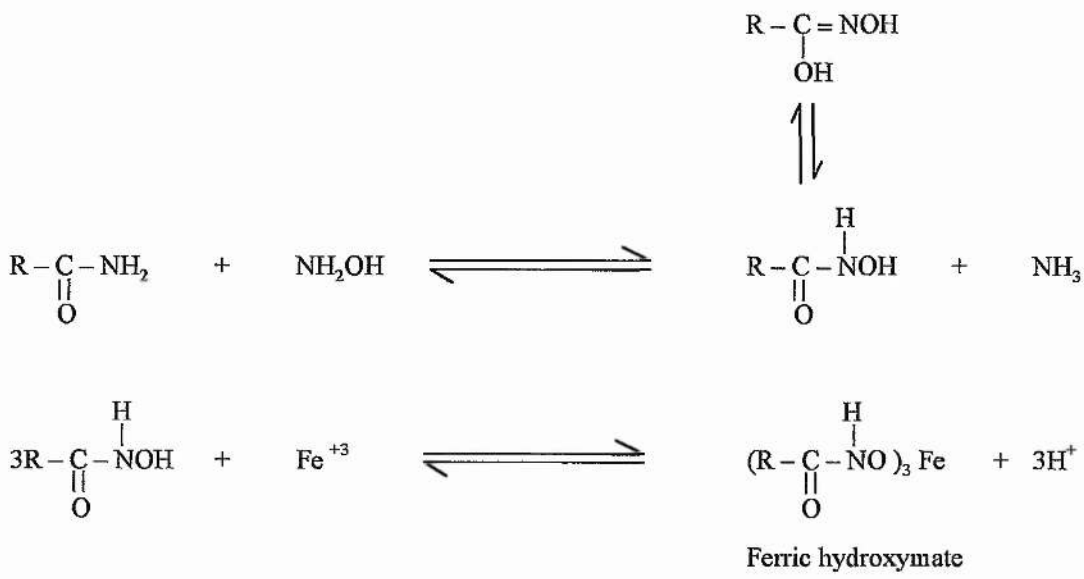


Fig. 2.1 Schematic representation of the reaction of amides and hydroxylamine; adapted from Soloway and Lipschitz, 1952.

In chemical terms, hydroxylamine reacts with certain carboxylic acid derivatives, e.g. anhydrides, imides and amides forming hydroxamic acids which on reaction with ferric ions undergo a colour reaction.

The substrate solutions were prepared immediately prior to the start of the assay. KCN solution (0.1M) was prepared in 0.05M Tris-HCl buffer (pH 8), and the cyanide solution was adjusted again to pH 8 using concentrated HCl (BDH Chemicals Limited) with vigorous stirring using a magnetic stirrer. The crude fungal filtrate (1ml) was mixed with 1ml of the above buffered KCN solution and incubated at 25 °C for 2 hours.

For the formamide analysis, the formamide concentration was measured colorimetrically by the modification of the method published for amides determination, as used by Fry and Millar, 1972.

Freshly prepared alkaline hydroxylamine reagent {1:1 mixture of 3.5M NaOH and 2.3M hydroxylamine hydrochloride} (2 ml) was mixed with 1ml of the reaction mixture. This was then re-incubated in a water-bath at 60°C for 10 mins followed by the addition of 2 ml of 1:1 mixture of 4M HCl and 1.23M $FeCl_3$ (BDH Chemicals Limited) in 0.075M HCl. The absorbance was recorded at 540 nm, within 5 mins, since the colour of the reaction fades with time.

Boiled extracts and substrate and enzyme blanks were prepared in a similar way to the rhodanese enzyme assay. They were then analysed separately for formamide following the same protocol as described above.

Standards were set-up using formamide at a concentration of 3.33 µmoles/ml. A reagent blank control was also set-up containing R.O. water instead of the formamide. Standards and the controls were included in each experiment in duplicate.

The CHT activity was obtained and expressed as the amount of formamide formed hr^{-1} . mg $protein^{-1}$ and calculated after subtracting the activity of the assay samples from the reagent blank control.

2.2.5.3 β-cyanoalanine synthase assay

The β-cyanoalanine synthase assay was based on the formation of methylene blue from the H_2S generated by the following enzymic reaction, as described by Bluementhal *et al*, 1968.

$$HSCH_2CHNH_2CO_2H + HCN \longrightarrow NCCH_2CHNH_2CO_2H + H_2S$$

The substrate solutions were again prepared immediately prior to use. KCN (0.05 M) and L-cysteine (0.01M) solutions were prepared using 0.1M Tris-HCl buffer, pH 8.5. The pH of both the solutions were then individually adjusted to 8.5.

After individually equilibrating the above solutions for 5 mins at 30°C, 0.5ml of buffered KCN solution was added to 1ml of the experimental sample (crude fungal extract) followed by 0.5ml L-cysteine. The reaction mixture tubes were then

immediately stoppered with a screw-cap and incubated in a water-bath at 30°C for 10 mins. A second set of experiments was also carried out where the incubation period was increased to 30 mins to account for low enzyme activity.

After the end of the incubation times, 0.5ml $FeCl_3$ (0.03M $FeCl_3$ in 1.2M HCl) was added, followed by the rapid addition of 0.5ml of N,N-dimethyl-p-phenylene diamine sulphate at a concentration of 0.02M in 7.2M HCl. The samples were then incubated in the dark for 25 mins, after which they were centrifuged, if necessary, in a bench-top Fisons Centaur 2 centrifuge for 5 mins at 4000rpm. The absorbance was recorded at 650nm.

Boiled extracts and substrate and enzyme blanks were again prepared in a similar way to the rhodanese enzyme assay. Each were then analysed separately for β-cyanoalanine synthase activity following the same protocol as described above. β-cyanoalanine synthase activity was obtained after subtracting the activity of the assay samples from the enzyme blank control.

A standard, sodium sulfide ($Na_2S.9H_2O$), dissolved in 0.1M Tris-HCl buffer at pH 8.5, ranging from 0 - 250nmoles sulfide ml^{-1} was also set-up. A linear calibration was obtained between 0 - 0.7 absorbance units corresponding to 0-200 nmoles sulfide ml^{-1}.

2.2.6 *Protein determinations*

Protein in the extracts of fungi was determined by the method of Miller, 1959, which was a modification of the Lowry method (Lowry *et al.*, 1951). The filtrate obtained above in Section 2.2.4 was used. For every experiment and assay, protein determinations were carried out. Bovine serum albumin (BSA) in concentrations ranging from 0-100 μg/ml was used as a protein standard. The protocol was as follows: Dilute fungal extract by 20-fold with 0.5N NaOH (May & Baker Limited).

An aliquot (1ml) was then taken and to it was added 1 ml of freshly prepared copper sulphate solution {1ml 5% (w/v) $CuSO_4 .5H_2O$ (Hopkins & Williams); 10ml 1% (w/v) sodium-potassium tartarate and 100ml 10% (w/v) sodium bicarbonate (Fisons Scientific Equipment) in 0.5 NaOH}. Tubes were left to stand for exactly 10 mins at room temperature. Folin-Ciocalteau (10x) (3ml diluted 1:10 in R.O.water) was added and the tubes immediately vortexed. Tubes were then incubated at 50 °C in a Grants Instruments water-bath for 10 minutes to allow colour to develop. After cooling the tubes to room temperature, the absorbance was read at 650nm, using Pye Unicam SP8-400 uv/vis spectrophotometer.

2.2.7 *Statistical analysis*

To estimate the statistical significance between the experimental and the control readings as well as between the different treatment groups, the Student's t-test statistical analysis was used throughout, in all the experiments.

2.3 RESULTS

2.3.1 *Reaction product standard curves*

The following enzyme reaction product curves were set up to establish the concentration ranges of each reaction product. Beer's law states that absorbance is directly proportional to the concentration was applied to calculate the respective concentrations from the curves.

2.3.1.1 *Rhodanese assay standard curve*

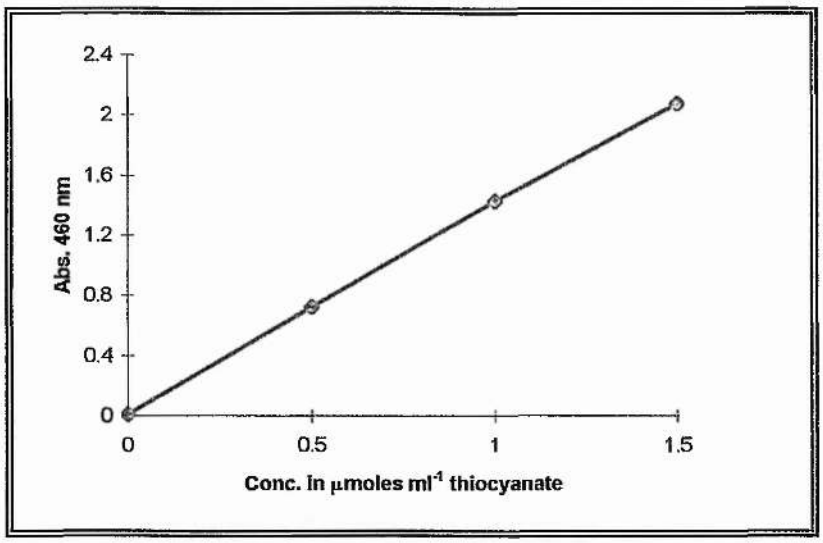


Fig. 2.2 Standard curve of absorbance at 460 nm against concentration of thiocyanate in µmoles ml^{-1}, which was used in the rhodanese assay. All the values are the means of two observations.

The above linear plot (Fig. 2.2) was used to determine the concentration of thiocyanate formed by the enzyme from each of the fungal strains used in the study.

The amount of thiocyanate formed was taken as an indication of rhodanese activity and the specific activity for each strain was calculated.

2.3.1.2 β-cyanoalanine synthase assay standard plot

The standard curve plotted below (Fig 2.3) was used to determine β-cyanoalanine synthase activities in the selected strains of *Trichoderma.* The formation of methylene blue from the hydrogen sulphide was taken as evidence of β-cyanoalanine synthase activity.

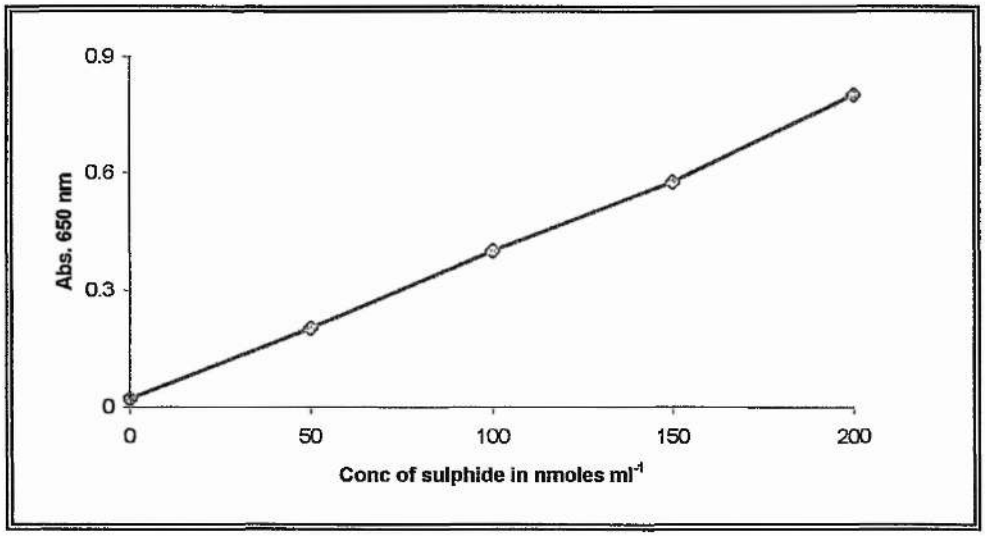


Fig. 2.3 Standard curve of absorbance at 650 nm against concentration of sulphide in nmoles ml^{-1} for the β-cyanoalanine synthase assay. All the values are the means of two observations.

2.3.1.3 Cyanide hydratase assay standard

Formamide at a concentration of 3.33 μmoles ml^{-1} was used as a standard reference in this assay for deriving the concentration of formamide liberated by the individual fungal strains. The average absorbance for 10 samples of the standard formamide (3.33 μmoles/ml) was 0.918 with a standard error of 0.07 and of the reagent blank was 0.475 with a standard error of 0.03.

2.3.2 *Enzyme activities*

2.3.2.1 *Cyanide hydratase activity*

Cyanide hydratase activities for all the five *Trichoderma* strains were calculated using the average of the standard values obtained in Section 2.3.1.3 and are plotted below (Fig. 2.4). Significant differences ($p < 0.001$) were observed between each of the strains.

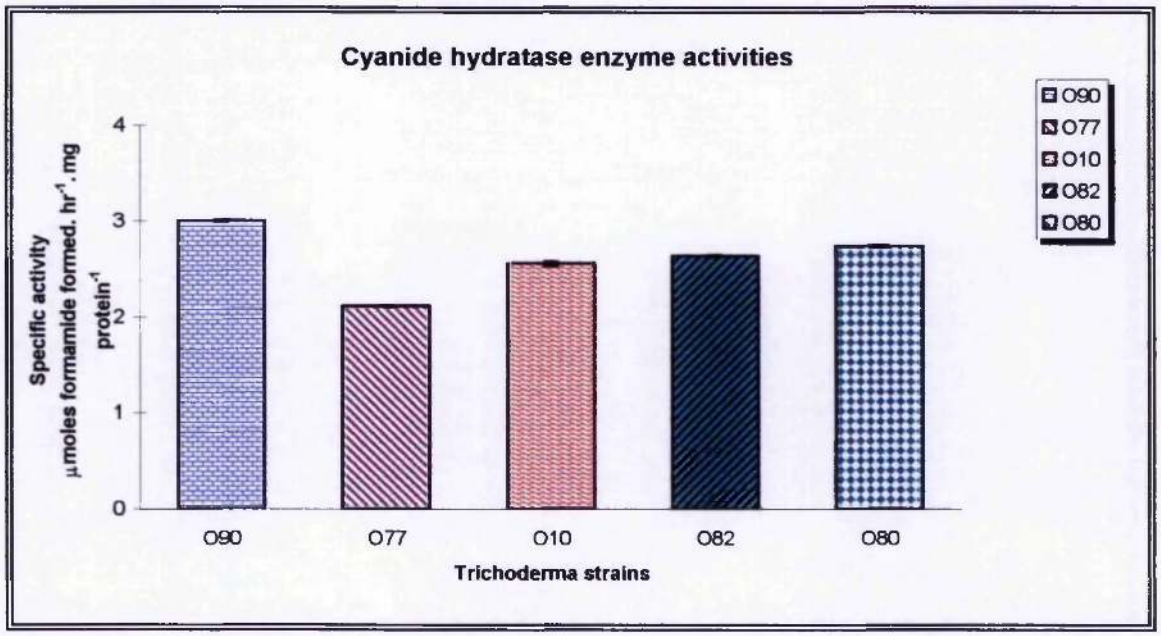


Fig. 2.4 Bar chart showing cyanide hydratase activity, in all the selected strains of *Trichoderma*. All the values are the means of five independent observations and the standard error bars are shown.

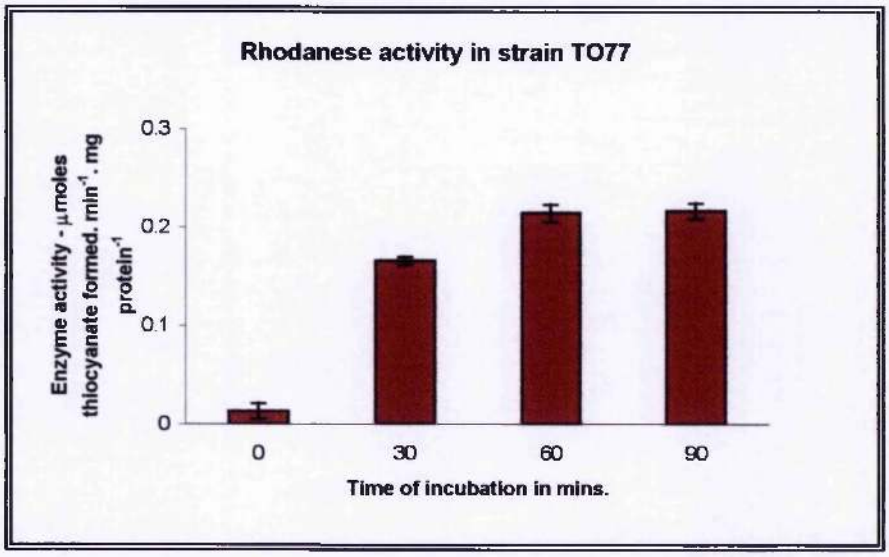


Fig. 2.6 Bar chart showing rhodanese activity in the *T. harzianum* strain O77 at the different time intervals of incubation. All the values are the mean of five independent observations and the standard error bars are shown.

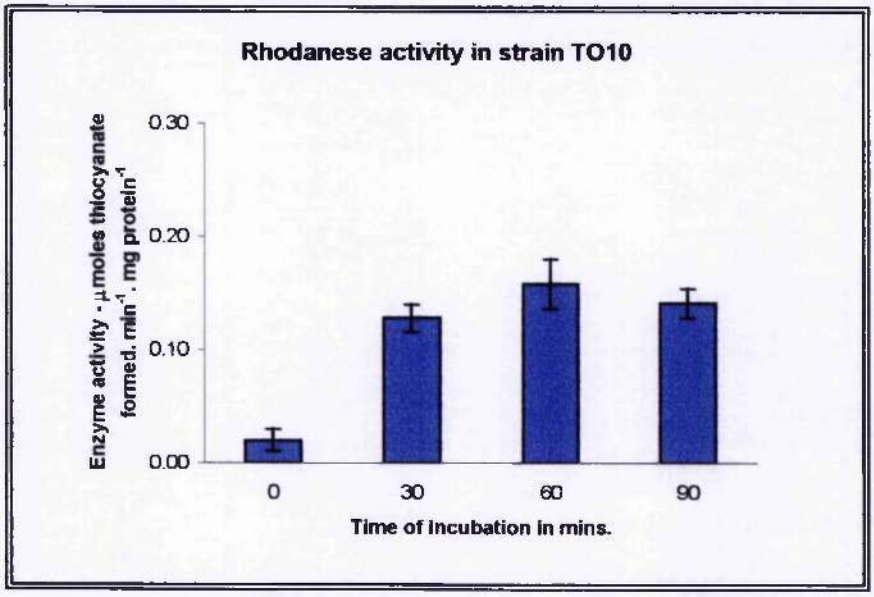


Fig. 2.7 Bar chart showing rhodanese activity in the *T. harzianum* strain O10 at the different time intervals of incubation. All the values are the mean of five independent observations and the standard error bars are shown.

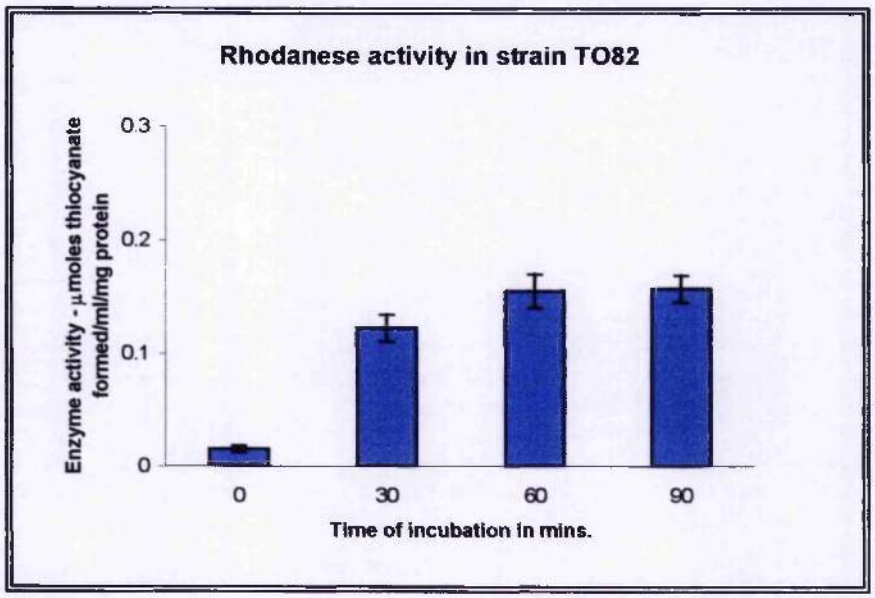


Fig. 2.8 Bar chart showing rhodanese activity in the *T. harzianum* strain O82 at the different time intervals of incubation. All the values are the mean of five independent observations and the error bars are shown.

