PHYSICO-CHEMICAL APPROACHES TO REDUCE DYES IN THE TEXTILE EFFLUENTS, PURIFICATION AND CHARACTERIZATION OF α-GALACTOSIDASE FROM *Pleurotus eous*

A PROJECT REPORT

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in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE
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APRIL 2008

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BONAFIDE CERTIFICATE

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(INTERNAL EXAMINER) (EXTERNAL EXAMINER)

Acknowledgement

First and foremost we express our praise and thanks to Lord Almighty for the blessings showered on us to complete our work successfully.

We extend our sincere thanks to our Principal **Dr.JOSEPH V THANIKAL,** Kumaraguru College of Technology, Coimbatore, for his incredible support for all our toil regarding the project.

We are deeply obliged to **Dr. P.RAJASEKARAN**, Head of the Department of Biotechnology, his concern and implication has been immensely helpful in the completion of the project course.

We articulate our thankfulness and deep sense of gratitude towards our class advisor and our project guide **Mr.S.SENTHIL KUMAR**, Department of Biotechnology, for his profound ideas, able guidance and continuing motivation. His kindness and invaluable guidance have been integral in the completion of this work. We consider this as an honour to have the opportunity to work under his guidance in this project work.

We are grateful to **Mr.RAMALINGAM,** Senior Lecturer, Department of Biotechnology, for lending a hand throughout the project path.

We thank all the **Non-teaching staffs** of our department for providing us the technical support for the project.

We also thank our **friends and family** who helped us to complete this project fruitfully.

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ABSTRACT

Due to rapid industrialization and urbanization, a lot of chemicals including dyes are manufactured and used in day-to-day life. Reactive dyes are important chemical pollutants in the textile waste water. Therefore innovative treatment technologies need to be investigated. We are reporting for the first time on the use of natural polyelectrolyte, i.e. chitin for the removal of reactive dyes present in the simulated textile waste water. In addition we have also made an attempt to use granular carbon for the removal of dyes in the simulated textile effluent. The use of powdered charcoal for the removal of textile dyes was discouraged due to the flux created. Also, the ability of eluting bound dyes from carbon and polyelectrolyte using hot water and other organic solvents were observed. An extracellular α-galactosidase from the mother spawn culture of fungus *Pleurotus eous* was purified by ammonium sulphate precipitation and dialysis with a 25.11 fold increase. The enzyme had an optimum temperature of 55°C and an optimum pH of 5. The enzyme activity was strongly inhibited by Ag^{+}. Raffinose and ribose inhibited the α-galactosidase. EDTA and 1, 10-phenanthroline did not inhibit the enzyme activity and hence it is not a metal dependent enzyme. The raffinose and stachyose content in the soymilk was degraded by α-galactosidase.

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LIST OF ABBREVATIONS

g	gram
ml	millilitres
mg	milligrams
h	hour
min	minute
M	molar
µl	microlitres
°C	degree Celsius
EDTA	Ethylene Di – amine Tetra Acetic acid
PNPG	P-nitro phenyl galactopyranoside
mM	milli molar
nm	nanometers

INTRODUCTION

1. Introduction

Introduction:

Increasing industrialization and urbanization leads to environmental pollution. The discharge of toxic effluents from various industries adversely affects water resources, soil fertility, aquatic organisms and ecosystem integrity. Among various industries textile-dyeing industry generates large quantities of wastewater after the dyeing process. It is a complex and highly variable mixture of many polluting substance ranging from inorganic compounds and elements to polymers and organic products. It induces persistent color coupled with organic load leading to disruption of the total ecological and symbiotic balance of the receiving water stream. Dyes with striking visibility in recipients may significantly affect photosynthetic activity in aquatic lives due to metals, chlorides, etc., associated with dyes or the dyeing process.

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography and cosmetics. Approximately 10,000 dyes and pigments are industrially used and over 0.7 million tons of synthetic dyes are produced annually worldwide. All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents and microbial attack. During processing up to 15%of the used dyestuffs are lost in industrial effluents. Major classes of synthetic dyes used are azo, anthraquinone and triphenylmethane dyes. In addition to their visual effects and adverse impact in terms of COD, many synthetic dyes show their toxic, carcinogenic and genotoxic effects. Conventional wastewater

treatment plants are unable to perform a complete dye removal, 90% of reactive dyes persist after activated sludge treatment. Therefore innovative treatment technologies need to be investigated. The textile industry waste water has been decolorized efficiently by physico-chemical methods adding granular carbon and polyelectrolyte such as chitin. Also, the ability of eluting bound dyes from carbon and polyelectrolyte using hot water and other organic solvents were observed. In the case of biological method of dye degradation, fungal metabolic activities are subjected to many studies.

Alpha-galactosidase catalyzes the hydrolysis of the alpha-1,6-linkages in disaccharides (melibiose), oligosaccharides (raffinose family sugars), and polysaccharides (galactomannan and galactoglucomannans) . Raffinose-family oligosaccharides are widely found in legumes and cruciferous vegetables, including beans, peas, broccoli, brussels sprouts and cabbage. These oligosaccharides escape digestion, because human digestive juice lacks a-galactosidase enzyme.

Against this background, our study was designed and carried out with the following major objectives:

1. Reducing dye concentration in the textile effluent using activated powdered and granular carbon.
2. Testing the ability of hot water and other organic solvents to elute the bound dyes from granular carbon.
3. Use of polyelectrolyte in reducing the dye concentration in the textile effluent and testing the ability of hot water and other organic solvents to elute the bound dyes polyelectrolyte.
4. To purify and characterize α-galactosidase enzyme from *Pleurotus eous*

LITERATURE REVIEW

2. Literature Review

2.1 Dyes

A dye can generally be described as a colored substance that has an affinity to the substrate to which it is being applied. The dye is generally applied in an aqueous solution, and may require a mordant to improve the fastness of the dye on the fiber (Zollinger, 2002). A mordant is a substance used to set dyes. A mordant is either inherently colloidal or produces colloids and can be either acidic or basic. Mordants include tannic acid, alum, chrome alum, and certain salts of aluminum, chromium, copper, iron, potassium, and tin.

In other words, dye is defined as a substance, usually organic, which is designed to be absorbed or adsorbed by, made to react with, or deposited within a substrate in order to impart color to the substrate with some degree of permanence. (Gregory , 1993).

2.2 Dye classification

The dyes were obtained from animal, vegetable or mineral origin with no or very little processing. By far the greatest source of dyes has been from the plant kingdom, notably roots, berries, bark, leaves and wood, but only a few have ever been used on a commercial scale.

2.2.1 Natural dyes

Animal Origin

Tyrian purple	Vat dye
Kermes	Mordant dye
Cochineal	Mordant dye

Vegetable Origin

Safflower	Direct (substantive) dye
Turmeric	Direct (substantive) dye
Indigo	Vat dye
Wood	Vat dye
Alizarin (Madder)	Mordant dye
Dyer's Broom	Mordant dye
Logwood	Mordant dye
Brazil wood	Mordant dye
Quercitron bark	Mordant dye
Weld	Mordant dye
Old Fustic	Mordant dye

2.2.2 Inorganic dyes

Iron Buff

Classification

The first man made organic dye, mauveine, was discovered by William Henry Perkin in 1856. Many thousands of dyes have since been prepared and because of vastly improved properties imparted upon the dyed materials quickly replaced the traditional natural dyes. Dyes are now classified according to how they are used in the dyeing process.

Acid dye

Water soluble anionic dyes that are applied to fibres such as silk, wool, nylon and modified acrylic fibres from neutral to acid dye baths. Attachment to the fibre is attributed, at least partly, to salt formation between anionic groups in the dyes and cationic groups in the fibre. Acid dyes are not substantive to cellulosic fibres.(Poots et al., 1976).

Basic dye

Water soluble cationic dyes that are mainly applied to acrylic fibres but find some use for wool, and silk. Usually acetic acid is added to the dye bath to help the take up of the dye onto the fibre. Basic dyes are also used in the coloration of paper.

Direct (Substantive) dye

Dyeing is normally carried out in a neutral or slightly alkaline dye bath, at or near the boil, with the addition of either sodium chloride (NaCl) or sodium

sulphate (Na_2SO_4). Direct dyes are used on cotton, paper, leather, wool, silk and nylon. They are also used as pH indicators and as biological stains.

Mordant dye

As the name suggests these dyes require a mordant. This improves the fastness of the dye on the fibre such as water, light and perspiration fastness. The choice of mordant is very important as different mordants can change the final colour significantly. Most natural dyes are mordant dyes. The mordant used is potassium dichromate applied as an after-treatment.

Vat dye

These dyes are essentially insoluble in water and incapable of dyeing fibres directly. However, reduction in alkaline liquor produces the water soluble alkali metal salt of the dye. In this leuco form these dyes have an affinity for the textile fibre. Subsequent oxidation reforms the original insoluble dye.