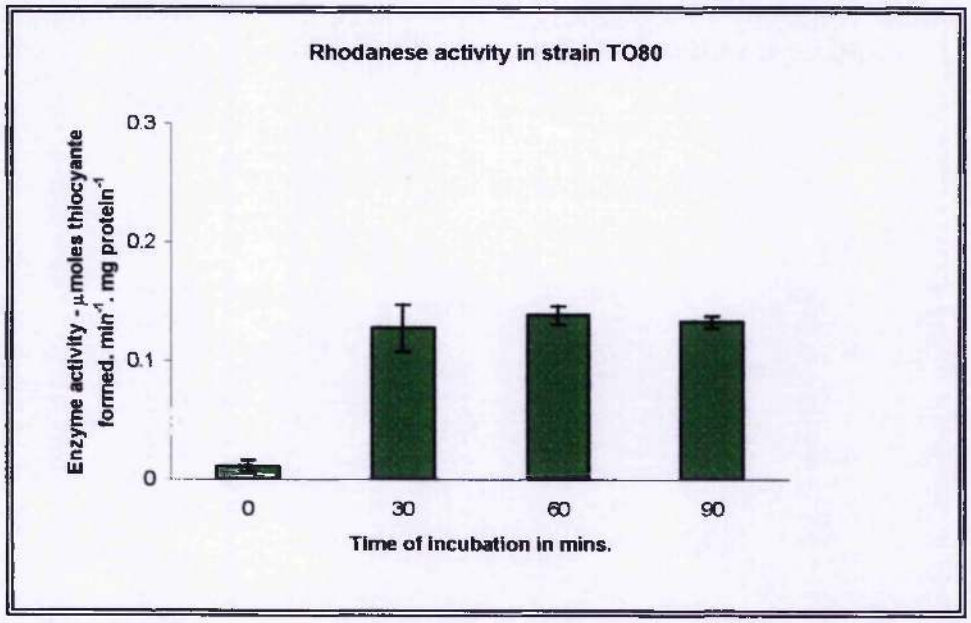


Fig. 2.9 Bar chart showing rhodanese activity in the *T. harzianum* strain O80 at the different time intervals of incubation. All the values are the mean of five independent observations and the error bars are shown.

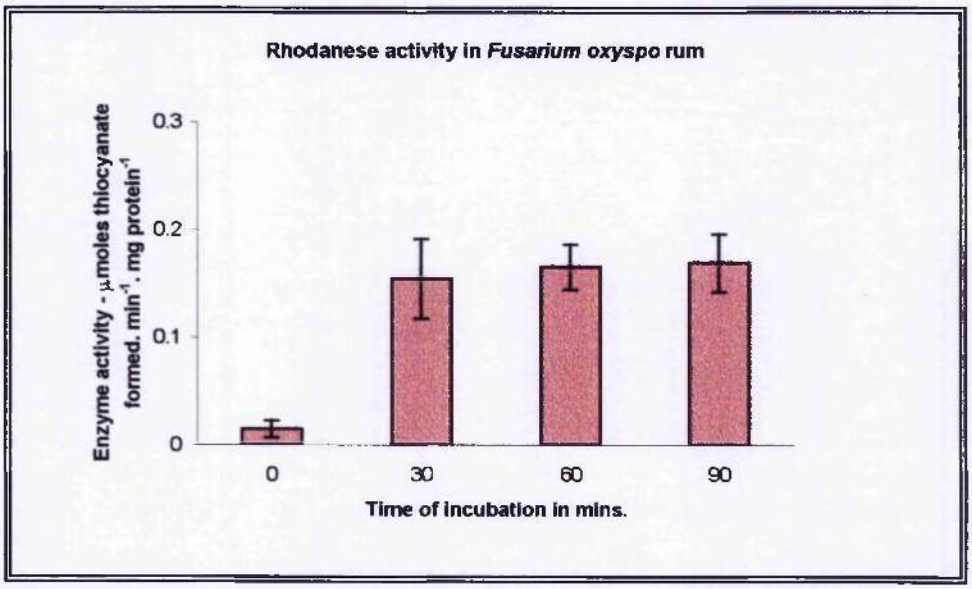


Fig. 2.10 Bar chart showing rhodanese activity in *the F. oxysporum* strain (FO) at the different time intervals of incubation. All the values are the mean of five independent observations and the error bars are shown.

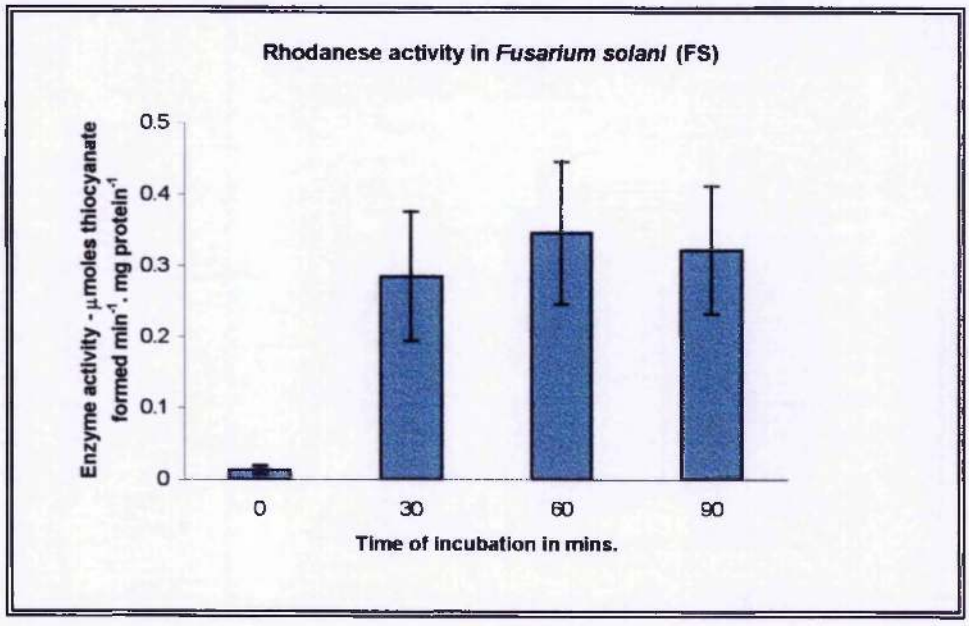


Fig. 2.11 Bar chart showing rhodanese activity in the *F. solani* strain (FS) at the different time intervals of incubation. All the values are the mean of five independent observations and the error bars are shown

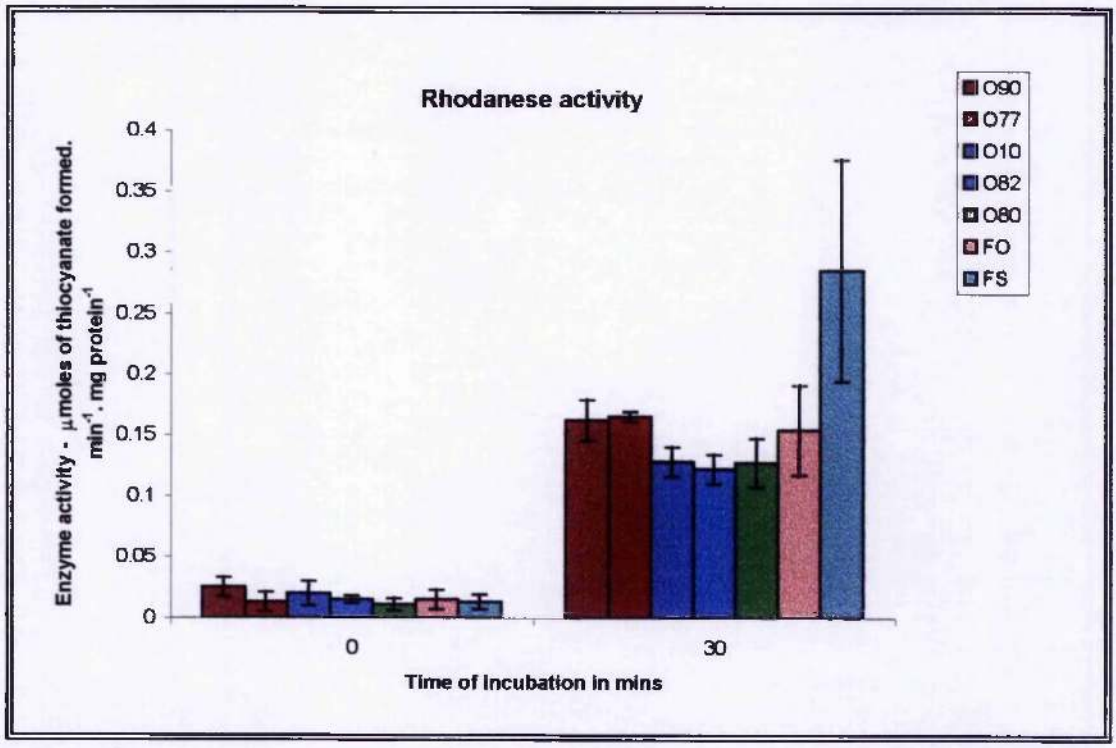


Fig. 2.12 Bar chart showing rhodanese activity, at different time intervals, in all the selected strains of *Trichoderma* and the *Fusarium* strains obtained from Oxford. All the values are the means of five observations and the standard error bars are shown.

2.3.2.3 β-cyanoalanine synthase enzyme activity

β-cyanoalanine synthase activity was not detected in any of the five selected strains of the fungal species, *Trichoderma*.

2.3.3 Studies on induction of the enzyme activities

2.3.3.1 Induction of cyanide hydratase enzyme activity

CHT activity was not found to be induced in any of the *Trichoderma* strains ($p > 0.1$) in the presence of cyanide (Fig 2.13 below). Therefore, cyanide does not appear to be an inducer of the cyanide hydratase enzyme, in any of the *Trichoderma* strains studied. On the other hand, in strains O90, O77 and O80, the addition of cyanide for induction significantly ($p < 0.001$) retards CHT activity when compared to the un-induced cultures.

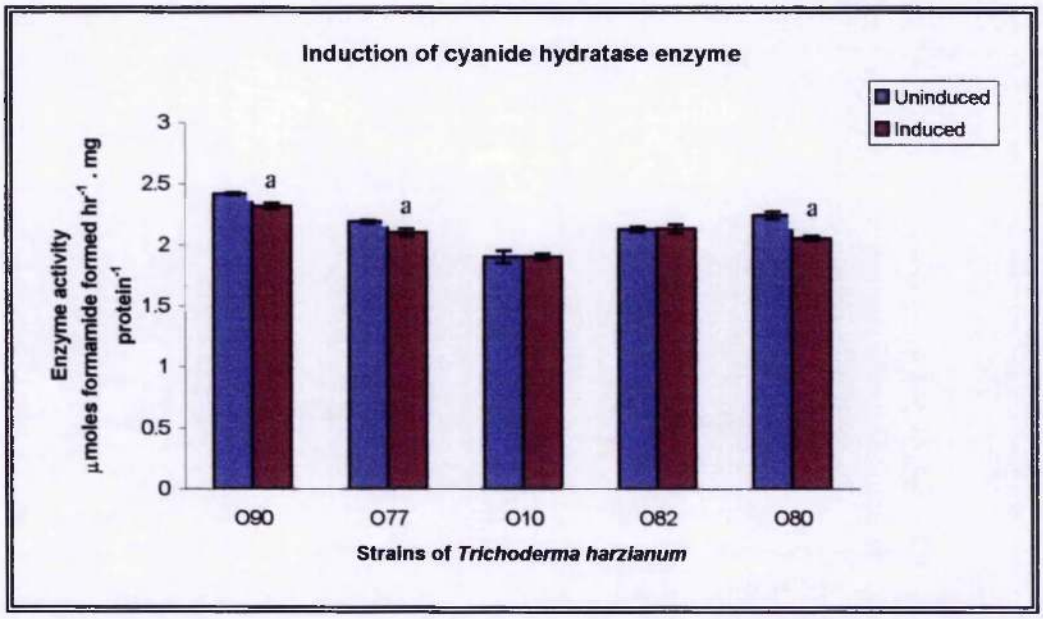


Fig.2.13 Cyanide hydratase activity in uninduced and induced strains of *T. harzianum*. All the values are the means of five independent observations and the standard error bars are shown. Cyanide at a concentration of 0.1mM was added 18 hours before the assay. a = significantly different at $p < 0.001$.

2.3.3.2 Induction of rhodanese enzyme activity

None of the *Trichoderma* strains ($p > 0.1$) showed the rhodanese activity to be induced in the presence of cyanide (Fig 2.14 below). Therefore, cyanide does not appear to be an inducer of the rhodanese enzyme, in any of the *Trichoderma* strains studied. Here, rhodanese activity is found to be significantly ($p<0.001$) retarded, in the presence of cyanide, in the strains O77, O10 and O82, when compared to the uninduced flasks.

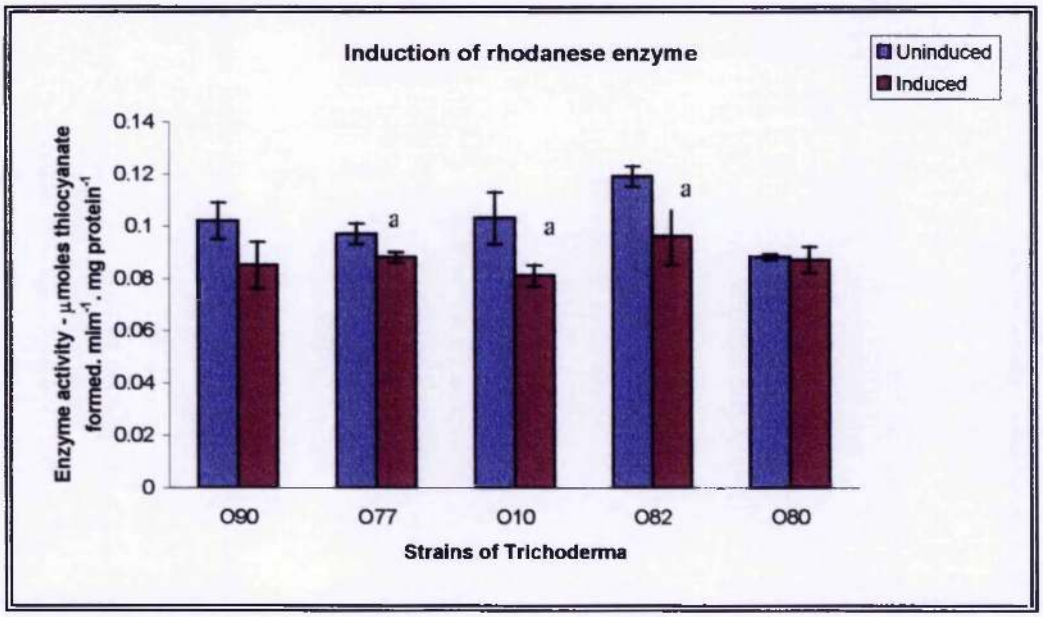


Fig. 2.14 Rhodanese activity in uninduced and induced strains of *T. harzianum*. All the values are the means of five independent observations and the standard error bars are shown. Cyanide at a concentration of 0.1mM was added 18 hours before the assay. a = significantly different at $p < 0.001$.

2.4 DISCUSSION

All the fungal strains investigated in this study were capable of metabolizing cyanide via both rhodanese and the cyanide hydratase (CHT) pathways. The results of the study also demonstrated the absence of any β-cyanoalanine synthase activity, which is in accordance to literature. β-cyanoalanine is not as commonly found in fungi as it is in higher plants and few bacteria (Miller and Conn, 1980; Bluementhal *et al*, 1968). On the other hand, literature cites different species of fungi possessing cyanide hydratase (Fry and Munch, 1975; Knowles, 1976).

Within the *Trichoderma* strains, the *T. harzianum* strains O90 and O80 consistently showed higher cyanide hydratase activity followed closely by O82 and the *T.pseudokoningi* strain (O10). Strain O77, on the other hand showed about 25% less activity than the former strains. This is in agreement with the experiments conducted by Lynch (unpublished), where, of these strains, O77 was found to degrade cyanide the slowest. The strain O77 was observed to degrade 50% cyanide after 25+ hours as against 3-9 hours by the other strains used, O90 and O80. Although this pattern was observed in most of the experiments, variations were observed. In the present experiment, controls, such as enzyme and substrate blanks that were run simultaneously along with the assay showed significant differences when compared to the experimental values, thus indicating true enzyme activity.

The average CHT activity of 2.5 μmoles formamide produced hr^{-1}mg $protein^{-1}$ in the present experiment, equivalent to 42nmole min^{-1}mg $protein^{-1}$, yields results very similar to the induced CHT activities of the fungi shown in Table 2.1, in experiments

carried out by Wang *et al*, 1992. The induced cultures were treated with 0.1mM KCN 4 hr prior to being assayed. Thus, in the *Trichoderma* strains studied, the constitutive CHT activities are similar to the induced activities in some of the pathogens reported.

	CHT activity in nmoles/ min/mg protein	
Pathogens	Uninduced	Induced
Pathogens of cyanogenic plants particularly sorghum		
Gloeocercospora sorghi	5.5	44.4
Helminthosporium sorghicola	0	34.6
Fusarium moniliforme	6.5	17.8
Colletotrichum graminicola	0.4	38.4
Pathogens of non-cyanogenic plants		
Stemphilium vesicarium	-	36.3
Helminthosporium turcicum	4.3	31.5
Aspergillus nidulans	0	0.9

Table 2.1 Cyanide hydratase activity in pathogens of cyanogenic and non-cyanogenic plants. CHT is expressed as nmole/min/mg. KCN (0.1mM) was used for induction and added 4hr before assaying. Adapted from Wang *et al*, 1992.

Fry and Evans, 1977, found constitutive CHT activity ranging from 0.1 to 1.2 μmoles. min^{-1}.mg $protein^{-1}$ and average induced specific activities to be 30.1 μmoles. min^{-1}.mg $protein^{-1}$, for pathogens of cyanogenic plants. However, in their study, Fry and Myers, 1981, have reported much higher induced activities of CHT of 129, 1240 and 158 μmoles formamide produced. hr^{-1}. mg protein $^{-1}$ in *Stemphilium loti*, *G. sorghi*, and *F. solani* respectively.

Initially, boiled controls were also considered. It was noted that the enzyme activities of these extracts when boiled were, in a few cases, similar to the assay samples, whilst sometimes the activity would be much higher than the assay samples. Yet at other times it would actually act as a control. One possible explanation would be that the 'over boiling' of the enzyme (crude extract) could have resulted in the 'denaturation' of the enzyme which could then possibly react chemically with the substrates. We decided to discontinue using these controls in later assays because of the inconsistent results obtained. The above observations were also similarly recorded for the rhodanese assay.

Rhodanese activity was found in all strains of *Trichoderma* and in the *Fusarium* strains. Results were again consistently found to be similar in replicated experiments and the values of one of the thrice-repeated experiments have been given here. A slightly different trend compared to the cyanide hydratase activity emerged for the rhodanese activities in the different strains. Here, both the O90 and O77 strains consistently showed higher activities when compared to the rest of the *Trichoderma* strains although overall the *Fusarium solani* strain (FS) showed the highest activity. Since saturation levels were observed to have reached at 30mins, the enzyme values for 0 and 30 mins only, were used in the comparision between the strains (Fig. 2.12). The values compared well with the reported value of 0.263 μmoles of thiocyanate formed/min/mg protein for the purified rhodanese enzyme in the *Thiobacillus* species, *Ferrobacillus ferrooxidans* (Tabita *et al*, 1969). Rhodanese activity in crude extracts of Tapioca leaf have been found to be 1.36 μmoles thiocyanate formed/min/ 75mg fresh wt (Chew and Boey, 1972). It was difficult to compare the values of enzyme

activities found in *Trichoderma* with those reported by Sorbo, 1955, of bovine rhodanese since the latter have been expressed in a different unit format.

Experiments conducted to assess the inducibility of the two enzymes in the presence of cyanide in the selected strains of *Trichoderma* gave negative results. There was no significant difference between the enzyme activities of the cultures incubated with and without cyanide in terms of enzyme induction. The enzymes, cyanide hydratase and rhodanese, in the *Trichoderma* strains studied here, therefore, appear to be constitutive. There was, in fact, evidence of inhibition of both the enzymes in a few strains of *Trichoderma* in the flasks with cyanide addition (Figs. 2.13 and 2.14). This non-inducibility of the two enzymes is in sharp contrast to literature which indicates most of the cyanide degrading enzymes to be inducible (Fry and Evans, 1977, Blumenthal *et al*, 1968, Rollinson *et al*, 1987). The cyanide hydratase (Fry and Evans, 1977) enzyme has been found to be inducible in a number of different microorganisms. Few fungi, like for eg. *Stemphilium loti, Colletotrichum graminocola, Fusarium moniliforme* (Fry and Evans, 1977), *F. lateritium* (Cluness *et al*, 1993) and *F. solani* f.sp. (Fry and Myers, 1981) do not have any detectable non-induced (constitutive) CHT activity.

On the other hand, experiments by Fry and Myers, 1981, demonstrated significant constitutive CHT activity in *G. sorghi* and *Helminthosporium maydis*. Fry and Evans, 1977 have reported constitutive levels of cyanide hydratase enzyme activity in a few fungal pathogens of non-cyanogenic plants e.g. R*hizoctonia solani* as well as in pathogens of cyanogenic plants such as *G. sorghi* and *H. turcicum*. However, the

induced mycelia showed increases of activity of CHT relative to that of the un-induced mycelium. This was not observed in the present study. One of the reasons could be that the cyanide concentration used in the study to induce CHT activity (0.1mM) was not sufficient for induction. Nazly and Knowles, 1981, used 0.5mM cyanide for induction of enzyme activity. In experiments by Fry and Evans (1977), maximal activity was obtained at 1-5mM HCN.

Rhodanese too has been reported to be inducible, for e.g. in *Thiobacillus denitrificans* (Bowen *et al*, 1965). Cyanide at a concentration of only 40 μM produced a 3-fold increase in activity.

As opposed to the above findings, in the present experiment, the level of rhodanese activity was not elevated by the incorporation of cyanide into the growth medium. Rhodanese in *Pseudomonas aeruginosa*, has also been found to be constitutive and addition of cyanide in the growth medium did not increase enzyme activity (Ryan and Tilton, 1977). *Bacillus subtilis* and *B. stearothermophilus* have been reported to produce rhodanese constitutively (Bowen *et al*, 1965; Atkinson 1975). In rodent tissues, too, rhodanese is found to be constitutive (Dubey and Holmes, 1995).

The need for a series of different blanks for an exact estimation and calculation of rhodanese activity has been stressed in a number of publications (Miller and Conn, 1980; Chew and Boey, 1972; Lieberei and Selmar, 1990). This is to take into account the thiocyanate formed due to the spontaneous non-enzymic reaction between cyanide and thiosulphate. In all the rhodanese assays carried out in the present project,

substrate and enzyme blanks were set-up and these values were subtracted from the assay readings before the enzyme activity was calculated.

In summary, all the *Trichoderma* and the *Fusarium* strains studied showed the presence of cyanide degrading enzymes, cyanide hydratase and rhodanese. β-cyanoalanine synthase, however, was found to be absent. Also, in contrast to most of the previous analyses reported, both these enzymes were found to be constitutively present and whereas their activities did not increase with incorporation of cyanide, in a few strains, however, inhibition of enzyme activities was observed.

CHAPTER 3:

CHARACTERISATION OF THE RHODANESE ENZYME

3.1 INTRODUCTION

Four strains of *Trichoderma harzianum* and one of *T. pseudokoningi* have been found to have novel cyanide degrading capability. Two enzymes facilitating this degradation, as presented in the previous chapter, have been found to be present in all the strains, viz. cyanide hydratase and rhodanese.

Although the enzyme rhodanese is ubiquitously found in nature and a number of bacterial and mammalian sources exist, there are very few reports on the characterization of rhodanese in fungi. Rhodanese catalyses the conversion of cyanide into a relatively less toxic product, thiocyanate, in the presence of thiosulphate. On the other hand, cyanide, in the presence of the other enzyme, cyanide hydratase, is hydrolysed to form formamide which eventually gets converted to carbon dioxide and ammonia.

The aim of this chapter was to characterize the rhodanese enzyme in terms of its intra- or extra-cellular activity and to assess the kinetics of the enzyme reaction including pH and temperature profiles. The relationship with the other rhodanese enzymes has also been evaluated. The *Fusarium* strains have also been analysed in this chapter.

3.2 MATERIALS & METHODS

3.2.1 Fungal Isolates

Crude extracts of rhodanese from all the seven isolates described in Section 2.2 were used for the characterization study.

3.2.1.1 Preparation of media and growth of fungi

The media was prepared and fungal strains were grown in conditions similar to that described in Section 2.2.3.

3.2.2 Characterisation of the rhodanese enzyme

The rhodanese enzyme assay was essentially similar to the one described in Section 2.2.5.1 and modified, where appropriate, to suit the different assay conditions involved.

3.2.2.1 Intra-/extra-cellular activity of the rhodanese enzyme

To investigate whether the rhodanese enzyme was present intra- or extra-cellularly, the fungal samples were prepared in the manner outlined in Fig 3.1 below. The flask cultures were prepared and grown as described in Section 2.2.3.3 and the fungal extract obtained as described in Section 2.2.4.

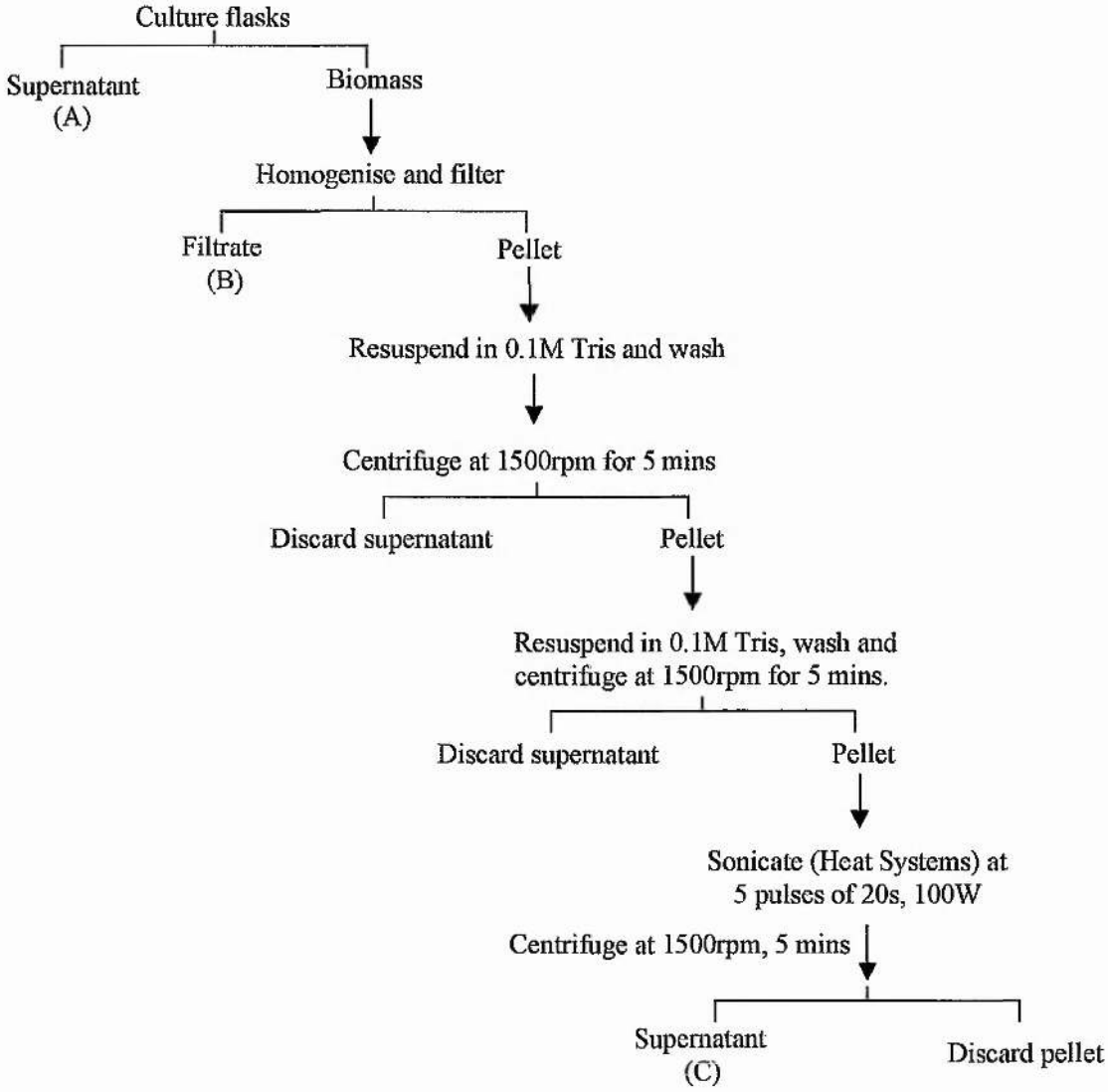


Fig. 3.1 The above protocol was followed for crude enzyme sample preparation to investigate the intra or the extra cellular activity of the rhodanese enzyme. Aliquots of (A), (B) and (C) were analysed for rhodanese activity

3.2.2.2 Rhodanese assay for the estimation of pH optima

Substrate solutions were prepared immediately prior to use. KCN and $Na_2S_2O_3$ were prepared at concentrations of 0.125 M each by dissolving them separately in 0.1 M

phosphate buffer prepared at a range of pHs. Phosphate buffer was used because it gave a wider range of buffering capacity. The pH of the substrate solutions were then individually adjusted to the required pH. The pH range used in the experiment was pH 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5.

After individually equilibrating the substrate solutions for 5 mins at 30 °C in a water-bath, prior to the start of the assay, 1.0 ml of buffered KCN at a particular pH was mixed with 1.0 ml of the corresponding buffered $Na_2S_2O_3$ solution and then 0.5 ml of the sample (crude fungal extract) was added.

The reaction mixtures were then incubated at 30 °C in a water-bath for 10 minutes. The reaction time was reduced to 10 mins from that of the previous chapter since the progress curves showed the increase in enzyme activity in the first 10 mins only (results demonstrated in this chapter). The reaction was stopped by the addition of 0.5ml of formaldehyde (40% w/v) and the colour developed by adding 2.5 ml of ferric nitrate reagent which consisted of $Fe(NO_3)_3.9H_2O$,25g; HNO_3 (sp. gravity 1.4), 262.5 mls; and R.O. water to make up to 1 litre. The samples were then centrifuged, if necessary, for 5 minutes at 4000 rpm. The absorbance was recorded at 460 nm.

Substrate blanks (R.O. water instead of the two substrates) and enzyme blanks (R.O. water in place of the enzyme) with the substrates at different pHs were also set-up. The rhodanese activity for each fungal strain was determined after subtracting the readings obtained from the corresponding substrate and enzyme blanks.

3.2.2.3 Rhodanese assay for the estimation of temperature optima

Substrate solutions were prepared immediately prior to use. KCN and $Na_2S_2O_3$ were prepared at concentrations of 0.125 M each by dissolving them separately in 0.1M Tris-HC1 buffer at pH 8.5. Water baths were set-up at different temperatures. The temperature range used in the experiment was 15, 25, 35, 45, 55, 65 and 75 °C.

After individually equilibrating the substrate solutions for 5 mins at each of the different temperatures, prior to the start of the assay, 1.0 ml of buffered KCN was mixed with 1.0 ml buffered $Na_2S_2O_3$ solution and then 0.5 ml of the sample (crude fungal extract) was added.

The reaction mixtures were then incubated at the various temperatures in a water-bath for 10 minutes. The reaction was stopped by the addition of 0.5ml of formaldehyde (40% w/v) and the colour developed by adding 2.5 ml of ferric nitrate reagent (prepared as in Section 3.2.2.2 above). The samples were then centrifuged, if necessary, in a Fisons Centaur 2 centrifuge for 5 minutes at 4000 rpm. The absorbance was recorded at 460 nm.

Substrate blanks (R.O. water instead of the two substrates) and enzyme blanks (R.O. water in place of the enzyme) at each of the different temperatures were also set-up. The rhodanese activity for each fungal strain was determined after subtracting the readings obtained from the corresponding substrate and enzyme blanks.

3.2.2.4 Estimation of Km and Vmax for the enzyme rhodanese

To determine the initial velocity, the rhodanese assay, using all the different fungal strains, was carried out at different time intervals of 2, 4, 6, 8,10, 12 and 14 mins. For the kinetic analysis of the enzyme activity, a slightly modified protocol was followed.

Substrates at different concentrations were used. Since the rhodanese assay reaction involved two substrates, a different experimental procedure to that followed for one-substrate reactions, needed to be pursued to obtain the Michaelis-Menten graphs and the enzyme constants. Here, one substrate was taken at a fixed concentration and this was assayed with varying concentrations of the second substrate. A range of the fixed concentrations of the first substrate was then assayed individually similarly. The values obtained were then plotted to get a primary plot from which secondary plots were made, as described below.

Cyanide was taken at a fixed concentration of 0.07M (assay concentration of 0.01 M CN^-) and assayed against varying final assay concentrations of sodium thiosulphate from 0.01, 0.045, 0.075, 0.1, 0.125 and 0.16 M. The two substrates (0.1ml each) were incubated with 0.5 ml of the enzyme. In the next set, cyanide concentration was taken at another fixed concentration of 0.42 M (assay conc. of 0.06 M CN) and assayed similarly with all the above concentrations of thiosulphate. Data was thus obtained for four sets of fixed assay concentrations of cyanide, viz. 0.01, 0.06, 0.11 and 0.16 M of cyanide (CN^-).

A reciprocal plot of 1/v (Enzyme activity) against 1/s (conc. of thiosulfate) at a

series of cyanide concentrations was thus obtained, which is called the primary plot. The intercepts obtained from these primary plots were then individually plotted against the inverse of the corresponding cyanide concentrations. The secondary plot is a straight line with a slope of Km^{CN}/V and the line intercepts the negative X-axis at a point, $-1/Km^{CN}$. The intercept on the y-axis equals 1/Vmax. This procedure was

followed for each of the seven different fungal strains and the Km and Vmax values, for rhodanese, for each individual strain was calculated from these plots.

For all of the above assays, the sample concentrations were determined from a standard graph which was plotted using sodium thiocyanate (NaSCN), ranging from 0-3 μmoles/ml, which gave a linear calibration plot.

3.2.3 *Protein determinations*

Protein in the extracts of fungi was determined as described in Section 2.5

3.3 RESULTS

3.3.1 *Intra-/extra-cellular activity of rhodanese*

The rhodanese enzyme was found to be extracellularly present in all the fungal strains evaluated as demonstrated in Fig. 3.2. The three supernatants were obtained as described in Fig. 3.1 and were tested for rhodanese activity using the same procedure as described in Section 2.2.5.1.

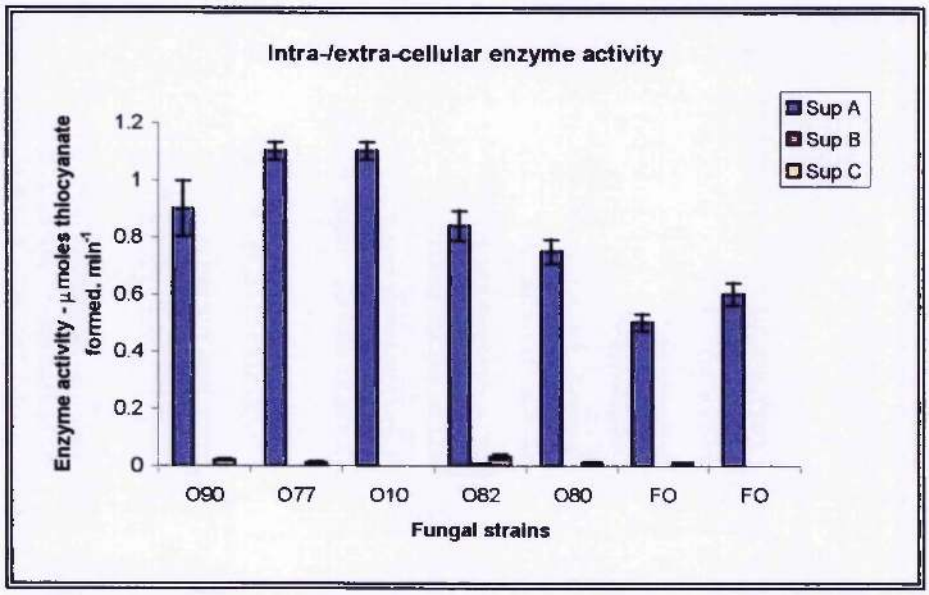


Fig. 3.2 Bar chart demonstrating the extracellular presence of the enzyme rhodanese in all the strains of fungi studied. The three supernatants were obtained as described in Fig. 3.1. Sup A was the first supernatant obtained without any treatment or centrifugation. Filtrate B was after homogenisation of the fungal pellet (biomass) and filtration. Sup C was obtained after washing and ultra-sonication of the pellet. The values are the means of five independent observations and the standard error bars are indicated.

3.3.2 Effect of pH

The relationship between the reaction rate (the rate of production of thiocyanate) and pH was studied. A broad pH optimum was obtained ranging from pH 8.5 to 10.5 for all the *Trichoderma* strains studied (Fig. 3.3).

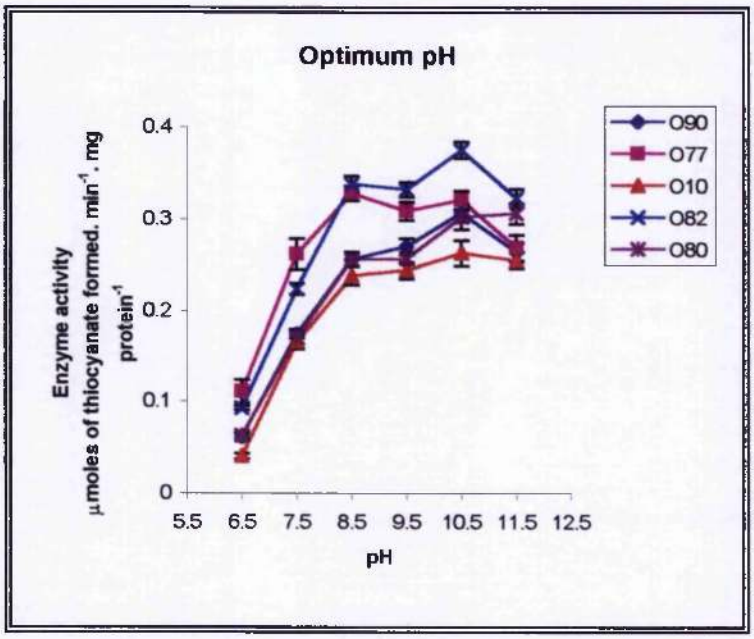


Fig. 3.3 Graph showing the optimum pH plot for each of the *Trichoderma* strains. The plotted values are after the subtraction of the enzyme and substrate blanks and are the means of five independent observations. Standard error bars are also shown.

3.3.3 *Effect of temperature*

The relationship between the reaction rate (the rate of production of thiocyanate) and temperature was studied. A broad temperature optimum was obtained, varying between all the *Trichoderma* strains studied and ranging from 35 °C to 55 °C (Fig. 3.4).

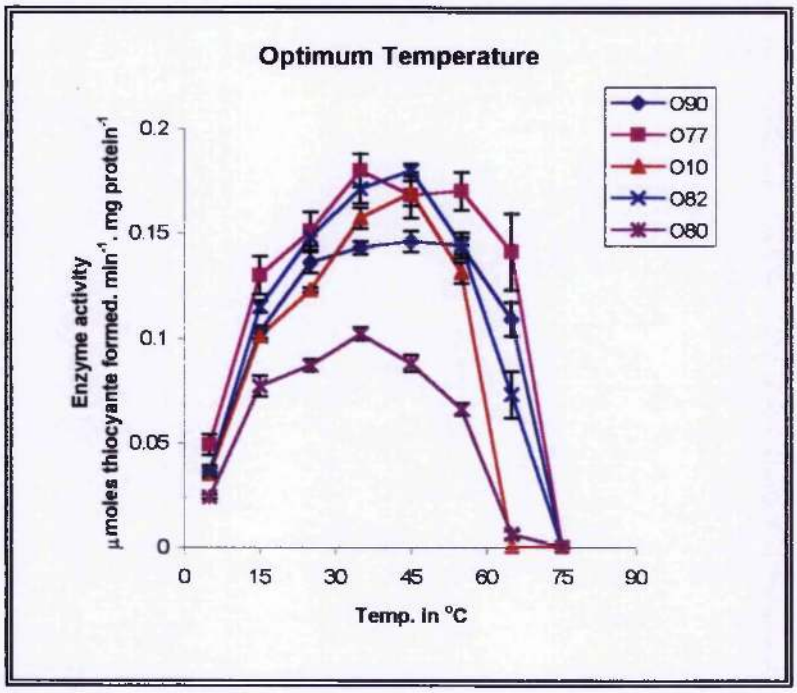


Fig. 3.4. Graph showing the optimum temperature plot for each of the *Trichoderma* strains. The plotted values are after the subtraction of enzyme and substrate blanks and are the means of five independent observations. Standard error bars are also shown.

3.3.4 *Effect of substrate concentration*

The effect on changing of substrate concentrations on the activity was evaluated. The Michealis-Menten constant, Km and Vmax were also obtained for all the strains studied.

3.3.4.1 *Initial velocity*

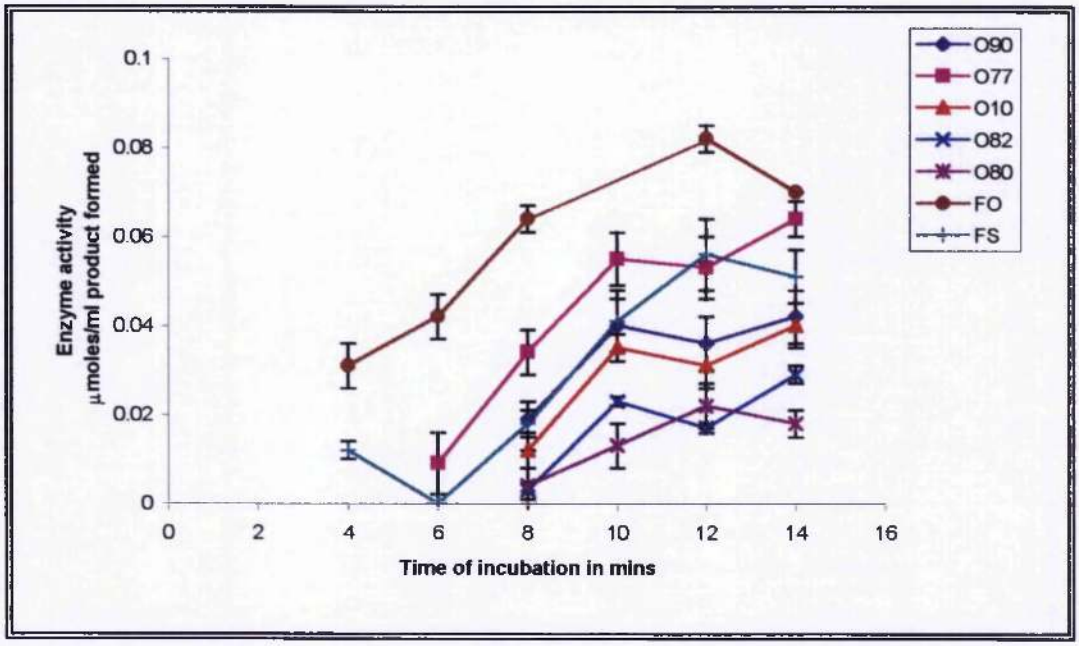


Fig. 3.5 Graph showing the preliminary progress curves for all the different strains.