Reactive dye

First appeared commercially in 1956, after their invention in 1954 by Rattee and Stephens at the ICI Dyestuffs Division site in Blackley, Manchester, UK. They are used to dye cellulosic fibers. The dyes contain a reactive group, either a haloheterocycle or an activated double bond, that, when applied to a fiber in a weakly alkaline dye bath, forms a chemical bond with a hydroxyl group on the cellulosic fiber. Reactive dyeing is now the most important method for the coloration of cellulsic fibers. Reactive dyes can also be used to dye wool and nylon, in the latter case they are applied under weakly acidic conditions.

Disperse dye

Originally developed for the dyeing of cellulose acetate. They are substantially water insoluble. The dyes are finely ground in the presence of a dispersing agent then sold as a paste or spray dried and sold as a powder. They can also be used to dye nylon, triacetate, polyester and acrylic fibers.

Azoic dye

A dyeing technique in which an insoluble azo dye is produced directly onto or within the fiber. This is achieved by treating a fiber with a diazo component and a coupling component. With suitable adjustment of dye bath conditions the two components react to produce the required insoluble azo dye. This technique of dyeing is unique in that the final color is controlled by the choice of the diazo and coupling components.

Synthetic

Synthetic dyes are extensively used for dyeing and printing in a variety of industries. Over 10 000 dyes with an annual production over 7 × 105 metric tones world wide are commercially available and 5- 10% of the dye stuff is lost in the industrial effluents (Sine, 2003). Therefore there is a need to remove dyes before effluent is discharged into receiving water bodies. The most popular treatment methods for textile wastewater are combinations of biological treatment, chemical coagulation and activated carbon adsorption.

Triphenyl methane dyes

Triphenylmethane, or triphenyl methane, is a hydrocarbon. It is a colorless to light brown powder. It is the basic skeleton of many synthetic dyes (Triarylmethane dyes); many of them are pH indicators, and some

display fluorescence. It is insoluble in water and soluble in chloroform. Its risks and safety phrases are R36 R37 R38.

2.3 Dyeing process

The dyeing of a textile fiber is carried out in a solution, generally aqueous, known as the dye liquor or dye bath. For true dyeing (as opposed to mere staining) to have taken place, the coloration must be relatively permanent; that is, not readily removed by rinsing in water or by normal washing procedures (Bennett, and Faison, 1997). Moreover, the dyeing must not fade rapidly on exposure to light. The process of attachment of the dye molecule to the fiber is one of absorption; that is, the dye molecules concentrate on the fiber surface.

There are four kinds of forces by which dye molecules are bound to fiber: (1) ionic forces, (2) hydrogen bonding, (3) van der Waals' forces, and (4) covalent chemical linkages. In the dyeing of wool, which is a complex protein containing about 20 different a-amino acids, the sulfuric acid added to the dye bath forms ionic linkages with the amino groups of the protein. In the process of dyeing, the sulfate anion (negative ion) is replaced by a dye anion (Carliell *et al.*, 1996). . In the dyeing of wool, silk, and synthetic fibers, hydrogen bonds are probably set up between the azo, amino, alkyl amino, and other groups, and the amido -CO-NH-, groups. Van der Waals' forces (the attractive forces between the atoms or molecules of all substances) are thought to act in the dyeing of cotton between the molecular units of the fiber and the linear, extended molecules of direct dyes. Covalent chemical links are brought about in the dye bath by chemical reaction between a fiber-reactive dye molecule, one containing a chemically

reactive centre, and a hydroxy group of a cotton fiber, in the presence of alkali.

2.4 Impacts of dyes

Dyes cause severe contamination in river and ground water in the vicinity of dyeing industries. The impact of dyes in food industry and their degraded products on human health has caused concern over a number of years, inspite of legislation controlling their use in several countries.

2.5 Dye removal techniques

The majority of physical, chemical and biological color removal techniques work either by concentrating the color into sludge, solid supports, or by the complete destruction of the dye molecule. It is expected that decoloration systems involving destruction technologies will prevail, as the transfer of pollution from one part of the environment to another is prevented (Thurston, 1994). Currently, the major methods of textile wastewater treatment involve physical and/or chemical processes as membrane filtration, coagulation/flocculation, precipitation, flotation, adsorption, ion exchange, ion pair extraction, ultrasonic mineralization, electrolysis, chemical reduction and advanced chemical. The advanced oxidation processes include chlorination, bleaching, ozonation, Fenton oxidation, photo catalytic oxidation and wet-air oxidation Such methods, are often very costly and accumulation of concentrated sludge creates a disposal (Rigling, and van Alfen, 1993).

There is also the Possibility that a secondary pollution problem will arise due to excessive chemical use. Biological and/or mixed treatment systems that can effectively remove dyes from large volumes of wastewater at

a low cost are a preferable alternative. Biological techniques include biosorption and biodegradation in aerobic, anaerobic, anoxic or combined anaerobic/aerobic treatment processes with bacteria, fungi, plants, yeasts, algae and enzymes. Textile dye effluents are complex, containing a wide variety of dyes, natural impurities extracted from the fibers and other products such as dispersants, leveling agents, acids, alkalis, salts and sometimes heavy metals (Leatham and Stahman, 1981). In general, the effluent is highly Colored with high biological oxygen demand (BOD) and chemical oxygen demand (COD), has a high conductivity and is alkaline in nature. For this reason, several factors determine the technical and economic feasibility of each single dye removal technique as dye type, wastewater composition, dose and costs of required chemicals, operation costs (energy and material), environmental fate and handling costs of generated waste products. Usually, the use of one individual process may not be sufficient to obtain complete decolourisation because each technique has its limitations. Dye removal strategies consist therefore mostly have a combination of different methods.

2.5.1 Physico-chemical methods

2.5.1.1 Absorption

The use of any adsorbent, whether ion-exchanger, activated carbon or high-surface-area inorganic material, for removing species from a liquid stream depends on the equilibrium between the adsorbed and the free species. Standard ion exchange systems have not been widely used for treatment of dye effluents due to the high cost of organic solvents to regenerate the ion-exchanger, and due to the extremely large inorganic load of the effluent. Activated carbon is reasonably effective at removing many different dyes from aqueous streams (Churchley, 1994).

Granular activated Carbon (GAC) absorption has been used successfully for the treatment of dyes. GAC is used to absorb the relative quantities of soluble organics such as dyes and inorganic compounds. Absorption occurs when the molecules adhere to the internal walls of pores in carbon particles. (Chen and Lin, 2001 ; Sastri, 1942).

Carbon regeneration is accomplished by addition of organic solvents such as acetone. The bound dyes within the pores of carbon are removed to an extent by this method and hence GAC can be reused. This method saves up to 50 percent of the carbon cost. (Kannan and Sundaram, 2001; McKay, 1982).

2.5.1.2 Polyelectrolyte

Polyelectrolytes are polymers whose repeating units bear an electrolyte group. These groups will dissociate in aqueous solutions (water), making the polymers charged. Polyelectrolyte properties are thus similar to both electrolytes (salts) and polymers (high molecular weight compounds), and are sometimes called **polysalts**. Like salts, their solutions are electrically conductive. Like polymers, their solutions are often viscous. Charged molecular chains, commonly present in soft matter systems, play a fundamental role in determining structure, stability and the interactions of various molecular assemblies. Theoretical approaches to describing their statistical properties differ profoundly from those of their electrically neutral counterparts, while their unique properties are being exploited in a wide range of technological and industrial fields. One of their major roles, however, seems to be the one played in biology and biochemistry. Many biological molecules are polyelectrolytes. For instance, polypeptides (thus all proteins) and DNA are polyelectrolytes. Both natural and synthetic polyelectrolytes are used in a variety of industries.(Vorontsov and Lyubartsev 1987)

Chitin is a polymer that can be found in anything from the shells of beetles to webs of spiders. It is present all around us, in plant and animal creatures. It is sometimes considered to be a spinoff of cellulose, because the two are very molecularly similar. Cellulose contains a hydroxy group, and chitin contains acetamide. Chitin is unusual because it is a "natural polymer," or a combination of elements that exists naturally on earth. Usually, polymers are man-made. Crabs, beetles, worms and mushrooms contain large amount of chitin.

Chitin is a very adaptive material for a creature. Insects and animals with chitin coats usually shed these coats, or molt, at least once a year. Chitin is a very firm material, and it help protect an insect against harm and pressure. Depending on its thickness, chitin can be rigid or yielding. Often, insect coats contain thick, stiff layers of chitin. The areas around legs and face contain very thin, pliable layers.

In addition to being a naturally-occurring molecule, chitin is also notable for the process in which it is broken down. Because many sea creatures shed shells of this material every year, one would expect the ocean to be full of chitin. This is not so. There are tiny bacteria in the ocean that can sense small chains of sugars sent from a discarded chitin shell. Once these bacteria (Vibrio furnisii) have determined that they have found a shell, they begin to create enzymes that can be used to break down the shell. In a series of nine steps, these bacteria reduce chitin to chains of simple sugars and ammonia. The implications that this holds are great. Using this process, scientists may be able to trigger breakdown and responses in other materials besides chitin. They may also be able to trigger an immune responses in plants, using the molecules that chitin releases when shed.

2.5.2 Biological methods

2.5.2.1 Bacterial

The ability of bacteria to metabolize azo dyes has been investigated by a number or research groups. Under aerobic conditions azo dyes are not readily metabolized, although the ability of bacteria with specialized reducing enzymes to aerobically degrade certain azo dyes was reported .In contrast, under anaerobic conditions many bacteria reduce azo dyes by the activity of unspecific, soluble, cytoplasmic reductase, known as azo reductases. The anaerobic reduction degrades the azo dyes that are converted into aromatic amines (Yaropolov, et al., 1994). , which may be toxic, mutagenic, and possibly carcinogenic to mammalians. Therefore, to achieve complete degradation of azo dyes, another stage that involves aerobic biodegradation of the produced aromatic amines is necessary. Bacterial biodegradation of non-azo dyes has only recently been studied. It has been observed that several bacteria can degrade anthraquinone dyes. Aerobic decolourisation of triphenylmethane dyes has also been demonstrated. In phtalocyanine dyes, reversible reduction and decolorization under anaerobic conditions have been observed. (Call and Mucke, 1997).

2.5.2.2 Fungal

The most widely researched fungi in regard to dye degradation are the ligninolytic fungi. White-rot fungi in particular produced enzymes as lignin peroxidase, manganese peroxidase and laccase that degrade many aromatic compounds due to their non-specific activity. Large literature exists regarding the potential of these fungi to oxidize phenolic, non-phenolic, soluble and non-soluble dyes. In particular laccase from Pleurotus ostreatus,

Schizophyllum commune, Sclerotium rolfsii and Neurospora crassa, seemed to increase up to 25% the degree of decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using enzyme preparations (Barr, and Aust, 1994) . On the contrary, manganese peroxidase was reported as the main enzyme involved in dye decolorization by Phanerochaete chrysosporium chrysosporium (Pitter, and Chudoba, 1990) and lignin peroxidase for Bjerkandera adusta. Some non-white-rot fungi that can successfully decolorize dyes have also been reported.

2.6 *Pleurotus eous*

Mushrooms are nutritionally functional food and a source of physiologically beneficial and nontoxic medicines. They have been used in folk medicine through-out the world since ancient times. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments. The most significant medicinal effect of mushrooms and their metabolites that attracted the attention of the public is their anti-tumour property. Mushroom metabolites are usually used as adaptogens and immunostimulants and they are now considered to be one of the most useful antitumour agents for clinical uses. Hence, search for new anti-tumour substances from mushrooms has been a matter of great importance. *Pleurotus* species are commonly called Oyster mushrooms. There are about 40 species of this mushroom. They enjoy worldwide distribution, both in temperate and tropical parts of the world. Oyster mushrooms now rank second among the important cultivated mushrooms in the world (Chern and Huang, 1998 ; Kumar et al, 2003).

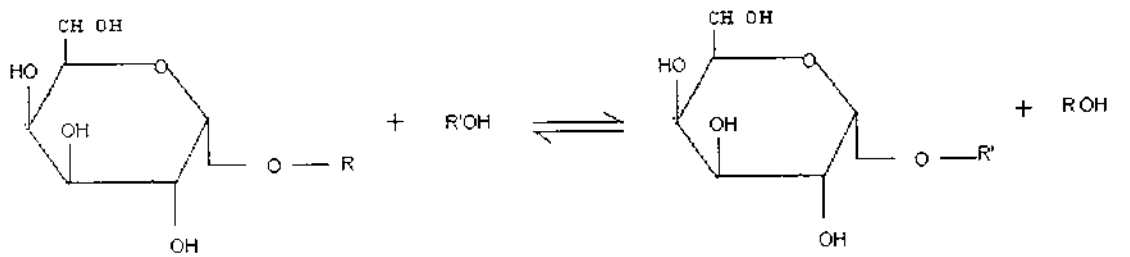
2.7 α-Galactosidase [α-D-galactoside galactohydrolase, EC 3.2.1.22]

Alpha-galactosidase catalyzes the hydrolysis of the alpha-1,6-linkages in disaccharides (melibiose), oligosaccharides (raffinose family sugars), and polysaccharides (galactomannan and galactoglucomannans) (Dey and Pridham, 1972; Varbarnets et al., 2001). Raffinose-family oligosaccharides are widely found in legumes and cruciferous vegetables, including beans, peas, broccoli, brussels sprouts and cabbage. These oligoscharides escape digestion, because human digestive juice lacks α-galactosidase enzyme. Microorganisms present in the large intestine ferment these sugars lead to gas production **(flatus)** (Steggerda et al., 1966).

The hydrolytic action of α-galactosidase on various sugars is shown as below:

1. Melibiose yields D-galactose and D-glucose,
2. Raffinose yields one molecule of galactose and sucrose,
3. Stachyose yields two molecules galactose and sucrose,
4. Verbascose yields three molecules galactose and sucrose.

α-Galactosidase catalyses the following reactions:



the hydroxylic acceptor molecule, R'OH, is commonly water, although R and R' can be aliphatic or aromatic groups. This clearly indicates that the enzyme

α-galactosidase may hydrolyze a number of simple α-galactosides to more complex polysaccharides.

2.7.1 Specificity:

α-Galactosidase exhibited a broad range of specificity and removes the terminal α-D-Galactose residues attached by α-1-2, α-1-3, α-1-4 and α-1-6 linkages (Dey and Pridham., 1972; Varbarnets et al., 2001).

α-Galactosidase exhibits two types of activity. They are as follows:

A. hydrolase activity, and

B. transgalactosylase activity

A. Hydrolase activity:

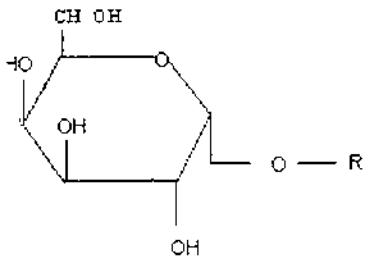
The configuration of hydrogen and hydroxyl group on single carbon atom of a glycosidic substrate plays an important role on the hydrolytic action of particular hydrolases. The hydrolytic action of α-galactosidase depends on two main factors, which are as follows:

i. The ring structure of the substrate must be pyranoid, and

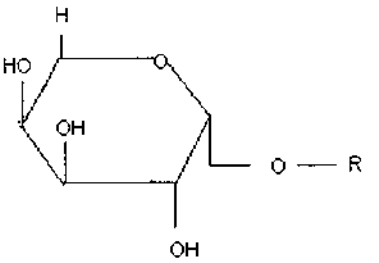
ii. The configuration of –H and –OH groups on carbon atoms 1, 2, 3 and 4 must be similar to that of α-D-galactose.

Like other carbohydrates, α-galactosidase can also tolerate changes at C-6 of glycosyl moiety of the substrate. Hence α-galactosidase from several sources has the capacity to hydrolyze β-L-arabinosides (Figure 1.1). However α-galactosidase from *Streptococcus bovis*, *Diplococcus pneumoniae* and *Calvatia cyathifomis* cannot act on arabinosides (Dey and Pridham, 1972). Dey and Pridham have reported that p-nitrophenyl- α-D-fucoside can be hydrolyzed by α-galactosidase, because p-nitrophenyl- α-D-

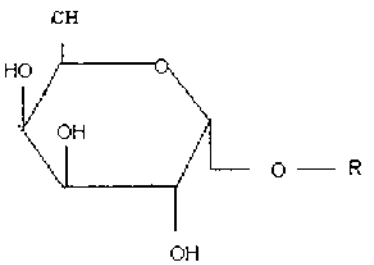
fucoside has the similar configuration with that of D-galactose (Figure 1.1). α-Galactosidase from sweet almond and yeast are not able to hydrolyze the α-isomer of D-glycero-D-glyceroheptoside (Figure 1.1).



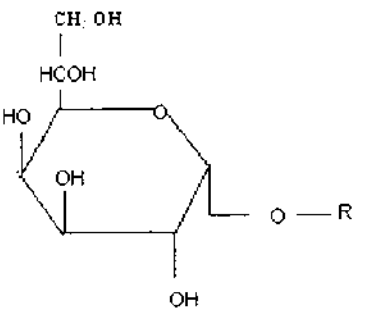
α-D-Galactoside



β-L-Arabinoside



α-D-Fucoside



D-Glycero-D-glyceroheptoside

Figure 2.7.1: α-D-galactopyranoside and related glycosides.