Fig. 3.5 (above) shows rhodanese activity to increase in the initial 10-11 mins but seems to plateau after that at 12-14 mins. It was therefore, decided to incubate the rhodanese enzyme reaction for 10 mins during the experiments to determine the effect of changing the substrate concentrations on the enzyme activity.

3.3.4.2 Km and Vmax of rhodanese enzyme in all the selected strains of fungi

The effect of cyanide and thiosulfate concentrations on the rate of thiocyanate formation, as a measure of the degradation of cyanide, is shown in the following sub-sections. The different strains were individually analysed and both the primary and secondary plots were plotted.

Common kinetic mechanisms for a two-substrate reaction of the type:

$$A + B \rightleftharpoons P + Q$$

gives rise to an equation of the general form given below which is very similar to the equation for single substrate reactions except that it contains a combined Michaelis constant term and a Michaelis constant for each of the substrates (Km^A and Km^B), which are true Michaelis-Menten constants (see Dixon and Webb, 1979).

$$v = \frac{Vmax}{[1 + Km^A/a + Km^B/b + Ks^A Km^B/ab]}$$

where a and b are the concentrations of the two substrates.

The method applied to determine the Km and Vmax was using the graphical procedure outlined by Florini and Vestling (see Dixon and Webb, 1979).
It is based on the reciprocal of the above reaction to produce the following equation:

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{Km^B}{b.Vmax} + \frac{Km^A}{a.Vmax} + \frac{Ks^A.Km^B}{a.b.Vmax}$$

where $B = CN^-$ and $A = S_2O_3$ and Km^A, Km^B, Ks^A and Vmax are constant for any line in the primary plot, 1/v vs 1/a.

3.3.4.2.1 Km and Vmax of rhodanese of strain O90

Analyses were carried out on the trend line (line of best fit) shown in Fig. 3.6. In the secondary plot (Fig.3.7), the intercept on the 1/v axis is the reciprocal of Vmax. Therefore, the Vmax was calculated to be $1/12.87 = 0.078\mu moles.\ min^{-1}.\ mg\ protein^{-1}$. The slope of the line in Fig.3.7 equals $Km^{CN}/$ Vmax. Therefore, Km^{CN} equals 16mM.

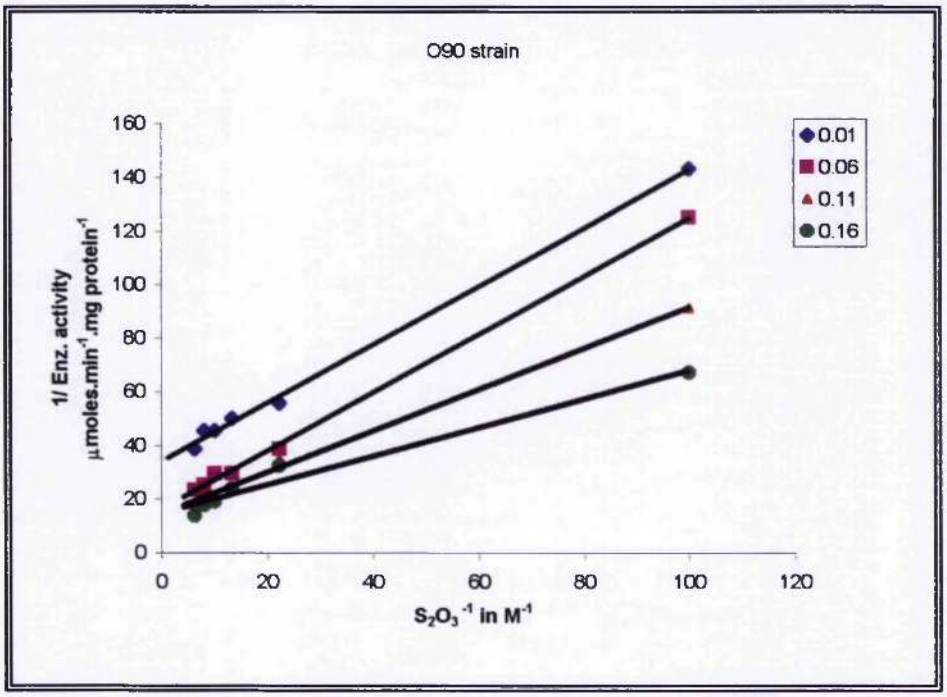


Fig. 3.6 Primary plot obtained with strain O90. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

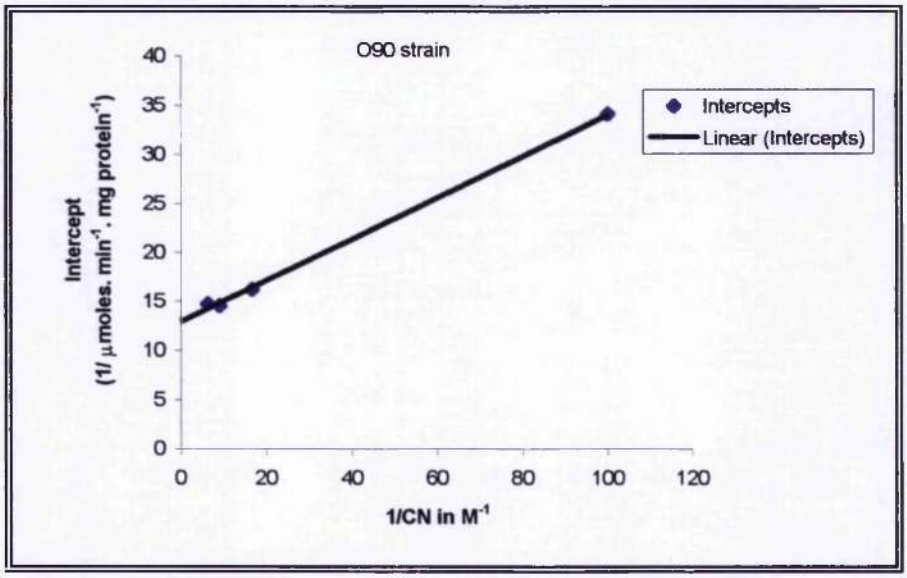


Fig. 3.7 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain O90.

3.3.4.2.2 Km and Vmax of rhodanese of strain O77

Analyses were carried out on the trend line (line of best fit) on the primary plot shown in Fig. 3.8. From the secondary plot (Fig.3.9), the Vmax was calculated to be 1/10.74 = 0.093 μmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 6mM (from Fig. 3.9).

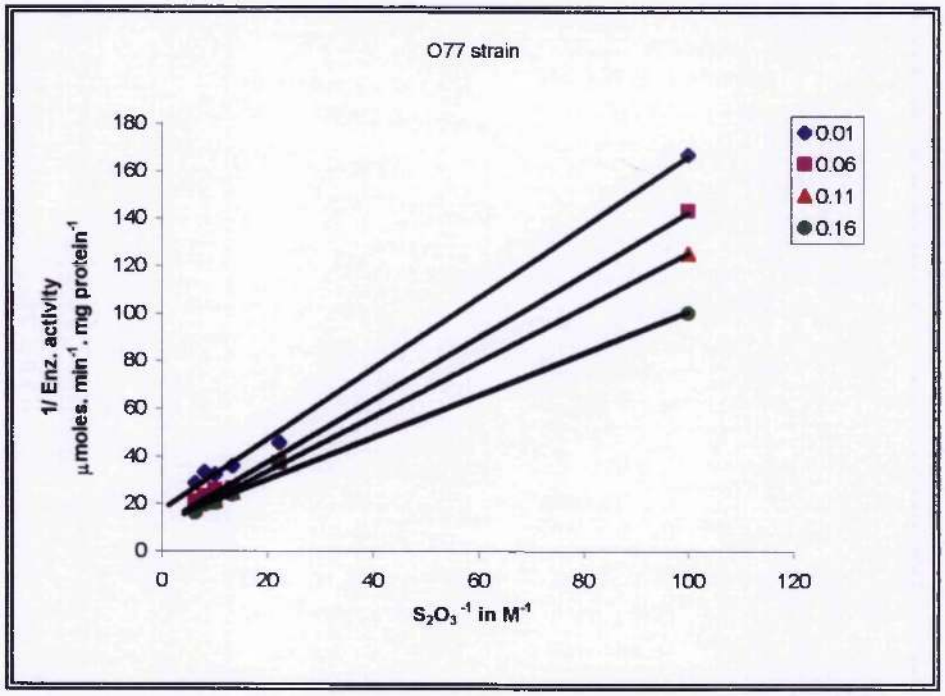


Fig. 3.8 Primary plot obtained with strain O77. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

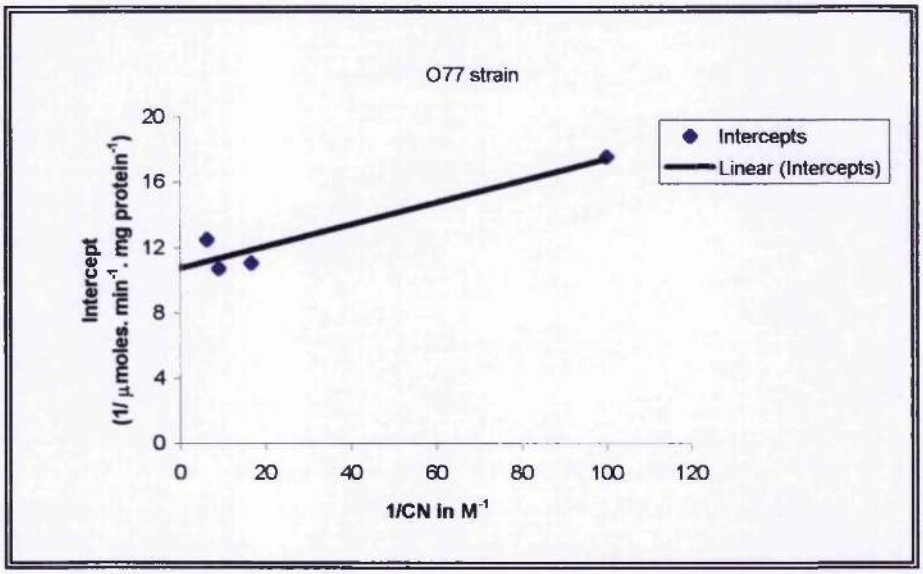


Fig. 3.9 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain O77.

3.3.4.2.3 Km and Vmax of rhodanese of strain O10

Analyses were carried out on the trend line (line of best fit) of the primary plot shown in Fig. 3.10. From the secondary plot (Fig.3.11), the Vmax was calculated to be $1/11.24 = 0.089$ μmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 16mM (from Fig.3.11).

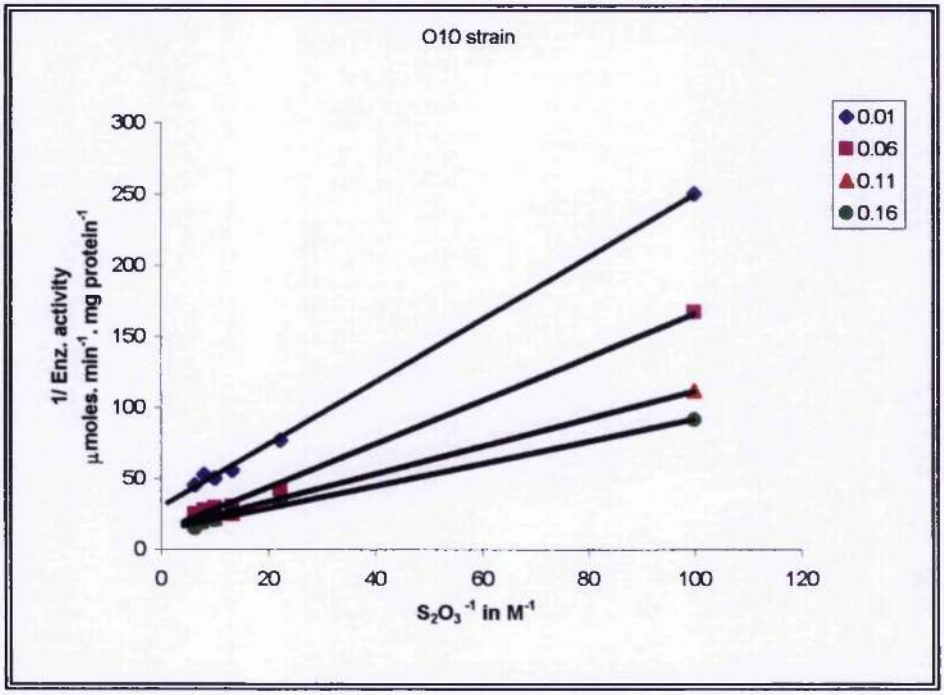


Fig. 3.10 Primary plot obtained with strain O10. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

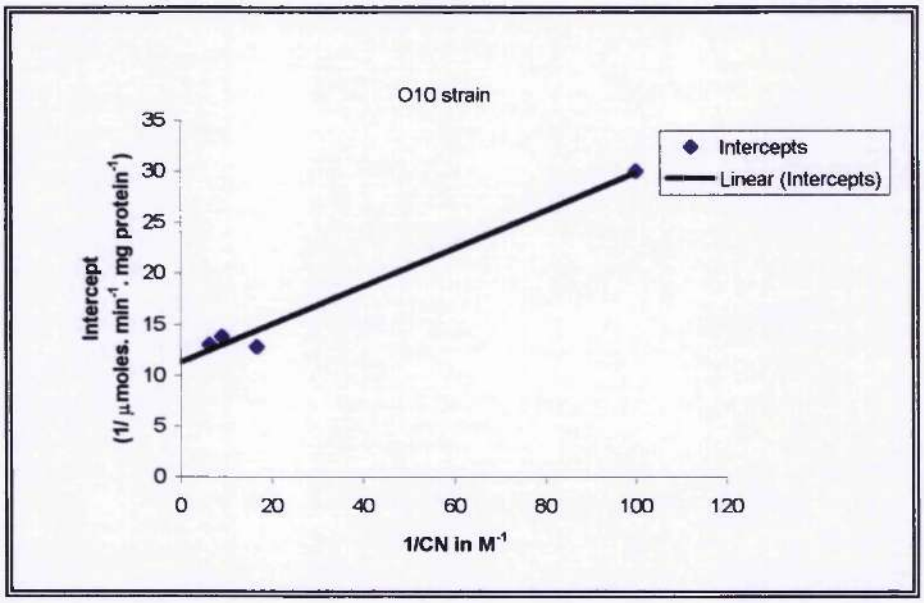


Fig. 3.11Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain O10.

3.3.4.2.4 Km and Vmax of rhodanese of strain O82

Analyses were carried out on the trend line (line of best fit) of the primary plot, shown in Fig. 3.12. From the secondary plot (Fig.3.13), the Vmax was calculated to be $1/14.55 = 0.069$ μmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 7mM (from Fig.3.13).

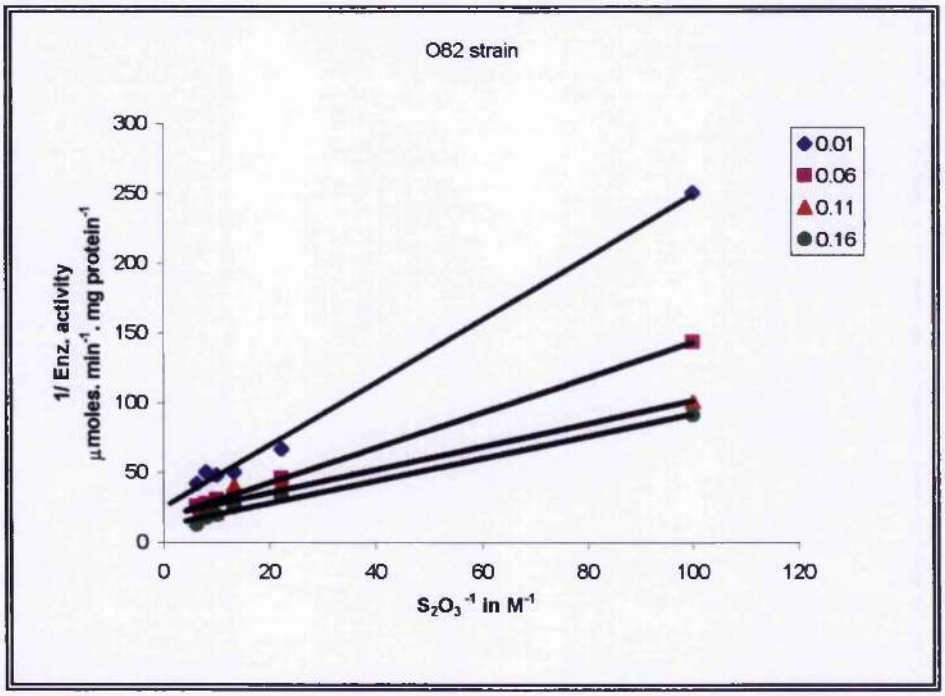


Fig. 3.12 Primary plot obtained with strain O82. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

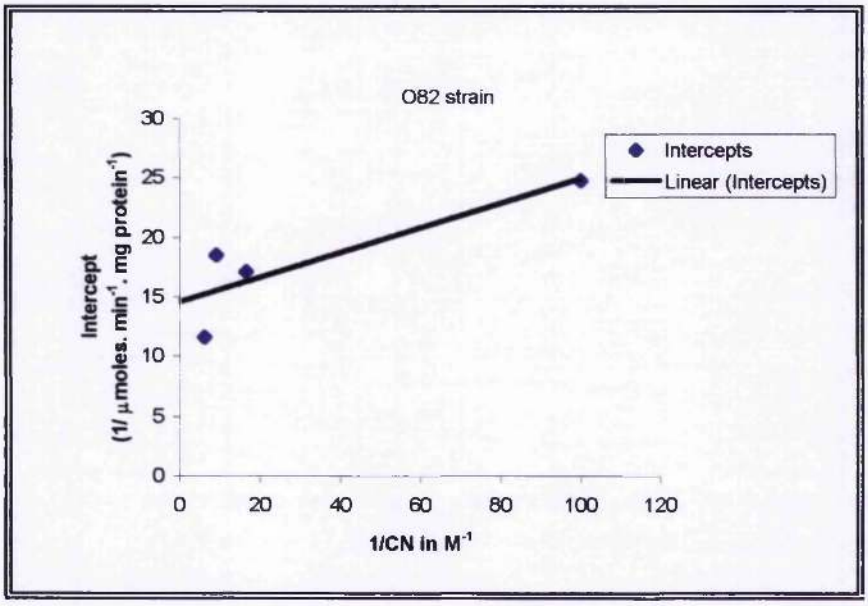


Fig. 3.13 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain O82.

3.3.4.2.5 Km and Vmax of rhodanese of strain O80

Analyses were carried out on the trend line (line of best fit) of the primary plot shown in Fig. 3.14. From the secondary plot (Fig.3.15), the Vmax was calculated to be $1/14.04 = 0.071$ μmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 9 mM (from Fig.3.15).