B. Transgalactosylase activity:

Blanchard and Albon (1950) for the first time reported the transferase properties of α-galactosidase from yeast. They found that galactose from one melibiose was transferred to a second acceptor molecule leading to the formation of mannanotriose. This transglycosylation property of α-galactosidase was studied extensively with respect to the specificity of galactosyl donor and acceptor specificity, donor and acceptor concentration, pH, temperature and source of enzyme.

Apart from hydrolase activity, α-galactosidase also exhibits transglycosylase properties, but relatively at high substrate concentrations (Ohtakara et al., 1984; Van Laere et al., 1999). Transglycosylation occurs in two steps:

1. transfer of glycon preferably α-D-galactose , from donor to enzyme, and 2. transfer of glycon from the enzyme to the acceptor.

Usually, in step 2. the glycon moiety is transferred to hydroxyl groups other than water. Thus glycosylation leads to the formation of a triple complex, ES'S, where S' is a glycoside residue from donor and S is a second substrate molecule to which S' is transferred. In transglycosylation reactions, glycon moiety from donor is accepted by hydroxyl group of methionine present at the active site of α-galactosidase from *Trichoderma reesi*. The nature of galactosidic bond [α-(1,3) or α-(1,4) or α-(1,6)] in transgalctosylation products depend on the donor and the source from which α-galactosidase is obtained. Because of competition in the transglycosylation reactions (self-condensation and condensation reactions), the end products are usually complex mixture. (Blanchard and Albon, 1950).

2.7.2 Polysaccharides:

Galactomannans are found as a major component of the endosperm in the seeds of many plants. Galactomannans consist of a backbone of β-(1,4)-linked D-mannosyl residues to which single α-(1,6)-linked D-galactosyl groups are attached. α-D-galactopyranosyl residues from galactomannan are released during seed germination and serves as energy source for the growing seedling. The removal of terminal α-D-galactopyranosyl moieties of blood group-B substances by α-galactosidase to type-O has been reported for a number of enzymes, including coffee bean α-galactosidase. (Van Laere, et al., 1999).

2.7.3 Mechanism of action:

When compared with bond fission by other glycosylases on their substrates, α-galactosidase is also believed to cleave the galactose-oxygen bonds of substrates. Nuclear magnetic resonance and polarimeter studies have clearly indicated that the anomeric configuration of the liberated galactosyl residues has the same configuration as that of its substrates. Dey has proposed two alternative mechanisms for the action of α-galactosidase i.e. two-step mechanism and one step mechanism. Chemical modification studies suggest the presence of two carboxyl groups, a tryptophan and a tyrosine, at or near active site of enzyme. Based on the foregoing results, they proposed a new mechanism in which the ionizing group is a carboxyl group present in ionized form. This carboxylate anion not only help to stabilize the carbonium ion intermediate but also would direct the neucleophilic attack on the carbonium ion from one direction so that the product retains the same configuration as the substrate. The galactose molecule can assume the half chair conformation when carbonium ion is formed, the perturbed carboxylic group that is present in the protonated form and is involved in the donation of a proton. The

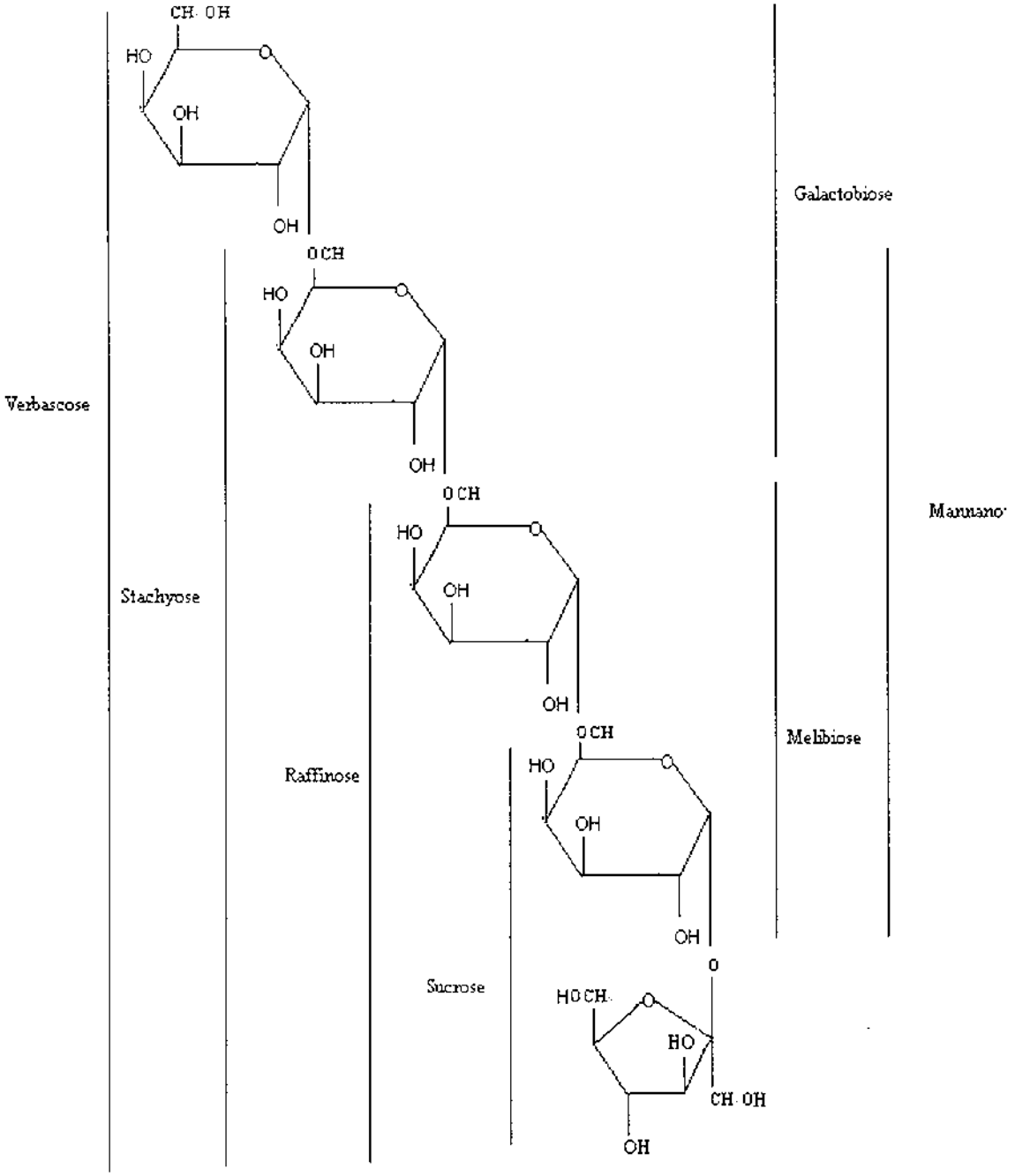


Figure 2.8.1. Raffinose-family sugars

2.9 Soy milk:

Soy milk (also called as soya milk, soybean milk, soy bean milk, soy drink, or soy beverage) is a milk-like product made from soybeans. Soy milk originated in Eastern Asia, China, a region where the soybean was native and used as food long before the existence of written records. Later on, the soybean and soybean foods were transplanted to Japan. Traditional soy milk, a stable emulsion of oil, water and protein, is simply an aqueous extract of whole soybeans. Soy milk contains about the same proportion of protein as cow's milk~ 3.5%; 2% fat, 2.9% carbohydrate and 0.5% ash.

Soy milk is nutritionally close to cow's milk, though most soy milk commercially available today contains artificially added vitamins such as vitamin B12 not naturally present. It naturally has about the same amount of protein as cow milk. Natural soy milk contains little digestable calcium as it is bound to the bean's pulp, which is insoluble in a human. To counter this, many manufacturers artificially enrich their products with calcium carbonate which can dissolve in the acid of the stomach. Notably it has little saturated fat, which many consider to be beneficial.

Soy milk is promoted as a healthy alternative to cow's milk for reasons including:

- contains fewer antibiotics, hormones, fat, cholesterol, excess protein, or links to cancer, diabetes, and other diseases
- Phytochemicals reduce the risk of cancer
- Soy protein reduces the levels of cholesterol and lessens the incidences of atherosclerosis
- Diabetes management through its ability to control blood sugar levels. However, diabetics should be aware that most brands of soymilk - even

those labelled "plain" or "organic" - are actually sweetened. Look for the word "unsweetened" on the label.

- Source of lecithin and vitamin E
- Lacks casein
- Soy milk is pareve and so may be consumed along with meat by Jews who keep kosher
- Safe for people with lactose intolerance or allergy to cow's milk
- Polyunsaturated and monounsaturated fats are good for your heart.
- Contains isoflavones, natural soy nutrients that are beneficial to health.