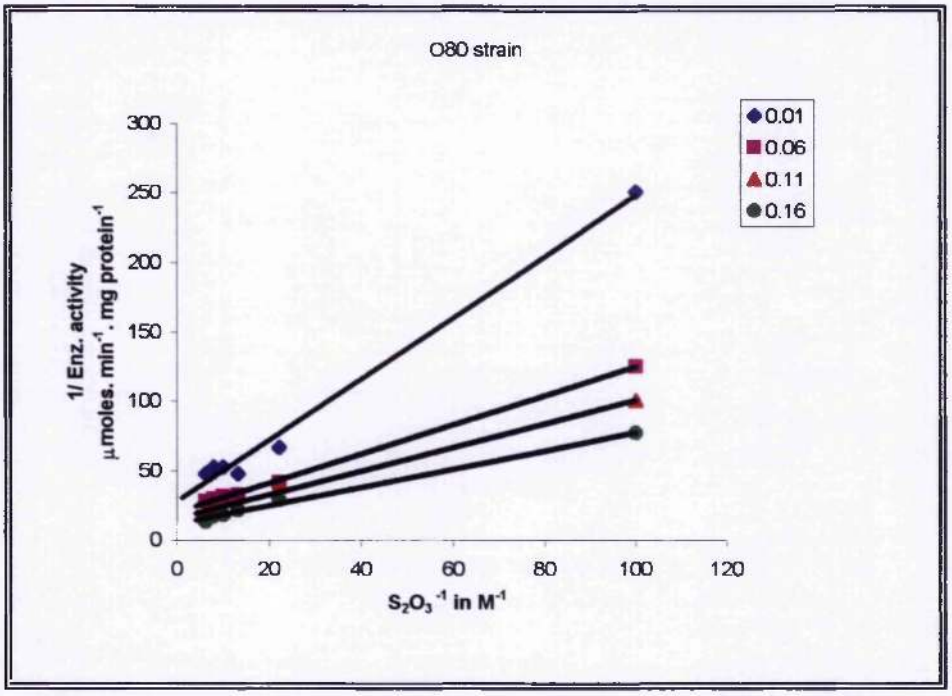


Fig. 3.14 Primary plot obtained with strain O80. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

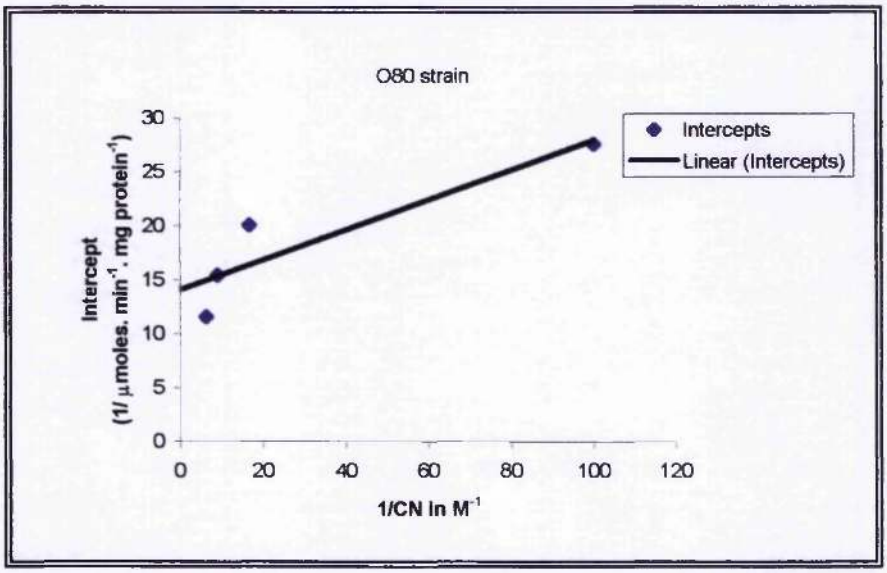


Fig. 3.15 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain O80.

3.3.4.2.6 Km and Vmax of rhodanese of strain F.O.

Analyses were carried out on the trend line (line of best fit) of the primary plot shown in Fig. 3.16. From the secondary plot (Fig.3.17), the Vmax was calculated to be $1/11.41 = 0.088$ µmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 3 mM (from Fig.3.17).

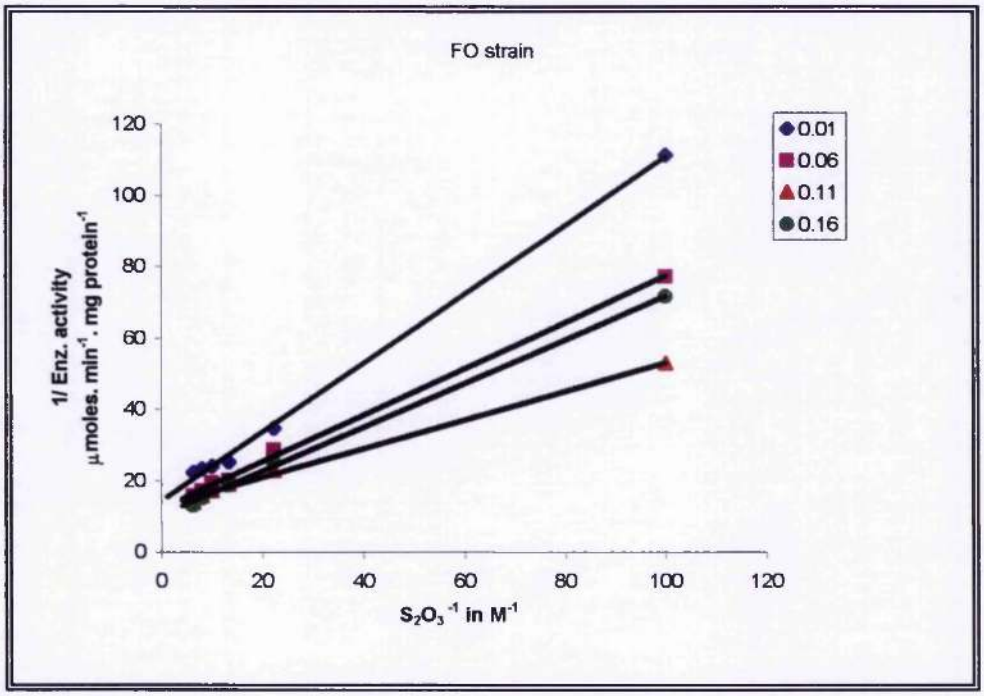


Fig. 3.16 Primary plot obtained with strain F.O. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

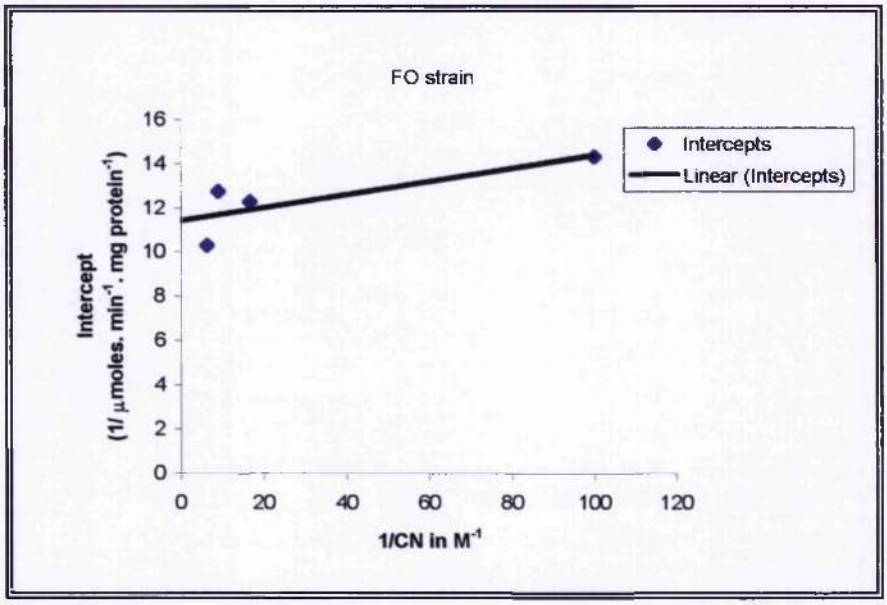


Fig.3.17 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain F.O.

3.3.4.2.7 Km and Vmax of rhodanese of strain F.S.

Analyses were carried out on the trend line (line of best fit) of the primary plot shown in Fig. 3.18. From the secondary plot (Fig.3.19), the Vmax was calculated to be $1/12.07 = 0.083$ µmoles. min^{-1}. mg $protein^{-1}$. Km^{CN} equals 2 mM (from Fig.3.19).

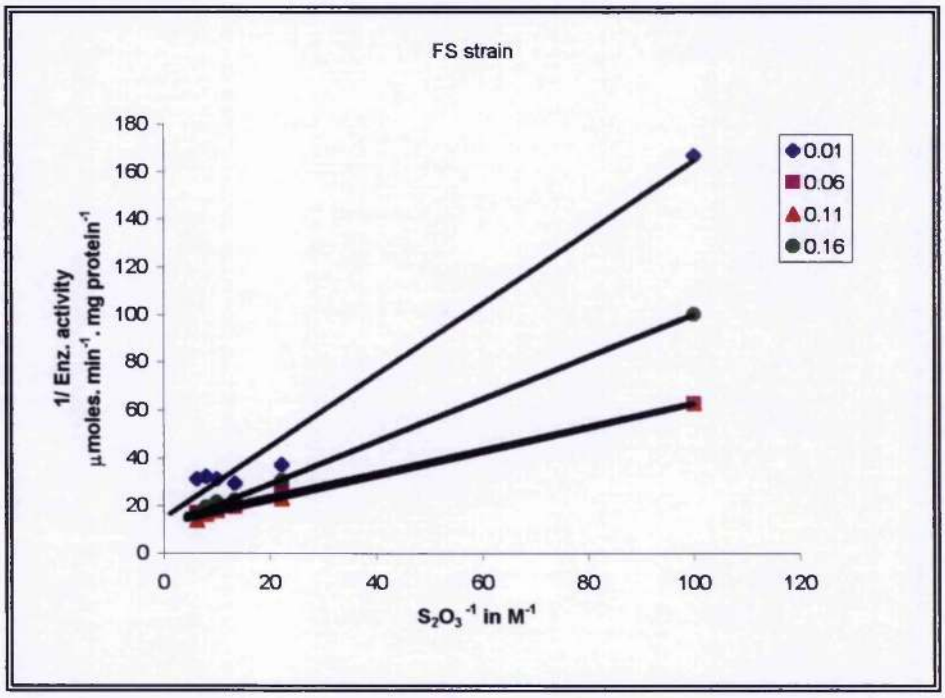


Fig. 3.18 Primary plot obtained with strain F.S. The cyanide concentrations were 0.16M, 0.11M, 0.06M and 0.01M.

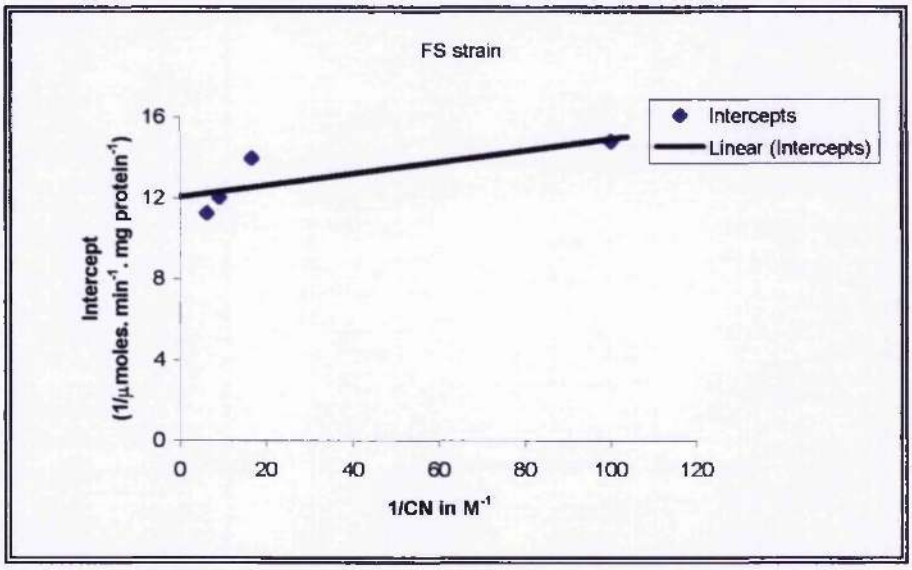


Fig. 3.19 Secondary plot of intercepts from the primary plot against reciprocal cyanide concentration for the rhodanese enzyme of strain F.S.

3.4 DISCUSSION

In the previous chapter, the enzyme rhodanese was found to be present in all the *Trichoderma* as well as the *Fusarium* strains from Oxford. The results found in this chapter go a step further to characterise the enzyme in terms of its presence (intra-/ extra-cellular), pH and temperature profiles and its kinetics.

Rhodanese was found to be present extra-cellularly. Of the fractions assayed for enzyme activity, the initial supernatant (A), without any homogenisation or disruption of cells, was found to contain most enzyme activity.

The time for incubation for the rhodanese assay was chosen as 10 mins based on the progress curves which showed increasing formation of product up to about 10 mins. Table 3.2 summarises the results of comparative investigations on rhodanese in different microorganisms as reported in the literature. The broad pH range and temperature optima of the *Trichoderma* rhodanese (Table 3.1) compare favourably with reports from other sources of the enzyme and in most cases the values obtained in the present study indicate much higher affinity of the enzyme (lower Km) towards cyanide.

The primary plots in all the *Trichoderma* strains follow the normal kinetic pattern with decreasing slope with increasing concentration of cyanide (from 0.01-0.16M). The plots of the *Fusarium* strains, however, appear to show a deviation from this trend but the values for the intercept secondary plot are very similar which may affect

the accuracy of the values for Km^{CN}. This suggests that the substrate concentrations for cyanide used in the experiment were probably very high resulting in all the points falling in the plateau of the Michealis curve.

Species	Optimum pH	Optimum Temp °C	Km^{CN} mM
Trichoderma harzianum strain O90	8.5 – 10.5	35 - 55	16
T. harzianum strain O77	8.5 – 10.5	35	6
T. harzianum strain O10	8.5 – 11.5	45	16
T. harzianum strain O82	10.5	45	7
T. harzianum strain O80	11.5	35	9
Fusarium oxysporum (FO)	-	-	3
F. solani (FS)	-	-	2

Table 3.1 Summary of the kinetic characteristics of all the strains used in the present study

Rhodanese from both the *Fusarium* strains showed maximum affinity (least Km) towards cyanide followed by the *Trichoderma* strain O77; the latter showing the highest maximum velocity. This was a surprising observation since as discussed in the previous chapters, the particular strain O77 was shown to be one of the slowest degraders in the study by Lynch (unpublished). However, the conditions in the latter study, were different in that it was carried out in soil, at a temperature of 25 °C.

Species	Optimum pH	Optimum Temp	Km	Reference
Ferrobacillus ferrooxidans	7.5 – 9	25 °C	10mM	Tabita *et al*, 1969
Chromatium	8.7	-	20mM	Smith and Lascelles, 1969
Chloradizon-degrading bacterium E	11	-	38mM	Layh *et al*, 1982
Thiobacillus denitrificans	8 – 9	-	-	Bowen *et al*, 1965
E. coli	-	-	24mM	Alexander and Volini, 1987
E.coli	-	-	17mM	Ray *et al*, 2000
Desulfotomaculum nigrificans	7.5	-	120mM	Burton and Akagi, 1971
Beef liver	8.6	50 °C	-	Sorbo, 1955
Tapioca leaf (*Manihot utilissima*)	10.2 – 11	57 – 59 °C	-	Chew and Boey, 1972

Table 3.2 Kinetic parameters for rhodanese for some of the microorganims reported in literature

Although the values for Km for all the strains (Table 3.1) are lower than most of those found in literature (Table 3.2), the relatively high Km, in a few of the strains such as O90, O10 and O80, and therefore a lower affinity of the enzyme for cyanide seems to indicate that cyanide detoxification may be no more than an incidental function of the *Trichoderma* rhodanese in these particular strains. The values of Km^{CN} show the poor affinity of the enzyme for the substrate cyanide thus suggesting that cyanide is not the physiological acceptor of the sulfur atom split from the thiosulfate. Another

reason for the relatively high Km could be that the crude extract may have contained inhibitors.

Alexander and Volini, 1987, have reported the *E. coli* rhodanese to follow a double-displacement or the ping-pong mechanism wherein the primary plot results in a series of parallel lines. On the basis of the primary plots in the present results, since parallel lines were not obtained, it seems that the reaction follows a sequential pathway rather than the ping-pong pathway in the present experiment.

A point in the experimental set-up to be criticised would be the choice of the thiosulfate concentrations. A clearer picture would probably have emerged if the points were evenly spread out when doing reciprocals. The system would then have been defined with more certainty. A different range of the cyanide concentrations too would probably have helped. The use of the same substrate range for all the enzymes with a range of Km values meant that those enzymes with a low Km values were likely to give unreliable plots as was found with the *Fusarium* strains in this study. For these latter strains a range of substrates centred around their Km value would probably have yielded better defined primary and secondary plots. Apart from the Line-weaver Burke plot, the Hanes equation was also analysed and plotted. No appreciable difference was observed.

Further studies need to be undertaken to fully understand the kinetics including purification of the enzyme, studies on the mechanism and mode of action and inhibition studies. An insight into the relationship of this enzyme with other rhodaneses, from other sources, would then be obtained.

CHAPTER 4:

BIODEGRADATION OF CYANIDE

4.1 INTRODUCTION

The aim of the work presented in this chapter was to find the rate of degradation of cyanide by certain selected strains of *Trichoderma* and *Fusarium*.

A number of reports on growth of microorganisms on cyanide as the sole source of nitrogen have appeared including growth on both free cyanides and metallo- cyanides (Kunz *et al*, 1992; Harris and Knowles, 1983; Knowles, 1976). Nickel cyanide was demonstrated to be utilized by a strain of *Pseudomonas* (Rollinson *et al*, 1987) whereas a mixed fungal culture was found to utilize iron or nickel cyanide at acidic or neutral pH's as sole nitrogen sources by Barclay *et al*, 1998. *P. fluorescens* NCIMB 11764 has been found to utilize cyanide as a nitrogenous substrate (Harris and Knowles, 1983; Kunz *et al*, 1992). Furuki *et al*, 1972, have reported a bacterium utilizing 40-50μg/ml of cyanide as a nitrogen source with an additional source of carbon.

On the other hand, a *Bacillus pumilus* strain (Skowronski and Strobel, 1969) was found to grow on cyanide as the sole source of carbon and nitrogen. Two facultative autotrophs, both actinomycetes, of the genus *Nocardia* and in another case a gram-positive filamentous organism, probably again an actinomycete converting cyanide to ammonia, have also been found to be capable of growing on cyanide as a carbon and nitrogen source (Knowles, 1976).

The present chapter is a combination of two sets of experiments. In the first experiment, cyanide was used as the sole carbon and nitrogen source whereas in the second, along with cyanide, glucose was provided as the source of carbon and energy and its effect on the rate of degradation as a co-metabolite was evaluated.

4.2 MATERIALS & METHODS

4.2.1 Fungal isolates

All the fungal isolates, described in Section 2.2.2, viz. the five strains of *Trichoderma* and the two *Fusarium* strains from Oxford were used in this experiment.

4.2.1.1 Preparation of media and growth of the fungi

Preparation of the PDB and agar PDA media and the growth of all the fungal strains were similar to that described in Section 2.2.3.

4.2.2 Cyanide degradation using cyanide as the sole carbon and nitrogen source

After incubation of the fungi in PDB for 3 days, a 1% (w/v) inoculum was transferred into 500 ml flasks containing minimal salts medium without any carbon source. The minimal medium was made of the following four constituents which were prepared and sterilized separately by autoclaving and then mixed aseptically (Lynch and Harper, 1974): (i) $MgSO_4.7H_2O$, 0.5g/l. (ii) $CaCl_2$, 0.1g/l. (iii) the mineral salts mixture consisting of (g/l): $ZnSO_4.7H_2O$, 0.05; $MnCl_2.4H_2O$, 0.05; $CuCl_2.2H_2O$, 0.005; $Na_2MoO_4.2H_2O$, 0.005; $Na_2B_4O_7.10H_2O$, 0.002; $CoCl_2.6H_2O$, 0.0002 and NaOH, 0.432. (iv) KH_2PO_4 buffer, 5g/l, to give a final pH in the medium of 6.5. It is necessary to sterilize the four constituents separately to avoid precipitation of the salts.

Cyanide (2.5ml) was added from a stock solution to give a final concentration of 2000 ppm CN^- in the flasks. The total volume in the flask was 75ml. The flasks were sealed with autoclaved silicone rubber bungs (Fisher Scientific). A sterilized 9-inch, 16 gauge luer lock needle (Apollo Stainless Tubes and Needles) was passed through the bungs such that the end tip of the needle was just dipping in the media in the flasks. The other (top) end of the needle was blocked with an empty syringe in order to prevent any escape of cyanide/cyanide vapours. All the flasks were incubated at 25 °C on a shaker at 220 rpm.

Two controls, one without any inoculum and the other with autoclaved cultures were also set up in triplicates in a similar way as described above.

Samples (9 totally of approx. 1ml each) were taken at regular intervals as follows and tested for cyanide reduction. The existing syringe on each flask was first removed and a fresh one then put on; the air in this new syringe was first pushed into the flask. Aliquots of samples were taken out in these syringes and the former syringes replaced back on the needle head on the respective flasks. The incubation was carried on for 90 days.

4.2.2.1 Dry weight determination

Since the dry weight estimation involved destructive sampling, a different set of flasks containing minimal media at the same concentrations of cyanide and inoculum was set up to check for growth. Duplicates for each culture as well as the controls were set up. These flasks were left on the shaker at 220 rpm for the whole 90-day period.

At the end of the assay period the medium was filtered using Whatman No.1 filter papers and dry weights of the cultures in the flask determined by drying the filter papers in the oven at 90 °C overnight and then weighing them. Only two readings were taken; an initial 0-day and one after 90-days of incubation.

4.2.3 *Cyanide degradation using cyanide as the nitrogen source and glucose as a co-metabolite*

A slightly modified procedure was followed to obtain the inoculum in this experiment. Fungi were grown on potato dextrose agar (PDA) plates and incubated at 25 °C, for approximately 10 days until sporulation was observed. On the day of start of the assay, a spore suspension (containing an average of approx. $3x\ 10^8$ cfu ml^{-1} of each individual fungal strain), was obtained from the plates containing the grown sporulating cultures, using sterile R.O. water. A 5% (v/v) suspension was inoculated into 500 ml flasks containing minimal salts. The sterile minimal medium was made up similarly as described in Section 4.2.2 above. Glucose, at a final concentration of 25mM (4.5 g l^{-1}), in the medium, was filtered through a 0.2µm filter (Sartorius) and then added to the flasks. Additional amounts of glucose at the same concentration were added at days 10 and 19.