Using soybeans to make milk instead of raising cows is said to have ecological advantages, as the amount of soy that could be grown using the same amount of land would feed more people than if used to raise cows. Because the soybean plant is a legume, it also replenishes the nitrogen content of the soil in which it is grown.

MATERIALS & METHODS

3. Materials and Methods

3.1 Effect of activated powdered charcoal on reducing dye concentration

1. 10 mg of three different dyes (congo red, reactive orange, reactive blue) were dissolved individually in 100 ml of distilled water.
2. About 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 g activated powdered carbon was added to test tubes and to each test tube 10 ml of the dye solution was added.
3. The contents were stirred intermittently for 30 min. The test tubes were allowed to stand for 1h and the contents were filtered through Whatman filter paper no.1.
4. The optimal density in the filtrate was measured and compared with the untreated dye solution.
5. Absorbance of congo red dye - 450 nm
   Absorbance of reactive orange dye - 454 nm
   Absorbance of reactive blue dye - 600 nm

3.2 Effect of granular activated charcoal (GAC) on reducing dye concentration

1. 10 mg of three different dyes was dissolved in 100 ml of distilled water
2. 1 g of activated granular carbon was taken in separate test tubes to which 20 ml of dye was added and the contents were stirred intermittently for 30 min.
3. The test tubes were allowed to stand for 1 h and the contents were filtered through Whatman filter paper no.1.

4. The optimal density in the filtrate was measured and compared with the untreated dye solution.

3.3 Testing the ability of hot water and other organic solvents to elute the bound dyes from granular carbon

1. Commercially available activated granular carbon was washed with distilled water. The contents were stirred for 15-20 min. The contents were filtered through Whatman filter paper no.1. and the carbon retained in the filter paper was dried in a hot air oven at 120°C for 30 min to remove the moisture.
2. 20 ml of dye solution was added to a series of test tubes containing 1 g of granular carbon.
3. The test tubes were stirred for 15 min and then allowed to stand for 30 min. Then the contents were filtered through Whatman filter paper no.1.
4. The residue in the filter paper was transferred back into the test tubes to which 10 ml of hot water and other organic solvents like ethanol, methanol, ethyl acetate and acetone were added.
5. The contents were stirred intermittently for 10 min and then filtered through Whatman filter paper no.1.
6. The absorbance value in the filtrate was measured. The residue obtained was reused and the experiment was repeated thrice

3.4 Testing the effect of polyelectrolyte concentration for the removal of dyes

1. 1 g and 10 g of chitin were washed with 100 ml distilled water. The contents were mixed thoroughly and decanted.

2. 20 ml of three different dyes- CBB, Malachite green and Bromophenol blue were added individually to the chitin (1g and 10g). The mixture was stirred continuously for 15 min and then filtered through Whatman filter paper no.1.
3. The absorbance of the dye solution from 1g and10g treated were measured with the corresponding wavelengths and was compared with the untreated dye solution.

3.5 Testing the ability of hot water and other organic solvents to elute the bound dyes from polyelectrolyte (chitin)

1. Commercially available chitin was washed with distilled water. 20 ml of dye solution was added to a series of test tubes containing 1 g chitin.
2. The test tubes were stirred for 15 min and then allowed to stand for 30 min. Then the contents were filtered through Whatman filter paper no.1.
3. The residue in the filter paper was transferred back into the test tubes to which 10 ml of hot water and other organic solvents like ethanol, methanol, ethyl acetate and acetone were added.
4. The contents were stirred intermittently for 10 min and then filtered through Whatman filter paper no.1.
5. The absorbance value in the filtrate was measured. The residue obtained was reused and the experiment was repeated thrice

3.6 Partial purification, characterization and applications of α-galactosidase from *P.eous*

3.6.1 Source and enzyme extraction

The white-rot fungus *Pleurotos eous* was procured from the Department of Mushroom, TNAU, Coimbatore, Tamil Nadu. Whole fungi was ground to a fine paste using mortar and pestle .The resultant fine paste was dissolved in acetate buffer (0.2 M, pH 5.0) and was allowed for sedimentation. The supernatant was centrifuged at 5000 rpm and 15°C for 15 min. The supernatant served as source of enzyme and stored at 4°C.

3.6.2 Assay of α-galactosidase

1. Assay of α-galactosidase was carried out by incubating 100 µl of a diluted enzyme, 800 µl of acetate buffer (0.2M, pH 5), 100µl of 0.2 M PNPG for 15 min at 55°C.
2. The reaction was stopped by the addition of 3 ml of 0.2 M Na_2CO_3 and the absorbance was measured at 405 nm.
3. One unit of enzyme activity is defined as the amount that liberated one micromole of product per min under assay conditions.

3.7 Purification of α-galactosidase

3.7.1 Ammonium sulphate fractionation

1. The supernatant was brought to 0-50 percent saturation of ammonium sulphate by the addition of required quantity of the salt (29.5g/100ml) and stirring continuously on ice for three hours.

2. The solution was centrifuged (10000 rpm, 10 minutes, 4°C), the supernatant was saved and brought from 50 to 75 percent saturation (16.1g ammonium sulphate/100ml).
3. The insoluble proteins were collected by centrifugation (10000 rpm, 15 minutes, 4°C) and dissolved in minimum volume of 0.2 M acetate buffer pH 5.0.

3.7.2. Dialysis

1. The dialysis tubing was cut into pieces of convenient length (10-20cm).
2. The tubes were boiled for ten minutes in larger volumes of 10% sodium bicarbonate and 1mM EDTA and rinsed with double distilled water.
3. The tubes were allowed to cool and stored at 5°C, care was taken to see that tubes were always submerged in 30% alcohol.
4. Before use, the tubes were washed inside and outside with double distilled water. The tubes were always handled with gloves.
5. Then the enzyme is poured into the bag and it is submerged into phosphate buffer pH 5.7 (0.1M) and the bag was kept at 4°C for overnight.

3.8 Characterization of partially purified enzyme

3.8.1 Effect of pH on α-galactosidase activity

For pH studies using PNPG as substrate, the reaction mixture contained 100 µl crude supernatant, 100 µl of 0.2 M PNPG and 800 µl of 0.2 M buffer at different pH values of [(acetate buffer 3.5, 4.0, 4.5, 5.0, 5.5) and (phosphate buffer 6.0, 6.5, 7.0) and (Tris-HCl buffer 7.5 and 8.0)]. The substrate blank consists of 100 µl 0.2 M PNPG and 900 µl of appropriate

buffer. The contents were mixed well and incubated at 55°C for 15 minutes. The color formed was spectrophotometrically read at 405 nm using Beckman Spectrophotometer.

3.8.2 pH stability

The pH stability tests were performed by incubating the enzyme at pH 5 and 6 for 1 h. At 15 min. interval 0.1ml of enzyme was withdrawn and its activity was measured under optimal conditions

3.8.3 Effect of temperature on α-galactosidase activity

For temperature studies using PNPG as substrate, the reaction mixture contained 100 µl crude supernatant, 100 µl of 0.2 M PNPG and 800 µl of 0.2 M acetate buffer at pH 5.0 were incubated at different temperature 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C for 15 minutes. The color formed was spectrophotometrically read at 405 nm using Beckman Spectrophotometer.

3.8.4 Thermal Stability

Thermal stability tests was performed by measuring the absorbance at intervals of 15 min for enzyme placed in varying temperatures ranging from 55°C to 65°C for a total of 1 hr.

3.8.5 Effect of metal ions , sugars and reagents

Enzyme assays were performed in the presence of various metal ions(1 mM and 5 mM) such as K^+,Ca^{2+},Cu^{2+},Cd^{2+} and Fe^{3+} as chloride salts, Ag^+ as nitrate salt, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+} as sulphate salts. Sugars such as fructose, lactose, sucrose, glucose, galactose, raffinose, ribose and xylose

(final concentration 10 mM). Some reagents (sodium azide, EDTA, 1,10-phenanthroline, N-bromosuccinimide and PMSF, final concentration indicated) were incubated for 15 min at 55°C and α-galactosidase activity was determined after adding 0.1ml of 10mM α-PNPG. The activity was expressed as a percentage of the activity level in the absence of the compound.

3.9 Protein estimation using Folin's Ciocalteau reagent

1. Pipetted out 0.2 ml of the working standard solution (concentration varying from 40-200 microgram) into a series of test tubes.
2. Pipetted out 0.4 ml of the unknown sample to a test tube.
3. To all the test tubes added distilled water to make up the volume to 1 ml. Took 1ml of distilled water in another test tube to serve as blank.
4. Pipetted out 2.1 ml of alkaline copper reagent into all the test tubes and incubated at room temperature for 10 minutes.
5. Then added 0.2 ml of FCR to all the tubes and kept at room temperature for 20 minutes.
6. Measured the blue color developed spectrophotometrically at 660 nm.The intensity of the color developed was directly proportional to the amount of protein present in it.