Cyanide (2.5ml) was added from a stock solution to give a final concentration of 2000 ppm CN^- in the flasks. The total volume in the flasks was 75 ml. The flasks were sealed with autoclaved silicone rubber bungs and then set-up for incubation in a way similar to that described in Section 4.2.2.

Two controls, one without any inoculum but with glucose and the other with autoclaved cultures and glucose were also set up in triplicates in a similar way as described above.

Samples were taken at regular intervals and tested for cyanide and glucose. The sampling method was similar to that described in Section 4.2.2. The incubation was carried out for 32 days.

4.2.3.1 Dry weight determination

Since the dry weight estimation involved destructive sampling, as in the previous experiment, here too, a different set of flasks containing minimal media and the same concentrations of cyanide and inoculum as in the assay flasks were set up to check for growth. Duplicates for each culture as well as the controls were set up. Five sets of such flasks were incubated under similar conditions as the assay flasks. One set was removed each at days 0, 7, 12, 28 and 32 and dry weights estimated. The medium along with the cultures was filtered through Whatman No. 1 filter papers and dry weights determined by drying the filter papers in the oven at 90°C overnight and then weighing them.

4.2.4 Cyanide assay

Cyanide was determined using the γ-picoline-barbituric acid method as described by Nagashima, 1977. The constituents of the assay were prepared as follows:

***Buffer solution (pH 5.2)*:** Potassium dihydrogen phosphate (13.6 g) was dissolved with 0.28 g of disodium hydrogen phosphate in water and diluted to 1litre.

***γ-picoline-barbituric acid reagent*:** Barbituric acid (3 g) was taken in a 50 ml volumetric flask along with a small quantity of R.O. water. To this was added 15ml of γ-picoline (4-methyl pyridine) and 3ml of concentrated hydrochloric acid (d=1.18), while stirring to dissolve the acid. After cooling, the mixture was diluted to 50 ml with R.O.

water. The reagent obtained was light yellow in colour.

***Cyanide assay procedure*:** In a dried reaction tube, diluted sample solution (10 ml) (approx. 5μg CN^-) was taken and to it added 5ml buffer solution (from above) and 0.25ml of 1% (w/v) chloramine-T solution. The tube was shaken gently and stoppered. After 2 min, 3ml γ-picoline-barbituric acid reagent was added, the tube re-stoppered and mixed and left at 25°C for 5 min. The absorbance was read at 605nm.

A standard curve for cyanide was obtained which was linear until 1.3 ppm CN^- and the sample values calculated from it.

4.2.5 *Glucose assay*

The change in glucose concentration of the culture medium during the growth of the fungi was determined using the Glucose (Trinder) reagent (Sigma Diagnostics). The principle of the assay is based on the following reaction:

$$\text{Glucose} + H_2O_2 + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyridine} + \text{p-hydroxybenzene sulphonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. The latter reacts in the presence of horse-radish peroxidase with 4-

aminoantipyridine and *p*-hydroxybenzene sulphonate to form a quinone-imine dye with an absorbance maximum at 505nm. The intensity of the colour is directly proportional to the glucose concentration in the sample.

The procedure used in the assay was as follows (Sigma Diagnostics):

a) The glucose Trinder reagent was prepared by dissolving the entire powder from the vial with 100 ml of R.O. water. The solution was then immediately mixed several times by inversion.

b) The spectrophotometer was set at a wavelength of 505nm and the absorbance reading to zero with water as reference.

c) A series of labelled cuvettes for reagent blank, standards, controls and the assay samples were set up.

d) Glucose (Trinder) reagent (1ml) was pipetted into each cuvette.

e) At timed intervals, 0.005 ml (5 μl), R.O. water, glucose standards, controls and assay samples were added to the respective cuvettes.

f) Each cuvette was incubated at ambient room temperature for exactly 18 mins.

g) The absorbance of all the cuvettes was read and recorded at 505nm using the same timed intervals as in step (e) above.

h) The absorbance of the reagent blank was subtracted from the assay samples and the controls.

i) A standard curve for glucose was plotted and the concentration of glucose from the assay samples was derived from it.

4.3 RESULTS

4.3.1 *Cyanide as the sole source of carbon and nitrogen*

Cyanide was used in the medium as the sole source of nitrogen and carbon for growth. All the selected fungal strains were studied for both, the growth and degradation studies as well as the biomass estimations.

4.3.1.1 *Cyanide degradation studies*

Cyanide degradation was followed over a period of 90 days and samples analysed for cyanide (Fig. 4.1). Appropriate dilutions of the samples were carried out to ensure the values detected fell within the linear range of the cyanide standard curve.

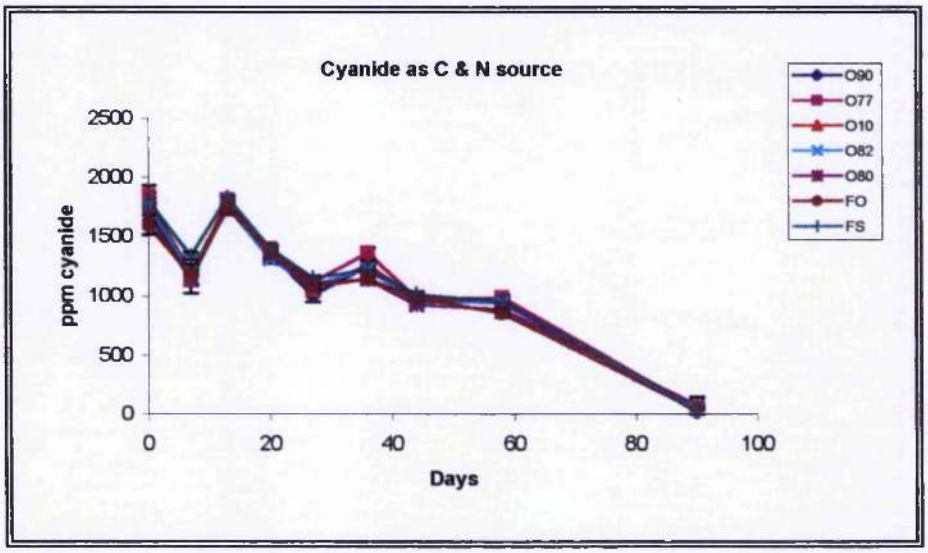


Fig. 4.1. Plot showing a decrease in cyanide concentration. The values plotted are the means of individually set-up triplicates and the standard error bars are plotted.

4.3.1.2 ***Biomass estimation***

Growth was visible in the form of pellets in all the assay flasks. The medium, however, did not turn turbid. Growth was measured by the increase in dry weight (Fig. 4.2) with almost all the strains except for one (strain O82) showing an approximately equal increase.

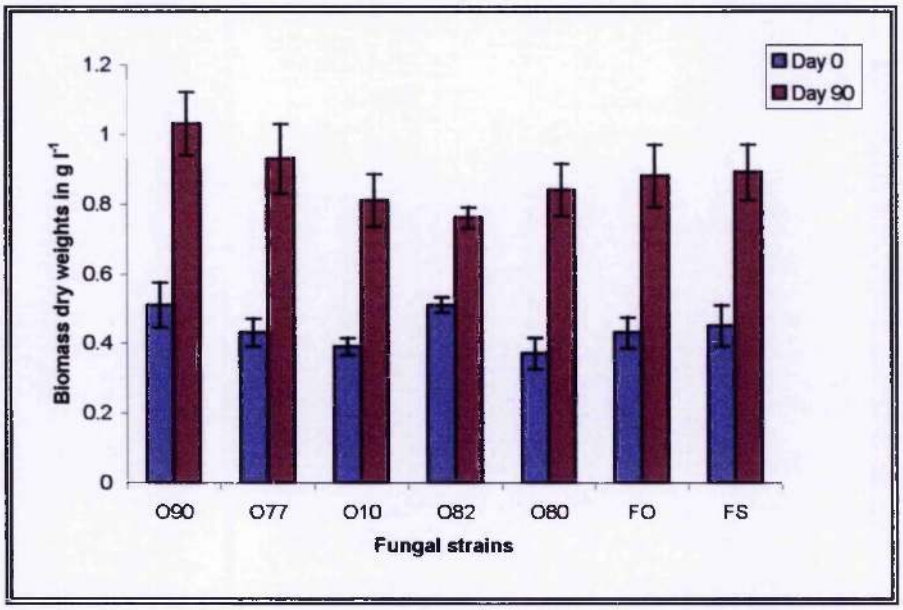


Fig. 4.2 Difference in dry weights of biomass over the entire assay period (at days 0 and 90). Values are the means of independent triplicates and standard error bars are plotted.

4.3.2 *Addition of glucose as the carbon source*

In this experiment, cyanide was added along with 25 mM (4.5 g l^{-1}) of glucose as a source of energy. All the selected *Trichoderma* and *Fusarium* strains were evaluated for their rates of cyanide degradation.

4.3.2.1 Studies on cyanide degradation and glucose consumption

Cyanide degradation was followed over a period of 32 days and samples analysed for reduction in concentrations of cyanide (Fig. 4.3) over the incubation period. Glucose consumption was also recorded during this entire period and has been shown in Fig. 4.4 below.

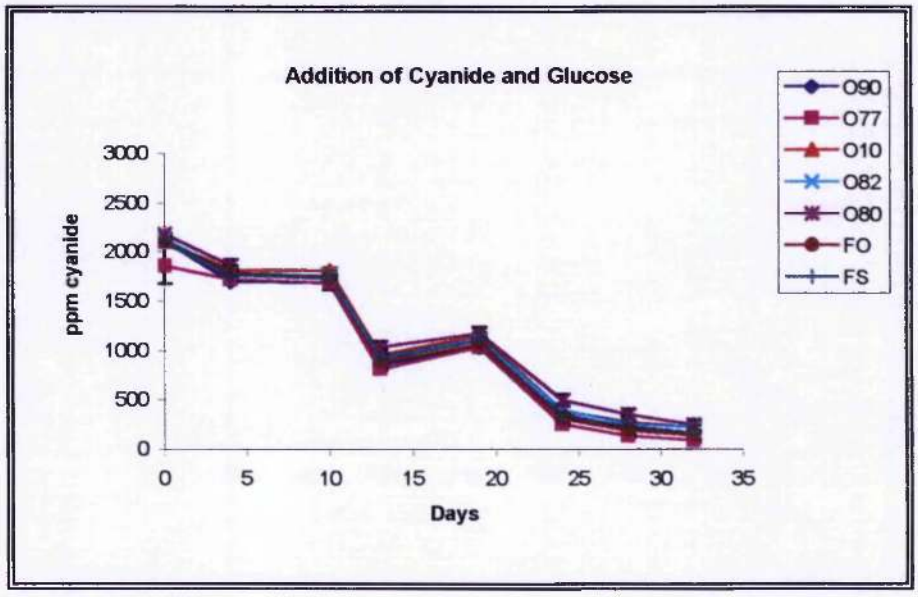


Fig. 4.3. Decrease of cyanide observed over a period of 32 days using glucose as a source of energy. All the values are the means of three independent observations and the standard error bars are plotted.

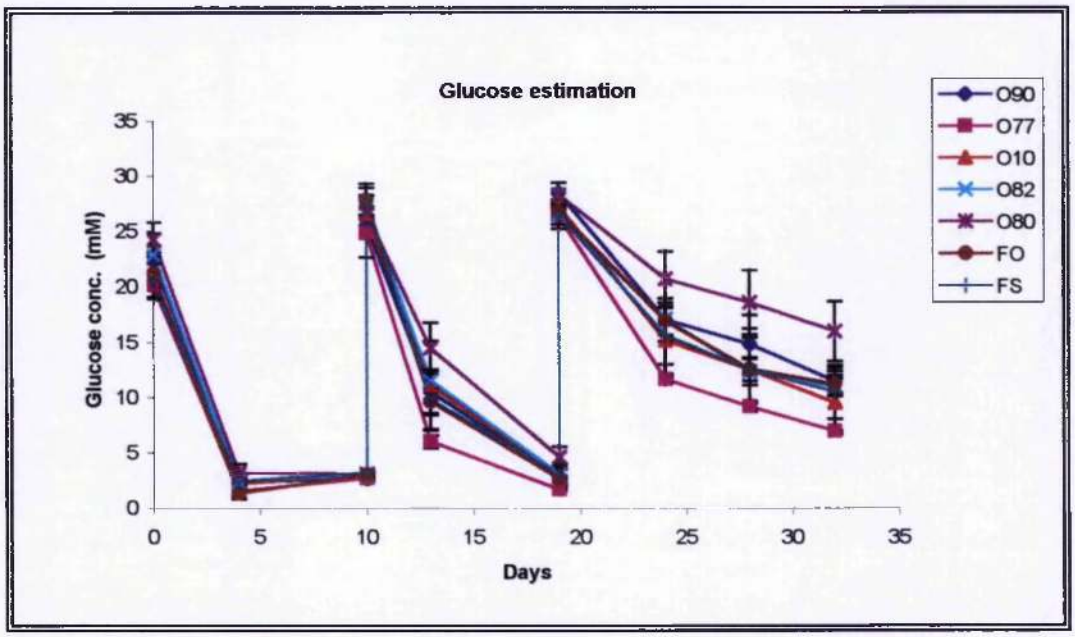


Fig.4.4. Amount of glucose consumed during the 32-day assay. 25 mM (4.5 g l^{-1}) of glucose was additionally added at days 10 and 19. Standard error bars are plotted.

4.3.2.2 Growth study – biomass estimation

Growth was visible in some of the flasks again in the form of pellets. Biomass formation was considered as indication of growth and was measured by the increase in dry weight (Fig.4.5) with all the strains showing varying increases.

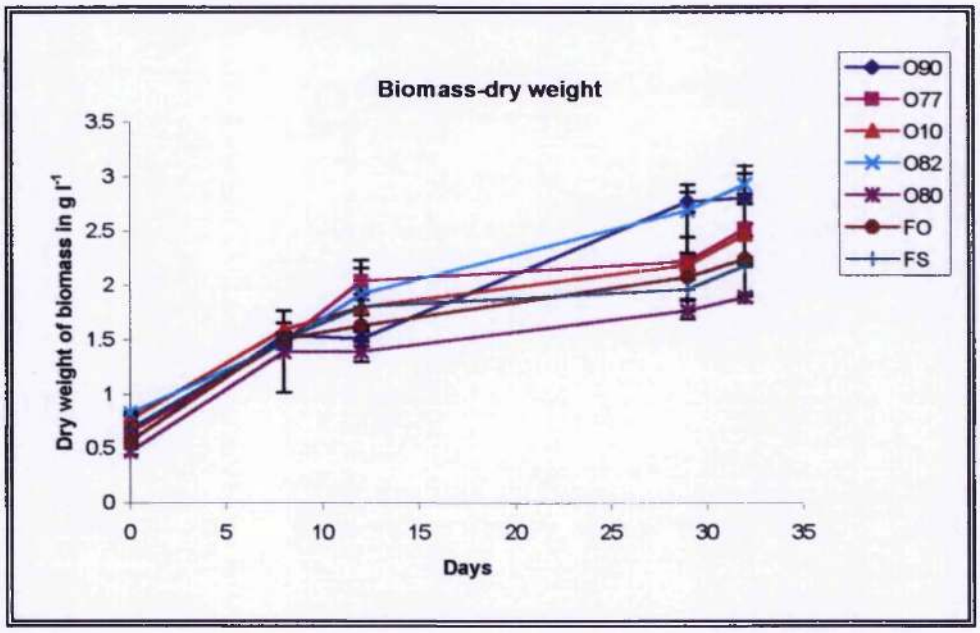


Fig. 4.5 Dry weight of biomass over the 32-day assay period. Values are the means of independent triplicates and standard error bars are plotted.

4.3.3 *Calculation of growth yield (Y)*

Growth yields from both experiments were calculated as follows:

In the first experiment where cyanide was used as the sole source of carbon and nitrogen, 2000ppm (2 g l^{-1}) of CN^- added to the medium, produced a highest dry weight biomass of approximately 0.5 g l^{-1}. Variations were, however observed between the different strains.

Growth yield is given as: $Y = \Delta x / \Delta S$ (Pirt, 1975)

where Δx = amount of biomass produced and ΔS = total substrate utilized.

$\therefore Y = 0.5 / 2 =$ **0.25 g dry weight/g.**

Now, if we consider just the cyanide-carbon as the substrate, 2 g l^{-1} CN^- is equivalent to 0.92 g C l^{-1} of cyanide-carbon.

$\therefore Y = 0.5 / 0.92 =$ **0.54 g dry weight biomass / g of cyanide-C.**

Similarly, in the glucose-inoculated experiment, 2000ppm (2 g l^{-1}) of CN^- and 4.5 g l^{-1} glucose added to the medium, produced approx. 2 g l^{-1} dry weight biomass (approx. average for all strains).

$\therefore Y = 2 / 6.5 =$ **0.31 g dry weight/g** (Total substrate = 6.5 g l^{-1} from 2 g l^{-1} CN^- and 4.5 g l^{-1} glucose).

Again, considering only the cyanide and glucose-carbons, we have 0.92 g C l^{-1} of cyanide-carbon and 1.8 g C l^{-1} of glucose-carbon to give a total of 2.72 g C l^{-1}.

$\therefore Y = 2 / 2.72 =$ **0.74 g dry weight biomass / g of cyanide and glucose C**

If the additional glucose added during the assay is considered, then Y would equal 0.15g dry weight/g substrate and 0.34 g dry weight biomass/ g of cyanide and glucose C.

4.4 DISCUSSION

This is the first instance of the fungal species, *Trichoderma* having been reported to utilize cyanide as a sole carbon and nitrogen source for growth. Evidence of the presence of cyanide degrading enzymes in these species has been presented earlier in this thesis.

There are relatively very few reports of studies on growth of microorganisms on cyanide as sole nitrogen and/or carbon source. In the present experiment, when cyanide was provided as the sole source of carbon and nitrogen, an unusually long period of 90 days of incubation was required to reach very low levels of cyanide. One of the reasons could be due to the fact that cyanide was used in very high concentrations coupled with the fact of it being the only exogenous carbon source provided. Another point to be noted here is the possibility of cyanide reaching low levels in the period between 60 and 90 days when cyanide was not estimated.

Skowronski and Strobel, 1969, have reported a strain of *Bacillus pumilus*, to grow in a media containing 0.1M KCN (2600ppm CN). Also, Kunz *et al*, 1992, found washed-cell suspensions of cyanide-grown cells of *P. fluorescens* NCIMB 11764 could remove KCN concentrations at 100mM. Cyanide was the sole source of carbon and nitrogen in the former whereas glucose was also provided in the latter study.

In the present study, glucose, when provided, remarkably increased the rate of degradation of cyanide. Cyanide concentrations reached low levels within 32 days of incubation as against 90 days observed earlier in experiments without glucose.
An interesting observation recorded in the present study was the apparent link

between the decrease in cyanide concentrations and the reduction in the levels of glucose in the medium. This is very well illustrated at Days 10 and 19 when comparing Figs. 4.3 & 4.4. Additional glucose was added to the medium at these days. Barclay *et al,* (1998), have reported a 90% reduction in cyanide levels after 34 days of incubation using *Fusarium solani*, the results of which were confirmed in this study though here, in the presence of free cyanide but at a much higher concentration. The former study used 20mM (3.6 g l^{-1}) glucose as a carbon source and 0.5mM $K_4Fe(CN)_6$ as the nitrogen source.

Dumestre *et al,* (1997), have observed rapid losses of cyanide in glucose minimal medium at high pH. The explanation given by them, for this phenomenon is that the loss is not due to the action of microbes but by the well-documented Kiliani reaction (Miltzer, 1949), wherein glucose reacts with free cyanide. In the present study, however, in the controls set up without any inoculation of microbial cultures, the concentration of glucose remained almost constant throughout the study with a negligible loss of glucose recorded. This indicates that glucose was indeed used by the fungi for growth along with the cyanide.

The utilization of glucose in the presence of cyanide, by all the strains of *Trichoderma* and *Fusarium* used in this study, is an indication of the presence of metabolic activity of these strains and energy production by them. Also the increase in biomass dry- weight, in both instances (with and without glucose), confirms the utilization of cyanide for growth and in the former case, i.e in the presence of glucose, proves that the increase was not merely due to resistance of the fungi to cyanide.

The biomass dry-weights in the glucose-inoculated study, although varying between strains, increased from an average of 0.7g l^{-1} to an average of 2.3g l^{-1}. This is much more than those observed by Barclay *et al*, (1998), who reported an increase of approx. 1g l^{-1} biomass dry-weight over the 34-day incubation period in her study. However, a higher concentration of both glucose and cyanide was used in the present study.

Further, in the glucose inoculated study, a saturation biomass seems to be attained at Day 10. Further addition of glucose appears to act as a source of energy in sustaining that biomass so that the cyanide degrading capability is maintained, although there is no increase in growth in the form of biomass. With the concentration of cyanide decreasing over time, nitrogen limiting conditions may have also arisen (since cyanide was the sole source of nitrogen) and therefore reducing production of biomass.

In the absence of glucose, however, the biomass dry weight increased by an average of about 0.5 g l^{-1} in all the strains. This value is similar to that found by Barclay et al, 1998, in their studies on $K_4Fe(CN)_6$ using *F.solani* where they recorded a growth of 0.57g l^{-1}, in studies without the inoculation of glucose.