3.10 Raffinose calibration curve:

A standard calibration curve of raffinose was constructed according to the method of Tanaka et al. (1970). A standard raffinose solution was prepared with a concentration of 1mg/2ml of distilled water, different volumes of raffinose solution 0.2, 0.4, 0.6, 0.8 and 1 ml was taken in a series of test tubes and the volume was made up to 1 ml with distilled water to each

tube. One ml of 0.2 M thiobarbituric acid and 1 ml of concentrated HCl were added and tubes were kept in boiling water bath for exactly 6 minutes. Then, the test tubes were kept in ice-cold water. The optical density of yellow color formed was measured at 435nm against blank in spectrophotometer. A graph was drawn.

3.11 Kinetic studies (K_m and V_{max}):

α-Galactosidase assay was performed by varying the concentrations of PNPG from 10 µl to 100 µl. The absorbance was measured at 405nm and readings were tabulated.

3.12 Applications of α-galactosidase :

3.12.1 Preparation of soymilk.

Soymilk was prepared by boiling 5g of whole dry seeds of soyabean in 100mL of distilled water for 60min to soften it and was allowed to attain room temperature. The boiled seeds were ground to fine paste in mortar and pestle and about 125mL of distilled water was added to the above paste, stirred thoroughly. The soymilk was obtained by filtering the above mixture. The filtrate was centrifuged at 6000rpm for 15min and the supernatant was used for enzymatic (α-galactosidase) treatment.

3.12.2 Treatment of soymilk by crude α-galactosidase:

The enzymatic treatment of soymilk was performed as follows: 2ml of soymilk and 1 ml of α-galactosidase was taken and incubated at 55°C for 2 hours. Samples were collected at different time intervals (30 min, 1hr, 1hr 30 min and 2 hrs). The samples collected at different intervals was arrested by adding 0.2ml of 0.2M $ZnSO_4$ and 0.18M $Ba(OH)_2$. The protein

precipitate formed was removed by centrifugation. The amount of reducing sugars present in the supernatant was determined by DNS method.

RESULTS & DISCUSSION

4. Results and discussion

4.1 Dye degradation using powdered charcoal

The dye concentration decreases with an increase in activated charcoal concentration.

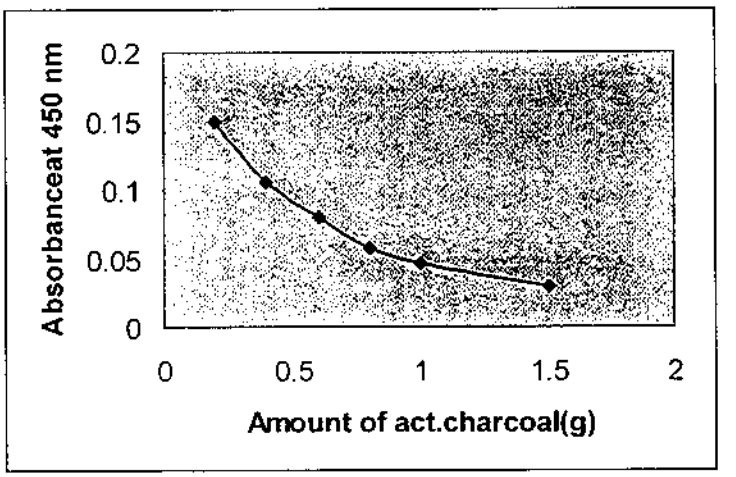


Fig 4.1.1: Removal of congo red dye using powdered charcoal

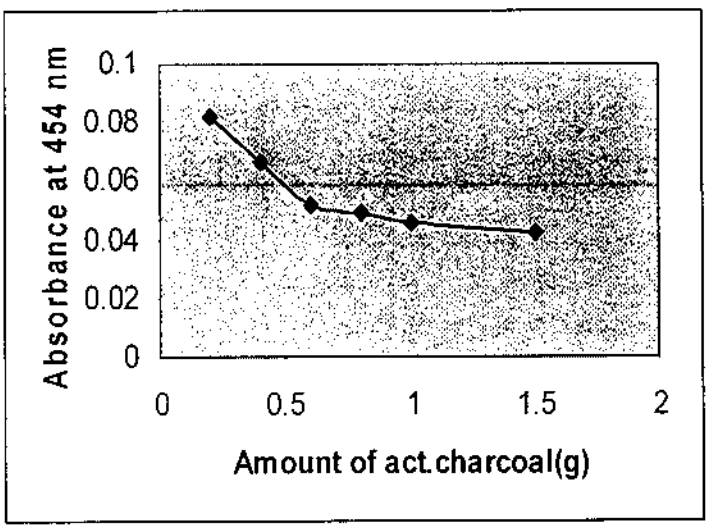


Fig 4.1.2: Removal of reactive orange dye using powdered charcoal

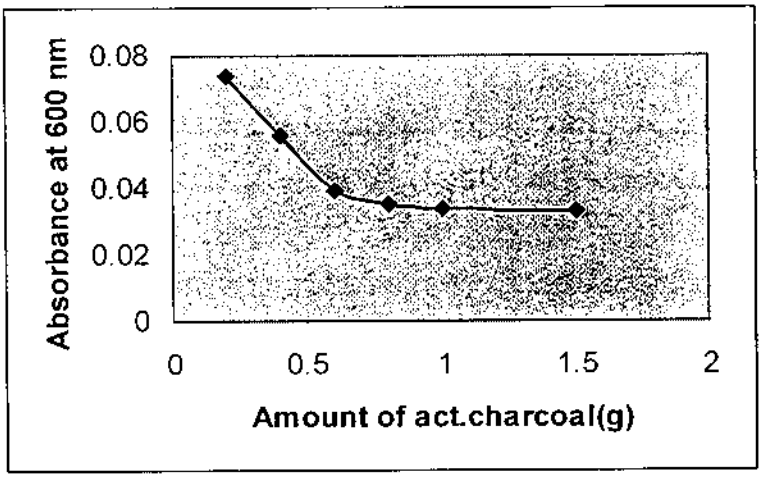


Fig 4.1.3: Removal of reactive blue dye using powdered charcoal

4.2 Dye degradation using Granular activated carbon

The dye concentration decreases with increase in activated granular carbon concentration.

TABLE 4.2.1:
CONGO RED DYE:

ACTIVATED GRANULAR CARBON(g)	OD AT 450 nm
Untreated	0.535
1.0	0.143

% reduction- 73.27%

TABLE 4.2.2:
REACTIVE ORANGE DYE:

ACTIVATED GRANULAR CARBON(g)	OD AT 454 nm
Untreated	0.103
1.0	0.079

% reduction- 23.30%

TABLE 4.2.3:
REACTIVE BLUE DYE:

ACTIVATED GRANULAR CARBON(g)	OD AT 600 nm
Untreated	0.073
1.0	0.028

% reduction- 61.64%

4.3 Ability of hot water and other organic solvents to elute the bound dyes from granular carbon

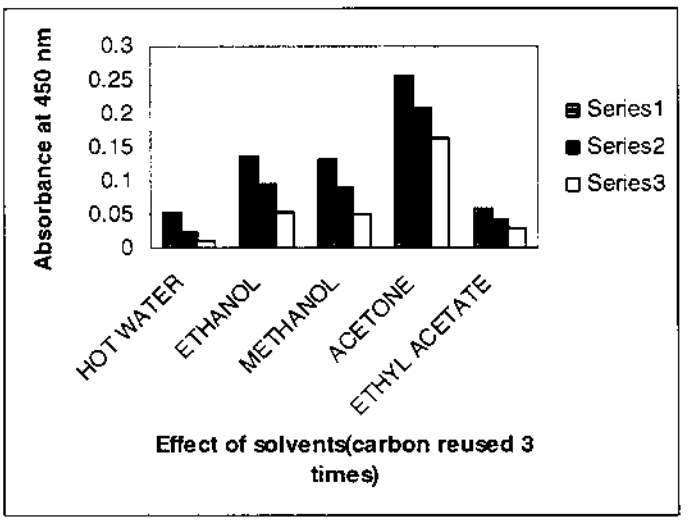


Fig 4.3.1: Ability of hot water and other organic solvents to elute the bound dyes (congo red) from granular carbon

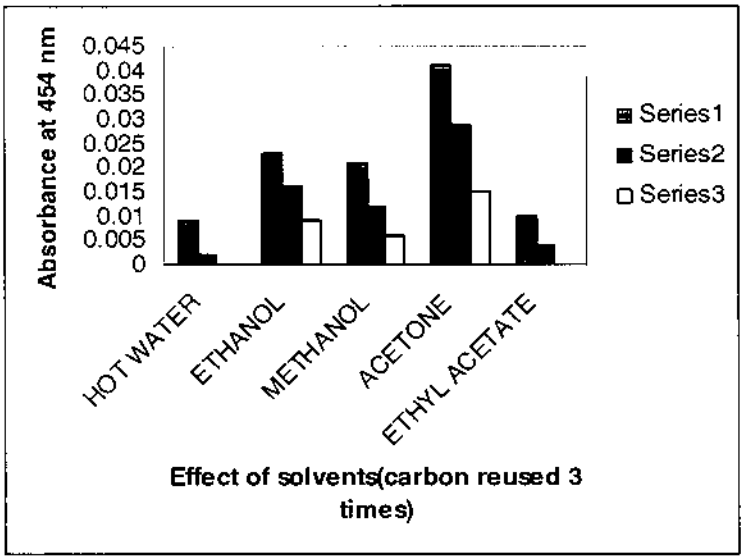


Fig 4.3.2: Ability of hot water and other organic solvents to elute the bound dyes (reactive orange) from granular carbon

It was observed from Fig 4.3.1 and 4.3.2 that the hot water and organic solvents eluted the bound dyes from activated granular carbon. It can be inferred that hot water and ethyl acetate had less effect, ethanol and methanol had medium effect while acetone had very high effect in eluting bound dyes from activated granular carbon.

4.4 Effect of polyelectrolyte concentration for the removal of dyes

TABLE 4.4.1: Testing the effect of polyelectrolyte concentration for the removal of dyes

DYES USED	UNTREATED	1 g CHITIN ADDED	10 g CHITIN ADDED
Coomassie Brilliant blue	0.466	0.020	0.017
Malachite Green	2.857	0.290	0.270
Bromophenol blue	2.849	0.876	0.847

It is clear from table 4.4.1 that addition of polyelectrolyte (chitin) to the dye solution decreased the dye concentration. It is also observed that percentage of removal of dye increases with an increase in chitin concentration.

4.5 Ability of hot water and other organic solvents to elute the bound dyes from polyelectrolyte (Chitin)

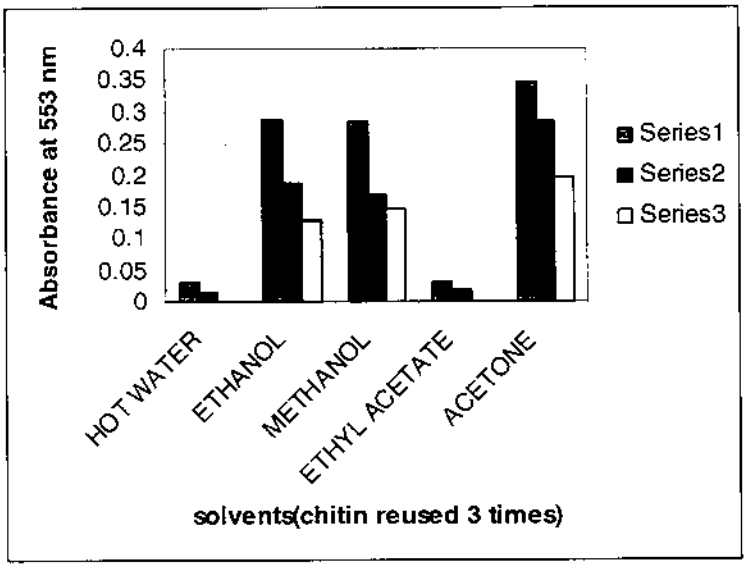


Fig 4.5.1: Ability of hot water and other organic solvents to elute the bound dyes (CBB) from polyelectrolyte (Chitin).

From Fig 4.5.1 it can be inferred that hot water and ethyl acetate had less effect, ethanol and methanol has medium effect while acetone had very high effect in eluting bound dyes from chitin. From the last row readings (supernatant), it was inferred that the concentration of dye adsorbed by chitin decreases gradually by repeated use. Hence, the absorbance value of supernatant increases respectively.

4.6 α-galactosidase assay

Enzyme assay was performed as mentioned in Chapter 3. (Materials and Methods). *Pleurotus eous* crude filtrates showed high activity of α-galactosidase (4.18 U ml^{-1}).

4.7 Estimation of protein

Protein content of the sample was estimated by Lowry's method. The concentration was found to be 200 mg/100 ml.

4.8 Purification of enzyme

α-galactosidase was partially purified from culture filtrate *P.eous*. using ammonium sulphate fractionation.

4.8.1 Culture filtrate

The culture filtrate served as a source of enzyme for the purification steps crude enzyme extract from the *Pleurotus eous* had a specific activity of 2.09 Units mg^{-1}.

4.8.2 Ammonium Sulphate fractionation

The crude extract was subjected to ammonium sulphate fractionation. Differential fractionation of protein in the crude extract was done by a range of ammonium sulphate fractionations (50% and 75%). The precipitates of each fraction were tested for α-galactosidase activity. In the precipitate α-galactosidase activity reached a maximum in the 75% saturation. Ammonium sulphate fractionation yielded a α-galactosidase preparation having 2.96 folds higher specific activity over the crude extract. The enzyme yield after ammonium sulphate precipitation was 25 %. (Table 4.8.1)

Table 4.8.1: Purification of α-galactosidase from *Pleurotus eous*

Enzyme fraction	Total volume (ml)	Protein conc. (mg)	Activity (Units)	Specific activity (Units/mg)	Enzyme Yield (%)	Purification (fold)
Culture filtrate	100	200	418	2.09	100.0	1.00
Ammonium sulphate precipitaton	30	17	105.25	6.19	25.11	2.96

4.9 Characterization of α-galactosidase

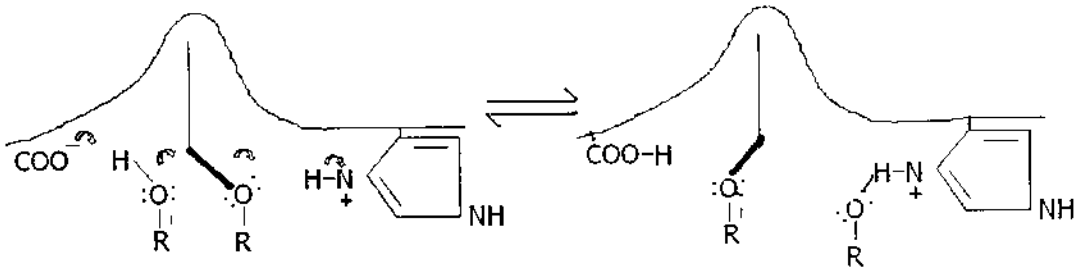
4.9.1 Effect of pH on α-galactosidase activity

The α-galactosidase enzyme activity assays was carried out at pH range from pH 3.5 to 7.5, using PNPG as the substrate. The optimum pH for α-galactosidase activity was found to be at pH 5.0. (fig 4.9.1.1)

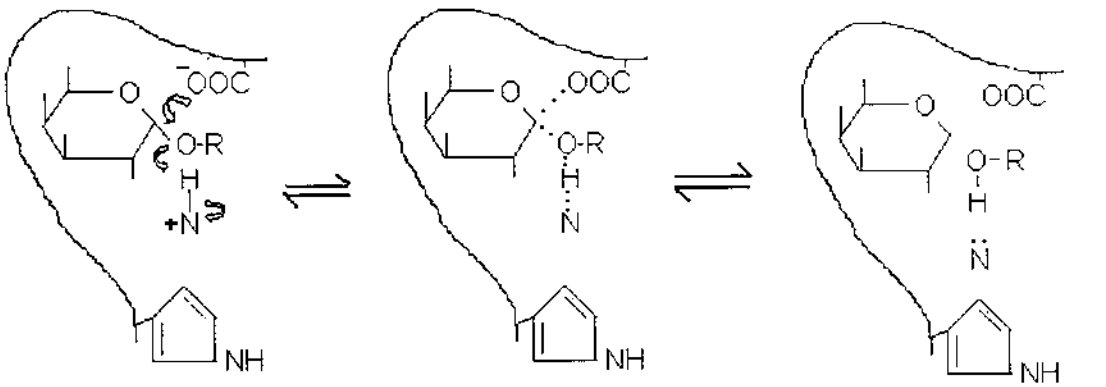
TABLE 4.9.1.1: Effect of pH on α-galactosidase activity from *P.eous*

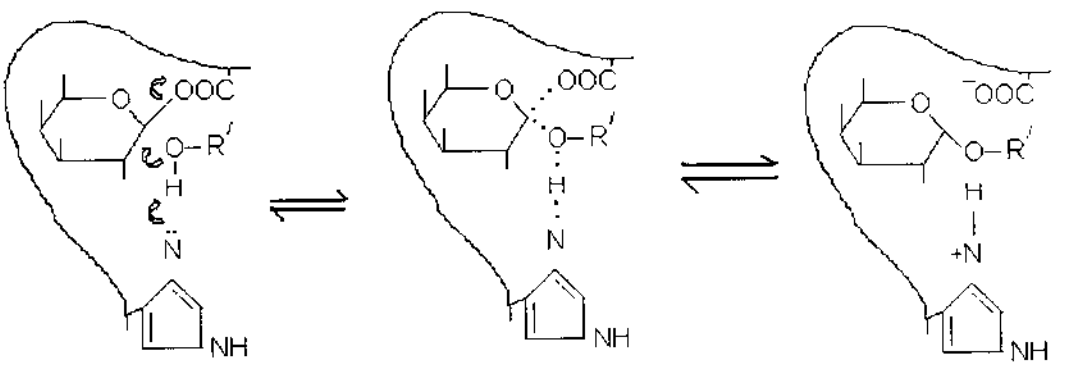
pH	RELATIVE ACTIVITY (%)
3.5	89.6
4	94.1
4.5	97.2
5	100
5.5	89.0
6	80.3

carboxyl group is perturbed due to the hydrophobic environment produced by the presence of tryptophan and tyrosine residues in its vicinity.