Growth yield calculations performed provided values that were comparable to those reported in literature for one-carbon substrates. Pirt, 1975, reported values ranging from 0.36 g l^{-1} for substrate and 0.9 g l^{-1} for substrate carbon for acetic acid in *Candida utilis* to 1.01 g l^{-1} to 1.34 g l^{-1}, respectively, for methane in *Methylococcus* spp. Also, values for glucose were 0.51g l^{-1} for substrate and 1.28 g l^{-1} for substrate carbon in *Candida utilis*. These overall yields include the requirements for cell carbon, energy for growth

and maintenance (Pirt, 1975). However, since the emphasis of this investigation was to stimulate the co-metabolism of the cyanide substrate, experiments on glucose-alone controls were not performed and thus a comparision would be difficult to make in this case.

In summary, preliminary experiments on the rate of degradation of cyanide have been carried out confirming the degradation of cyanide by the *Trichoderma* strains. Glucose appears to act as a co-metabolite, and in terms of time, was found to efficiently increase the cyanide rate of degradation by almost 3 times. Indeed, a proportional increase of biomass was obtained in the glucose-inoculated experiments. A different picture might have been obtained if glucose had been added at Day 4, instead of the first extra addition being delayed till Day 10.

CHAPTER 5:

PLANT MICROCOSM STUDIES

5.1 INTRODUCTION

The previous chapter establishes the ability of the selected strains of *Trichoderma* used in this study to degrade cyanide in flask cultures. In order to simulate environmental conditions, especially soil sites, microcosm studies were designed. *Trichoderma* has been studied as a biocontrol agent (Lynch, 1990, Lynch *et al*, 1991A; Lynch, 1987) and as a plant growth promoter (Lynch *et al*, 1991; Ousley *et al*, 1993A).

Lynch, 1990, has suggested that low concentrations of toxins formed in the rhizosphere could possibly retard plant growth. *Trichoderma* added to the compost may detoxify these toxins and thus promote plant growth. A number of reports have suggested toxins like cyanide to be produced in the rhizosphere by plants and/or due to microbial action (Conn, 1981; Timonim, 1941; Knowles, 1986). A range of microorganisms have been isolated which can catabolize cyanide (Knowles and Bunch, 1986) and/or its growth, enhanced in the presence of cyanide (Timonim, 1941).

The aim of this study was to evaluate the capability of the selected *Trichoderma* and *Fusarium* strains to degrade cyanide in soil under conditions very similar to a natural environment. Also, the effect of the presence of the fungi itself on plant growth was evaluated.

5.2 Materials & Methods

5.2.1 Fungal Isolates

All the *Trichoderma* and *Fusarium* isolates described in Section 2.2 were used in this study.

5.2.1.1 Preparation of media and growth of fungi

The media was prepared and fungal strains were grown in conditions similar to that described in Section 2.2.3.

5.2.1.2 Preparation of the fungal spore suspension

The spore suspension was prepared in a similar way to that described in Section 4.2.3. Plates inoculated with fungal cultures were incubated for approx. 8-10 days at 25°C until sporulation. The spores were then gently scraped off the surface of the plate using sterile R.O. water and under aseptic conditions. A spore suspension (containing an average of approx. 3×10^8 cfu ml^{-1} of each individual fungal strain) was thus obtained. A 5% (v/w) suspension was used for inoculation.

5.2.2 Preparation of the bran-sand mixture

A mixture of bran, sand and water was made in the proportion 1:1:2 (adapted from Lewis and Papavizas, 1984). This mixture taken in 500 ml flasks was then autoclaved twice at 121°C for 20 mins at a pressure of 15 lb/in^2.

5.2.3 *Microcosm set up*

The cultures were first grown on a mixture of bran and sand prepared as described above in Section 5.2.2. Autoclaved bran-sand was inoculated with individual spore suspensions obtained from Section 5.2.1.2 and was incubated at 25 °C for 8 days.

Microcosms were prepared by using an A4-sized transparency sheet, rolled to fit in a petri-plate at the bottom. The petri-plate and the transparency sheet were then taped at the bottom to form a cylindrical structure.

Pre-weighed (200g) non-sterile Merrist Wood soil (pH 7.5) was taken in individual polythene bags. Cyanide at a final concentrations of 50 ppm and 100 ppm CN^- (in the different experimental set-up's) was added to the soil and mixed thoroughly by vigorously shaking the bag. The bran-sand-spore combination (from above) was then inoculated at a concentration of 4 % (w/w). The whole mixture was again mixed thoroughly within the bag by vigorous shaking and added to the microcosms. Pea (*Pisum sativum* L.) and wheat (cv. *Axona*) seeds (5 in each microcosm) were finally added about a cm deep in the respective microcosms. Triplicates of each set-up were prepared.

Different controls were also set-up in a similar way as described above, viz. microcosms without any inoculation and containing only the pea or the wheat seeds and microcosms without any cyanide (R.O. water instead) but with fungal inoculations at the same concentrations as above and pea and wheat seeds in the respective microcosms.

In order to prevent evaporation of cyanide and for reasons of safety, the microcosm set-up had to be kept covered. Wide-necked (11.4cm diameter), screw-cap plastic containers with a volume of 3.75 L were used for this purpose. The microcosms were enclosed in inverted containers and the latter were, in turn, sealed by tape to ensure against any leakage of cyanide vapours (Fig. 5.1).

The microcosms were left to grow under controlled conditions in a Vindon Scientific growth chamber with photo-period specifications set at: 16hrs (day) – 8hrs (night); 25°C (day) - 21°C (night); 40 Klx and relative humidity at 70%. The microcosms were incubated for 21 days and watered periodically and in the process aerated by simple exposure of the microcosms to ambient air.

Fig. 5.1 Photo showing the experimental set-up in the Vindon Scientific growth chamber.

5.2.4 *Root/shoot lengths and dry weights*

At the end of the assay period, the growing plant with the root system intact was separated from the soil. The shoot was sliced-off from the root, and for each of the different experimental set-up, both root and shoot lengths were measured. Dry weights were obtained after drying in the oven overnight at 90°C.

5.3 RESULTS

5.3.1 *Demonstration of alleviation of phytotoxicity of cyanide*

The following figures (Figs. 5.2 to 5.9) demonstrate the growth of pea and wheat plants in microcosms inoculated with different treatments of cyanide and/or the selected fungal strain of *Trichoderma harzianum* O90 at the different concentrations of 50 and 100ppm CN^-. The most conspicuous observation was the absence of any seed germination in any of the controls containing cyanide without inoculation of any of the *Trichoderma* strains.

Fig. 5.2 Pea microcosms showing the control (left) inoculated with cyanide but without *Trichoderma* and on the right, pea with *Trichoderma* and cyanide at 50ppm.

Fig. 5.3 Pea microcosms showing from left: pea only; pea + Trichoderma; and pea with *Trichoderma* and cyanide at 50ppm.

Fig. 5.4 Pea microcosms showing the control (left) inoculated with cyanide but without *Trichoderma* and on the right, pea with *Trichoderma* and cyanide at 100ppm.

Fig. 5.5 Pea microcosms showing from left: cyanide at 100ppm without *Trichoderma*; pea only; pea + *Trichoderma*; and pea with *Trichoderma* and cyanide at 100ppm.

Fig. 5.6 Wheat microcosms showing the control (left) inoculated with cyanide but without *Trichoderma* and on the right, wheat with *Trichoderma* and cyanide at 50ppm.

Fig. 5.7 Wheat microcosms showing from left: wheat only; wheat + Trichoderma; and wheat with *Trichoderma* and cyanide at 50ppm.

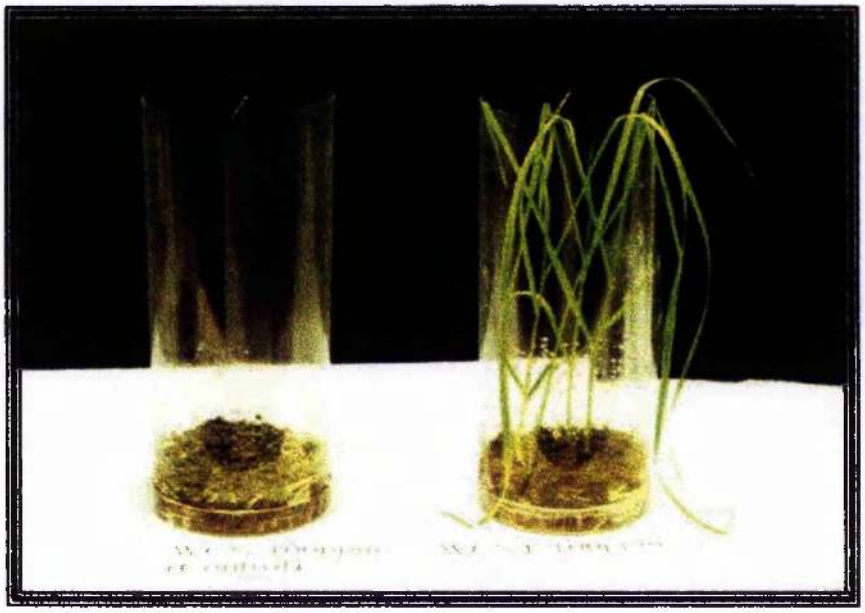


Fig. 5.8 Wheat microcosms showing the control (left) inoculated with cyanide but without *Trichoderma* and on the right, wheat with *Trichoderma* and cyanide at 100ppm.

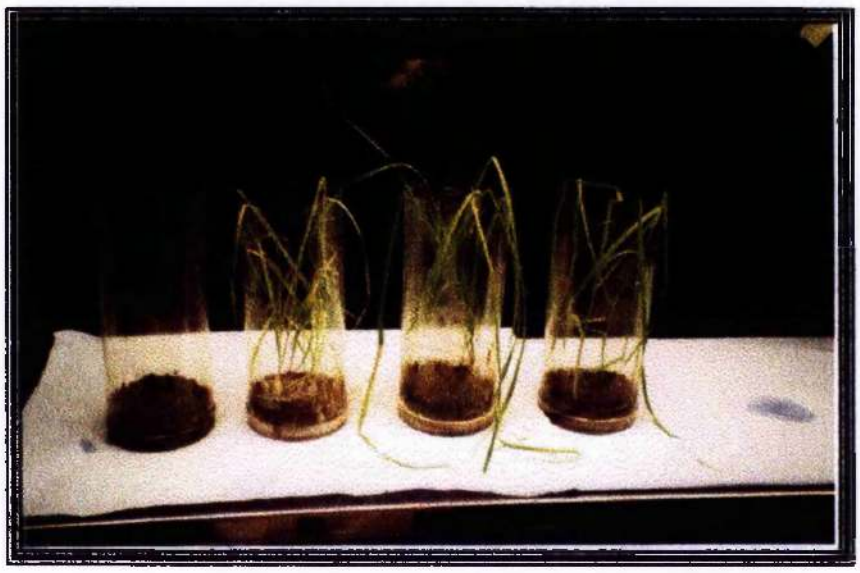


Fig. 5.9 Wheat microcosms showing from left: cyanide at 100ppm without *Trichoderma*; wheat only; wheat + *Trichoderma*; and wheat with *Trichoderma* and cyanide at 100ppm.

5.3.2 *Quantitative studies on pea and wheat plants*

The effects of cyanide and/or *Trichoderma* on pea and wheat plant growth have been evaluated. The *Fusarium* strains obtained from Oxford were also tested. Shoot and root length were measured and the dry weights of both the shoot and root were obtained.

5.3.2.1 *Shoot lengths of pea and wheat plants*

The lengths of shoots obtained from pea and wheat plants, that germinated in microcosms inoculated with cyanide (50 and 100 ppm), along with the individual fungal strains, are shown in Tables 5.1 and 5.2 below, respectively. Significant growth promotion in the presence of the fungal strains was observed in both pea and wheat plants. The presence of cyanide (at both 50 and 100ppm concentrations) too, improved growth.

Shoot length in pea plants (in cm)				
Fungal strain	**Pea only**	**Pea + fungi**	**PCNT/F 50ppm**	**PCNT/F 100ppm**
O90	13.69 ± 0.7	17.73 ± 0.57[a]	17.78 ± 1.42[b]	16.04 ± 0.35[c]
O77	13.69 ± 0.7	18.96 ± 0.66[ad]	16.54 ± 0.5[b]	15.5 ± 0.55[c]
O10	13.69 ± 0.7	17.93 ± 0.68[ad]	13.89 ± 0.5	18 ± 0.85[ce]
O82	13.69 ± 0.7	18.12 ± 0.76[a]	16.54 ± 0.65[b]	18 ± 0.76[c]
O80	13.69 ± 0.7	16.77 ± 0.59[a]	16.13 ± 0.4[b]	21 ± 0.78[ce]
FO	13.69 ± 0.7	17.54 ± 0.66[ad]	14.14 ± 0.51	17.75 ± 0.75[ce]
FS	13.69 ± 0.7	16.03 ± 0.31[ad]	14.7 ± 0.32	14.67 ± 0.67

Table 5.1. Shoot lengths from pea plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values with standard errors recorded. PCNT/F - 50ppm - microcosms with pea seeds, the respective fungi and cyanide at 50ppm. PCNT/F - 100ppm - microcosms with pea seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistical difference between the values for pea + fungi and pea. b= significant increase when compared to the un-inoculated controls. c = statistically significant increase in relation to pea-only controls. d = significantly higher than pea plants inoculated with fungi and 50 ppm CN^-. e = statistically higher than pea plants inoculated with fungi and 50ppm CN^-

Shoot length in wheat plants (in cm)				
Fungal strains	**Wheat only**	**Wheat + fungi**	**WCNT/F 50ppm**	**WCNT/F 100ppm**
O90	30.45 ± 1.87	48.69 ± 2.34^{ad}	37.5 ± 2.41^{b}	37.25 ± 1.28^{c}
O77	30.45 ± 1.87	45.0 ± 3.35^{ad}	35.64 ± 1.34^{b}	36.7 ± 1.63^{c}
O10	30.45 ± 1.87	48.06 ± 2.71^{ad}	36.96 ± 1.72^{b}	43.38 ± 3.98^{e}
O82	30.45 ± 1.87	53.33 ± 0.87^{ad}	37.29 ± 2.13^{b}	19.75 ± 5.75^{e}
O80	30.45 ± 1.87	42.93 ± 2.79^{a}	43.22 ± 1.22^{b}	45.67 ± 2.09^{e}
FO	30.45 ± 1.87	46.0 ± 1.4^{a}	43.44 ± 2.1^{b}	33.75 ± 7.25
FS	30.45 ± 1.87	43.18 ± 0.72^{a}	42.0 ± 1.45^{b}	58.25 ± 4.75^{ce}

Table 5.2. Shoot lengths from wheat plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. WCNT/F - 50ppm - microcosms with wheat seeds, the respective fungi and cyanide at 50ppm. WCNT/F - 100ppm - microcosms with wheat seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistical difference between the values for wheat + fungi and wheat. b= significant increase when compared to the un-inoculated controls. c = statistically significant increase in relation to wheat-only controls. d = significantly higher than wheat plants inoculated with fungi and 50 ppm CN^-. e = statistically different than wheat plants inoculated with fungi and 50ppm CN^-.

5.3.2.2 *Root lengths of pea and wheat plants*

The lengths of roots obtained from pea and wheat plants that germinated in microcosms inoculated with cyanide (50 and 100 ppm) and the individual selected fungal strains are shown in Tables 5.3 and 5.4 below, respectively. An increase in the root length of pea plants was only observed in a few of the *Trichoderma* strains in the combination treatments including cyanide and fungi, whereas in the wheat plants, the presence of cyanide and fungi resulted in the promotion of root length in almost all the strains.

Root length in pea plants (in cm)				
Fungal strains	**Pea only**	**Pea + fungi**	**PCNT/F 50ppm**	**PCNT/F 100ppm**
O90	26.31 ± 1.97	26.63 ± 1.35	32.06 ± 2.6^{bd}	17.57 ± 0.76^{ce}
O77	26.31 ± 1.97	20.54 ± 1.4	27.12 ± 1.27^{d}	32.3 ± 2.52^{ce}
O10	26.31 ± 1.97	28.2 ± 1.39	31.67 ± 1.32^{bd}	30.2 ± 2.93
O82	26.31 ± 1.97	26.08 ± 0.91	30.15 ± 1.36^{d}	31.33 ± 2.89
O80	26.31 ± 1.97	19.35 ± 1.76	30.03 ± 1.3^{d}	36.5 ± 4.71^{e}
FO	26.31 ± 1.97	27.63 ± 1.38	25.39 ± 1.13	36.5 ± 4.71^{ce}
FS	26.31 ± 1.97	25.73 ± 0.74	27.57 ± 1.51	31.33 ± 4.1

Table 5.3. Root lengths from pea plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. PCNT/F - 50ppm - microcosms with pea seeds, the respective fungi and cyanide at 50ppm. PCNT/F - 100ppm - microcosms with pea seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: b = significant increase when compared to the un-inoculated controls. c = statistically significant in relation to pea-only controls. d = significantly higher than pea plants inoculated with fungi only. e = statistically different than pea plants inoculated with fungi and 50ppm CN^-.

Root length in wheat plants (in cm)				
Fungal strains	**Wheat**	**Wheat + fungi**	**WCNT/F 50ppm**	**WCNT/F 100ppm**
O90	28.59 ± 1.82	25.42 ± 2.94	19.63 ± 1.89^{b}	16.25 ± 0.96^{c}
O77	28.59 ± 1.82	24.75 ± 2.28	24.23 ± 0.97^{bc}	16.1 ± 1.4^{c}
O10	28.59 ± 1.82	29.69 ± 2.31^{d}	21.65 ± 1.46^{bc}	16.5 ± 2.54^{c}
O82	28.59 ± 1.82	45.83 ± 5.58^{ad}	24.04 ± 1.3^{bc}	16.25 ± 1.25^{c}
O80	28.59 ± 1.82	25.64 ± 3.85	22.5 ± 0.98^{b}	24.33 ± 3.53
FO	28.59 ± 1.82	31.72 ± 1.06^{d}	24.11 ± 0.89^{bc}	19 ± 3.5^{c}
FS	28.59 ± 1.82	28.23 ± 1.33^{d}	22.89 ± 2.34^{b}	20.5 ± 0.5^{c}

Table 5.4. Root lengths from wheat plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. WCNT/F - 50ppm - microcosms with wheat seeds, the respective fungi and cyanide at 50ppm. WCNT/F - 100ppm - microcosms with wheat seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistically higher than wheat alone. b= significant difference when compared to the un-inoculated controls. c = statistically significant in relation to wheat-only controls. d = significantly higher than wheat plants inoculated with fungi and 50 ppm CN^-. e = statistically higher than wheat plants inoculated with fungi and 100ppm CN^-.

5.3.2.3 *Dry weights of shoots of pea and wheat plants*

The dry weights of shoots obtained from pea and wheat plants, that germinated in microcosms inoculated with cyanide (50 and 100 ppm), and the individual selected fungal strains, are shown in Tables 5.5 and 5.6 below, respectively. Although, growth promotion in terms of shoot lengths had been observed in all the strains, the dry weights of shoots of pea demonstrated a significant increase in only a few fungal strains. However, the dry shoot weights of the wheat plants showed significant increases in all the strains.

Dry weights of shoots of pea plants (in mg)				
Fungal strains	**Pea only**	**Pea + fungi**	**PCNT/F 50ppm**	**PCNT/F 100ppm**
O90	125.62 ± 10.22	137.09 ± 6.0	114.48 ± 15.02	152.88 ± 7.0^{ce}
O77	125.62 ± 10.22	168.89 ± 8.63^{ad}	138.38 ± 7.73	115.68 ± 5.22^{e}
O10	125.62 ± 10.22	148.55 ± 8.5^{a}	148.31 ± 11.18	142.78 ± 9.02
O82	125.62 ± 10.22	131.39 ± 12.68	139.12 ± 9.52	134.23 ± 1.39
O80	125.62 ± 10.22	151.05 ± 8.07^{ad}	122.35 ± 4.74	143.78 ± 9.14^{e}
FO	125.62 ± 10.22	152.96 ± 11.14^{ad}	121.64 ± 5.74	142.35 ± 9.75^{e}
FS	125.62 ± 10.22	141.91 ± 4.77^{d}	105.33 ± 5.02^{b}	123.83 ± 4.94^{e}

Table 5.5. Dry weights of shoots (in mg) from pea plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. PCNT/F - 50ppm - microcosms with pea seeds, the respective fungi and cyanide at 50ppm. PCNT/F - 100ppm - microcosms with pea seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistically higher than pea alone. b= significant difference when compared to the un-inoculated controls. c = significantly higher in relation to pea-only controls. d = significantly higher than pea plants inoculated with fungi and 50 ppm CN^-. e = statistically different than pea plants inoculated with fungi and 50ppm CN^-.

Dry weights of shoots of wheat plants (in mg)				
Fungal strains	**Wheat only**	**Wheat + fungi**	**WCNT/F 50ppm**	**WCNT/F 100ppm**
O90	23.2 ± 2.36	72.57 ± 4.79[a]	75.3 ± 10.58[b]	72.08 ± 3.59[ce]
O77	23.2 ± 2.36	85.89 ± 11.51[a]	70.16 ± 4.52[b]	50.68 ± 5.54[ce]
O10	23.2 ± 2.36	111.87 ± 10.33[ad]	75.33 ± 6.5[b]	68.03 ± 6.4[c]
O82	23.2 ± 2.36	118.57 ± 18.42[ad]	57.45 ± 3.37[b]	86.95 ± 13.25[ce]
O80	23.2 ± 2.36	84.75 ± 7.31[a]	88.19 ± 6.18[b]	78.43 ± 8.88[c]
FO	23.2 ± 2.36	83.52 ± 6.66[a]	77.81 ± 6.41[b]	107.85 ± 0.65[ce]
FS	23.2 ± 2.36	70.13 ± 2.91[a]	67.81 ± 6.7[b]	113.7 ± 27.9[ce]

Table 5.6. Dry weights of shoots (in mg) from wheat plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. WCNT/F - 50ppm - microcosms with wheat seeds, the respective fungi and cyanide at 50ppm. WCNT/F - 100ppm - microcosms with wheat seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistical difference between the values for wheat + fungi and wheat. b= significant increase when compared to the un-inoculated controls. c = statistically significant increase in relation to wheat-only controls. d = significantly higher than wheat plants inoculated with fungi and 50 ppm CN^-. e = statistically different than wheat plants inoculated with fungi and 50ppm CN^-.

5.3.2.4 *Dry weights of roots of pea and wheat plants*

The dry weights of roots obtained from pea and wheat plants, that germinated in microcosms inoculated with cyanide (50 and 100 ppm), and the individual selected fungal strains, are shown in Tables 5.7 and 5.8 below, respectively. The presence of cyanide resulted in a decrease in root weight of pea plants in a few strains and seemed to inhibit root growth. However, in the wheat microcosms almost all the individual strains demonstrated an increase in root weight in the combination experiments.

Dry weights of root in pea plants (in mg)				
Fungal strains	**Pea only**	**Pea + fungi**	**PCNT/F 50ppm**	**PCNT/F 100ppm**
O90	62.92 ± 3.79	67.34 ± 3.15^{d}	42.41 ± 5.32^{b}	63.96 ± 3.11^{e}
O77	62.92 ± 3.79	69.89 ± 3.48^{d}	59.95 ± 2.89	48.08 ± 3.64^{ce}
O10	62.92 ± 3.79	65.34 ± 2.95	67.5 ± 4.62	58.2 ± 2.28
O82	62.92 ± 3.79	102.81 ± 14.71^{ad}	61.99 ± 3.71	60.83 ± 1.28
O80	62.92 ± 3.79	57.58 ± 3.01	52.79 ± 2.38^{b}	67.8 ± 3.74^{e}
FO	62.92 ± 3.79	54.05 ± 3.17	51.01 ± 4.21^{b}	64.05 ± 6.25^{e}
FS	62.92 ± 3.79	59.81 ± 1.94^{d}	44.42 ± 2.27^{b}	49.37 ± 3.67^{e}

Table 5.7. Dry weights of roots (in mg) from pea plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. PCNT/F - 50ppm - microcosms with pea seeds, the respective fungi and cyanide at 50ppm. PCNT/F - 100ppm - microcosms with pea seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a =statistically higher than pea alone. b= significant difference when compared to the un-inoculated controls. c = statistically significant in relation to pea-only controls. d = significantly higher than pea plants inoculated with fungi and 50 ppm CN^-. e = statistically different than pea plants inoculated with fungi and 50ppm CN^-.

Dry weights of roots in wheat plants (in mg)				
Fungal strains	**Wheat only**	**Wheat + fungi**	**WCNT/F 50ppm**	**WCNT/F 100ppm**
O90	11.7 ± 1.04	17.25 ± 1.24^{a}	28.63 ± 2.81^{bd}	22.43 ± 1.29^{ce}
O77	11.7 ± 1.04	18.03 ± 2.38^{a}	22.63 ± 2.35^{b}	20.4 ± 2.12^{c}
O10	11.7 ± 1.04	25.16 ± 2.23^{a}	22.36 ± 2.29^{b}	19.43 ± 1.64^{c}
O82	11.7 ± 1.04	34.27 ± 5.39^{a}	16.39 ± 1.22^{bd}	31.55 ± 0.75^{ce}
O80	11.7 ± 1.04	19.89 ± 1.92^{a}	20.94 ± 1.55^{b}	17.4 ± 2.76^{c}
FO	11.7 ± 1.04	15.88 ± 1.33	20.51 ± 1.4^{b}	20.45 ± 0.95^{c}
FS	11.7 ± 1.04	17.4 ± 1.01^{a}	21.46 ± 1.9^{b}	25 ± 1.1^{c}

Table 5.8. Dry weights of roots (in mg) from wheat plants obtained from the microcosms exposed to the different treatments shown. Triplicates of each treatment were set-up with each containing 5 seeds and mean values and standard errors were recorded. WCNT/F - 50ppm - microcosms with wheat seeds, the respective fungi and cyanide at 50ppm. WCNT/F - 100ppm - microcosms with wheat seeds, the respective fungi and cyanide at 100ppm. Statistical differences were observed as follows; all differences being highly significant at $p < 0.001$: a = statistical difference between the values for wheat + fungi and wheat. b= significant increase when compared to the un-inoculated controls. c = statistically significant increase in relation to wheat-only controls. d = significant difference between the wheat and fungi and the wheat, fungi and cyanide at 50ppm. e = statistically different than wheat plants inoculated with fungi and 50ppm CN^{-}.

5.4 Discussion

All the fungal strains evaluated demonstrated the ability to degrade cyanide in soil and as a result permit growth of the pea and wheat plants in the resultant cyanide-free environment. Appropriate controls were set-up alongside the experimental microcosms. The most striking observation was recorded in the controls containing cyanide without *Trichoderma* or *Fusarium* inoculations. Cyanide was found to be extremely toxic to both pea and wheat plants at 50 and 100ppm CN^-. None of these control microcosms showed any seed germination throughout the assay period.

A different set of controls wherein microcosms, in conditions similar to the above controls were set-up and bran was additionally added to them. A loss of cyanide was detected in these bran-containing controls and seed germination too was observed, although the latter was delayed and occurred only in the final stages of the experiment, after Day 24. *Trichoderma* therefore, indeed increased the rate of degradation of cyanide thereby facilitating the germination of the pea and wheat seeds. The loss of cyanide from the controls could be partially attributed to auto-oxidation and/or incorporation into the humus (Bossert *et al*, 1984). Also, the soil bacteria in the presence of bran as an additional nutrient source could have adapted and contributed to cyanide loss by starting to degrade it. This assumes significance since there clearly was no plant growth in any of the non-inoculated microcosms without bran. A strong odour of cyanide was detected even at the end of the assay period in the latter microcosms.

Trichoderma and *Fusarium* seemed to promote shoot growth in that all the strains showed shoot lengths in the pea plants significantly higher ($p < 0.001$) than the uninoculated ones (Table 5.1). The dry weights of shoots of the pea plants too showed significant ($p < 0.001$) increases in all the strains except strains O90 and O82 (Table 5.5). Similarly, the shoot lengths (Table 5.2) and the dry weights of the shoots in the wheat plants were significantly higher ($p < 0.001$) in the inoculated microcosms (Table 5.6). This indicates plant growth promotion in the presence of the fungi. The potential of *Trichoderma* as growth promoters has earlier been reported (Lynch, 1987 and Lynch *et al*, 1991). In another study, however, Lynch (unpublished), in studies on lettuce germination in presence of cyanide reported fresh shoot weight to be significantly greater but not dry shoot weight.

However, when root lengths of the pea plants were considered, no significant differences were observed among the different fungal strains (Table 5.3). In the wheat plants, except for strain O82, which had a root length significantly more than the control (uninoculated), none of the other strains demonstrated any significant ability to have an effect on the root lengths (Table 5.4). In spite of that, the dry root weights of all the strains showed a highly significant increase in the wheat plants (Table 5.8) although, except for strain O82, the same was not observed in the pea plants (Table 5.7). Root lengths and dry weights have, generally been reported, earlier to be sometimes linked. The reason for there being no increase in root lengths but significant increases in dry weights here in the present study, could be attributed to the root architecture. Microbial influences have been known to change root architecture. These changes could be manifested in the thickening of the root or

especially, in the case of wheat, increased lateral root or root hair formation.

Addition of cyanide at 50 ppm and *Trichoderma* increased shoot length in the pea plants with four of the five *Trichoderma* strains, but it did not have any effect on the microcosms with *Fusarium* (Table 5.1). This may be due to the established role of *Trichoderma* as a growth promoter (Ousley *et al*, 1993; Ousley *et al*, 1994), which is demonstrated here, even in the presence of cyanide. *Trichoderma* including the strain O90, have previously been reported (Ousley *et al*, 1993A), to be efficient growth promoters. Thus a dual beneficial effect may have resulted here in this study, in the presence of some of the strains of *Trichoderma*. Growth promotion in both *Trichoderma* and *Fusarium* is a strain-dependant phenomenon where some strains result in promotion of growth whereas certain strains, especially in the case of *Fusarium* pathogens, actually inhibit plant growth. The apparent reduction in toxicity due to the simultaneous addition of *Trichoderma* or *Fusarium* and cyanide was also observed in the wheat plants with an increase in the shoot lengths only (Table 5.2). The lengths of wheat roots were shorter in the presence of 50ppm cyanide with *Trichoderma or Fusarium* when compared to the uninoculated controls (Table 5.4). Most of the pea plants too did not show any significant difference (Table 5.3). However, here too, as with the wheat and fungi-only microcosms, the dry root weights of the wheat plants with all the strains showed a significant increase in the combination microcosms (Table 5.8).

When comparing microcosms containing *Trichoderma* and cyanide at 50ppm with those containing *Trichoderma* only, cyanide had an effect in that shoot lengths of pea

plants were observed to be retarded in the former case (Table 5.1), so too was the dry shoot weight (Table 5.5). Root length however, increased in all the *Trichoderma* strains with cyanide (Table 5.3). In the wheat plants too, the shoot (Table 5.2) and root lengths (Table 5.4) were found to be less in the cyanide-containing microcosms with most of the fungal strains.

Even with cyanide at 100ppm along with fungi shoot length increased in pea (Table 5.1) and wheat plant microcosms (Table 5.2), with most of the fungal strains, when compared to controls. Although most of the fungal strains showed no significant difference in the dry weights of shoots (Table 5.5) and roots (Table 5.7) in the pea plants when compared to the controls, these were significantly ($p < 0.001$) higher in cyanide (at 100ppm) and fungi containing wheat microcosms (Tables 5.6 and 5.8).

In general, with most of the strains, there was no significant difference between the shoot (Table 5.1) and root (Table 5.3) lengths of pea plant microcosms with cyanide at 50ppm and 100ppm. Although, a decrease in the values of the root and shoot parameters would have been expected, considering the toxicity of cyanide at 100ppm, a couple of *Trichoderma* and the two *Fusarium* strains, however, showed slightly increased dry shoot weight in both the pea and wheat microcosms containing 100ppm CN^- (Tables 5.5 and 5.6). A similar observation was also recorded with the dry root weights in both sets of microcosms (Tables 5.7 and 5.8). The data obtained in this study could not be evaluated or compared with any other work since, except for the plant growth promotion studies, there has been no previous report in literature on any similar microcosm study.

In summary, this is the first reported instance of such a microcosm study been undertaken with plants and cyanide for the study of biodegradation of cyanide. The soil microcosm studies have helped establish the ability of the *Trichoderma* strains to successfully degrade cyanide in a simulated natural environment. The absence of any germination of the seeds in the non-inoculated controls, confirm that the loss of cyanide was not abiotic in the assay microcosms and that biological degradation of cyanide by the inoculated fungi did occur.

CHAPTER 6:

FINAL DISCUSSION

6.1 DISCUSSION

Cyanide is found to enter the environment through natural and anthropogenic sources. Natural sources of cyanide include a broad range of different life forms from simple forms of life such as bacteria to eukaryotes and the animal kingdom. Industrial wastes, however, are the major contributors to the cyanide in the environment. Former manufactured gas plant sites are commonly found to contain cyanide-contaminated soils.

A number of strains of *Trichoderma harzianum* had previously been identified as being resistant and capable of degrading cyanide by Prof. J.Lynch (unpublished). The first objective of the project was to identify enzymes in *Trichoderma*, involved in this biological degradation of cyanide by the fungi. Of the number of enzymes, which have been reported in literature, catalysing the degradation of cyanide, three were chosen, to get an idea of the distribution of the different enzymes involved in the degradation of cyanide in the species *Trichoderma*.

To my knowledge, this is the first reported instance of cyanide-catabolizing property recognised in strains of the fungal species, *Trichoderma harzianum*. The presence of the enzymes, cyanide hydratase (formamide hydrolyase) and rhodanese was demonstrated in all the selected strains of *Trichoderma*. Another enzyme assayed, β-cyanoalanine synthase, however, was not detected. The cyanide hydratase and rhodanese activities of all the strains compared favourably with previously reported values in other fungal systems. Strains of the fungal species, *Fusarium, F. oxysporum*

and *F. solani*, were obtained by the courtesy of Prof. C. Knowles, at Oxford. These strains have been reported to degrade metallo-cyanides due to the presence of cyanide hydratase and amidase (Barclay, 1997) and were used for comparisons and as a positive control system in this project.

An interesting observation was the absence of induction, in the presence of cyanide, of both enzyme activities, in any of the *Trichoderma* strains. This constitutive nature of the enzymes appears to be unique in that most of the cyanide degrading enzymes in fungi have been found to be inducible and where constitutive, have been found to demonstrate an increase in activity on induction with cyanide (Fry and Evans, 1977). Bacterial systems, however, have been reported to show constitutive rhodanese systems that do not demonstrate any increase in activity on induction with cyanide (Ryan and Tilton, 1977; Bowen *et al*, 1965). However, in the present study, in a few of the strains, evidence of inhibition by cyanide was also observed.

Characterisation of the crude extract of the rhodanese enzyme revealed the enzyme to have a Km similar to those reported in earlier studies in bacterial and fungal systems. Although free cyanide is a small lipophillic molecule and can pass through the cell wall, results indicate that the degradation occurs outside the cell since the enzyme rhodanese was found to be present extracellularly in the *Trichoderma* strains.

Compelling evidence of co-metabolism emerged from a set of experiments carried out as flask cultures. Decrease in concentration levels of glucose directly linked to the catabolism and decrease of cyanide levels in the medium. The rate of degradation of

cyanide increased dramatically with the addition of glucose as a co-metabolite; with cyanide being reduced to very low levels, three times faster in the glucose-inoculated systems. This may be due to the glucose acting primarily as an energy source for metabolism. The biomass too was found to increase almost three times as compared to the experiment without glucose. The experiment also demonstrated the ability of the *Trichoderma* strains to utilize cyanide for growth as a sole carbon and nitrogen source.

The microcosm studies provided further evidence of the viability and biodegradative capabilities of the *Trichoderma* strains in the natural environment. There are no reports in literature of any such microcosm experiment being carried out to evaluate cyanide degradation in a simulated natural ecosystem. Pea and wheat seeds were added to soil contaminated with cyanide and inoculated with *Trichoderma*. A cyanide-free environment, produced as a result of the action of removal of the cyanide from the soil by the *Trichoderma*, permitted the germination of the seeds. No germination was observed in controls without inoculation of *Trichoderma*. This provided conclusive proof of biodegradation occurring and authenticated the earlier experiments in the present project on the cyanide biodegradation capability of *Trichoderma*. Also, there was evidence of significant stimulation of plant growth in microcosms inoculated with the fungi alone.

A point to be noted is that the two enzymes found to be present in the *Trichoderma* have an alkaline pH optimum. High cyanide concentrations are found in the groundwater at sites with alkaline soils (clay) with pH above 7.5 as against much

lower concentrations (in sandy soils) at acidic pH's of about 4.5 (Meeussen *et al*, 1992). Leaching of complexed cyanide generally occurs at sites of former gas works. This complexed cyanide is not thermodynamically stable and tends to decompose to free cyanide, depending on pH and the redox potential as well as uv-radiation from sunlight (Meeussen *et al*, 1992). At present there is no data on the interactions of *Trichoderma* with complexed cyanides like Prussian Blue generally found on gasworks sites. Although, these sites are largely contaminated with high amounts of iron cyanide complexes and iron cyanide minerals (Prussian Blue), Meeussen *et al*, 1992 report that in most soils these complexes will tend to decompose to toxic free cyanide. Even under optimal conditions, the stability of iron cyanides is not sufficient to reduce free cyanide to sub-toxic levels (Meeussen *et al*, 1992). These considerations along with the extra-cellular presence of the cyanide catabolizing enzymes, taken together in perspective, present a new avenue with tremendous implications in the use of *Trichoderma*, in clean-up programmes of contaminated soil sites. Further detailed research would, however, be required on the scale-up to actual large field conditions.

In conclusion, this is the first reported instance of the fungal species *Trichoderma harzianum* degrading cyanide. All the strains of *Trichoderma* assayed in this project demonstrated resistance and degradative capability of very high concentrations of cyanide. Concentrations of up to 2000 ppm of CN^- in aqueous media and 100 ppm in soil systems were utilized in the experiments with positive results. The novel ability of the selected *Trichoderma* strains of utilizing cyanide as a sole source of carbon and nitrogen was also established. The mechanism of action of the two enzymes, from the

Trichoderma strains, are not clear and further research needs to be undertaken in terms of the degradation products of cyanide in the reactions catalysed by these enzymes or the possibility of the existence of another enzyme system. These investigations and understanding of mechanisms involved would then aid in setting-up treatment strategies for the treatment of cyanide containing wastes including aqueous and soil systems.

6.2 Future Research

Purification of both the enzymes would be one of the first steps in the follow-up to this project. This would then permit the evaluation and comparison of the enzymes present in *Trichoderma* and those in *Fusarium* or in other species producing cyanide hydratase and rhodanese. The need for purification of the enzyme was not felt in the present project since the eventual aim was to look for degradation of cyanide in a natural environment such as the rhizosphere in soils.

On the other hand, immobilization of the both the cyanide hydratase and rhodanese enzymes outside the cells may be employed in certain treatment processes, if so warranted, such as where production of biomass needs to be avoided.

Dubey and Holmes, 1995, have suggested that cyanidase and rhodanese could be used in the treatment of waste streams containing metallic cyanides and other forms of cyanide by using protective devices to keep the enzymes stable and reactive.

Interaction of *Trichoderma* with metal complexed cyanides should be evaluated and studies undertaken to check for viability and degradative capabilities of the *Trichoderma* strains in a metallo-cyanide environment.

Molecular biology studies on the genetics of the two enzymes in particular and *Trichoderma*, in general, could help in the development of probes for the enzyme systems. These probes can give an indication of the potential of the autochthonous microbial population of a contaminated site for the degradation of cyanide. On the other hand, *in situ* expression of catabolic genes and therefore, biodegradation, can be measured using mRNA probes from contaminated sites. Gene probes for the *Fusarium* strains from Oxford, used in this project, have been developed (C. Knowles, personal communication). A comparison of the *Trichoderma* and *Fusarium* strains can be carried out with respect to any homology in their enzymes of degradation.

Full scale aerobic biological treatment processes have been developed and implemented to effectively remove cyanide and metal cyanides. The concentrations of cyanide and metals in the solutions treated have, however, been low (Goncalves, 1998). The diverse characteristics of industrial effluents, especially its content, may influence process performance. In the present project, strains of *Trichoderma* have been shown to possess two constitutively present cyanide-catabolizing enzymes capable of degrading high concentrations of cyanide. This ability, in combination with the inherent versatile characteristics of biocontrol and plant growth promotion, make *Trichoderma* a serious candidate for clean-up solutions to contaminated land and water.

CHAPTER 7:

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REFERENCES

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APPENDIX

Control of Substances Hazardous to Health (COSHH) Assessment

All hazardous substances used in this study were carefully used in acordance with COSHH regulations, 1988. All the experiments were carried out using Good Laboratory Practice (GLP). A brief summary of some of the extremely hazardous substances used, their concentrations; the description and assessment of the hazard/risk and the measures adopted to prevent exposure and control risk have been tabulated in Table, below.

Hazardous substance and Concentrations	Description and assessment of risk	Measures adopted to prevent exposure
KCN - 0.05M, 0.1M and 0.125M	Extremely toxic; can enter body through exposure to skin, eyes, ingestion & inhalation. Should not come in contact with acids and oxidisers.	Fume cupboard, eye protection, two pairs of gloves and presence of first-aider's within ear-shot with anti-toxin kit & breathing apparatus during weighing of the stock solutions.
N,N-dimethyl-p-phenelene diamine sulphate - 0.02M in 7.2M HCl	Toxic; can get through the skin	Fume cupboard, eye protection, gloves
Hydroxylamine - hydrochloride 2M in 3.5M NaOH	Irritant; harmful. Avoid contact with oxidants. Violent/ explosive reaction with many oxidants.	Fume cupboard, eye protection, gloves
Formamide - 3.33 :moles/ml	Irritant, teratogen OES - 30 ppm	Fume cupboard, eye protection, gloves and storage at 4°C
Sodium sulphide – 250 nmoles ml^{-1}	Corrosive, irritant and poisonous	Fume cupboard, eye protection, gloves
Sodium thiocyanate 2 :moles ml^{-1}	Corrosive, irritant and poisonous	Fume cupboard, eye protection, gloves