A. One-step mechanism





B. Two-step mechanism

Figure 2.7.3.1 Mechanism of action of α-galactosidase.

2.8 Raffinose-family sugars:

The raffinose-family of oligosaccharides (RFO) are alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide, raffinose, the tetrasaccharide, stachyose and the pentasaccharide, verbascose. The RFO's are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to sucrose in abundance as soluble carbohydrates.

Humans and other monogastric animals (pigs and poultry) do not possess the alpha-galactosidase enzyme to break down these RFO and the oligosaccharides pass undigested through the stomach and upper intestine. In the lower intestine they are fermented by gas-producing bacteria that produce carbon dioxide, methane or hydrogen. Raffinose is broken down by the intestinal bacteria with the help of the enzyme α-galactosidase.

2.8.1 Stachyose

Stachyose [O-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-2)-β-D-fructofuranoside] was first isolated from the rhizomes of Stachys tubifera. Stachyose is an oligosaccharide (trisaccharide) consisting of two D-galactose units and one sucrose sequentially linked Its molecular weight is 666.6 g/mol. Stachyose is naturally found in many vegetables (e.g. green beans, soybeans and other beans) and plants.

Stachyose is less sweet than sucrose, with about 28% on a weight basis. It is mainly used as a bulk sweetener or for its functional oligosaccharide property. Stachyose is not completely digestible by humans

Raffinose:

Raffinose is a complex carbohydrate. It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. Raffinose is hydrolysed to D-galactose and sucrose by α-galactosidase (α-GAL) (1). α-GAL also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol [1-O-(α-D-galactosyl)-myoinositol], if present. The enzyme does not cleave β-linked galactose, as in lactose.

Raffinose is also known as melibiose and may be thought of as galactose + sucrose connected via an alpha-(1-6)-glycosidic linkage and so raffinose can be broken apart into galactose and sucrose via the enzyme alpha-galactosidase. Human intestines do not contain this enzyme.

2.8.2. Verbascose:

Verbascose [O-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl-(1-2)-β-D-fructofuranoside] was first isolated from the roots of Verbascum thapus by Bourquelot and Bridel (1910). In vitro experiment using an enzyme preperation from the mature seeds of Vicia faba demonstrated the transfer of galactinol to stachyose yielding verbascose. When raffinose is galactose acceptor, stachyose if formed. The synthesis of verbascose is inhibited to greater extent by raffinose.

6.5	74.9
7	0.00
7.5	0.00

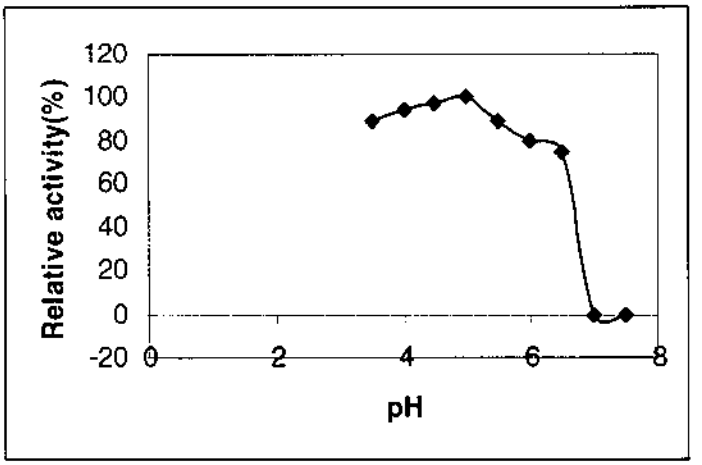


Fig 4.9.1.1: Effect of pH on α-galactosidase activity from *P.eous*

4.9.2 pH stability

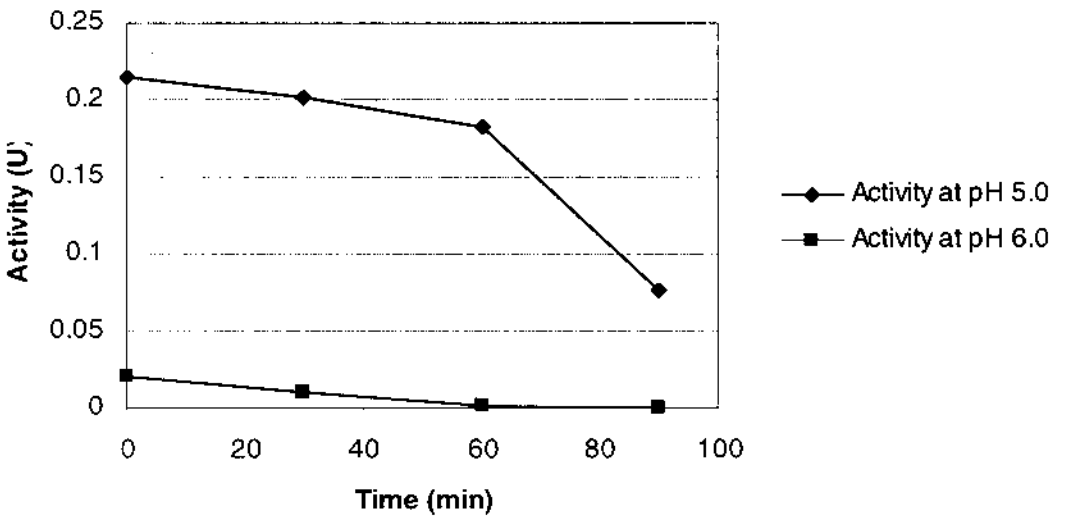


Fig.4.9.2.1 : Effect of pH on the stability α-galactosidase from *P.eous*

4.9.3 Effect of temperature on α-galactosidase activity

The α-galactosidase enzyme activity assays was carried out at a temperature range from 10°C-65°C, using PNPG as the substrate. The optimum temperature for α-galactosidase activity was found to be at 55°C.(fig 4.9.3.1)

TABLE 4.9.3.1: Effect of temperature on α-galactosidase activity from *P.eous*

TEMPERATURE (°C)	RELATIVE ACTIVITY (%)
10	26.9
15	28.5
20	33.3
25	40.8
30	48.4
35	61.3
40	73.8
45	82.9
50	89.8
55	100
60	91.6
65	74.4

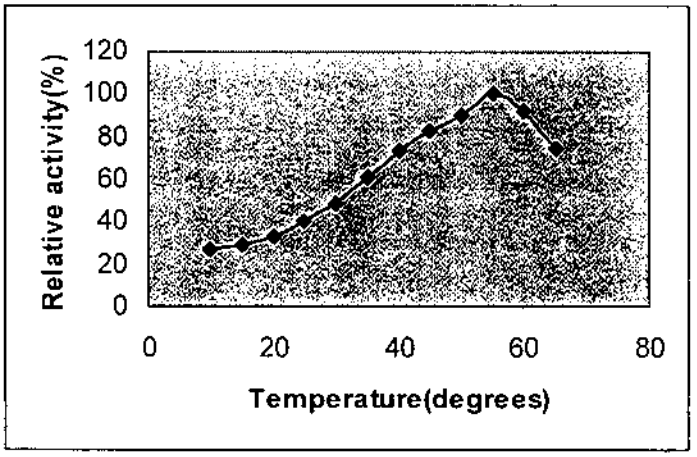


Figure 4.9.3.1: Effect of temperature on α-galactosidase activity from *P.eous*

4.9.4 Thermal stability

Thermal stability tests were done by measuring the absorbance at 15 min time intervals by placing the enzyme at 55°C and 65°C for 1h. The enzyme was thermo stable, i.e., 30% of its original activity is retained at the end of 1 h at 55°C.

Table 4.9.4.1 Thermal stability at 55°C

Time (min)	Enzyme activity (U/ml)
0	38.77
15	20
30	15
45	10.55
60	8.33

Table 4.9.4.2. Thermal stability at 65°C

Time (min)	Enzyme activity (U/ml)
0	38.88
15	2.66
30	1.11
45	0.55
60	0

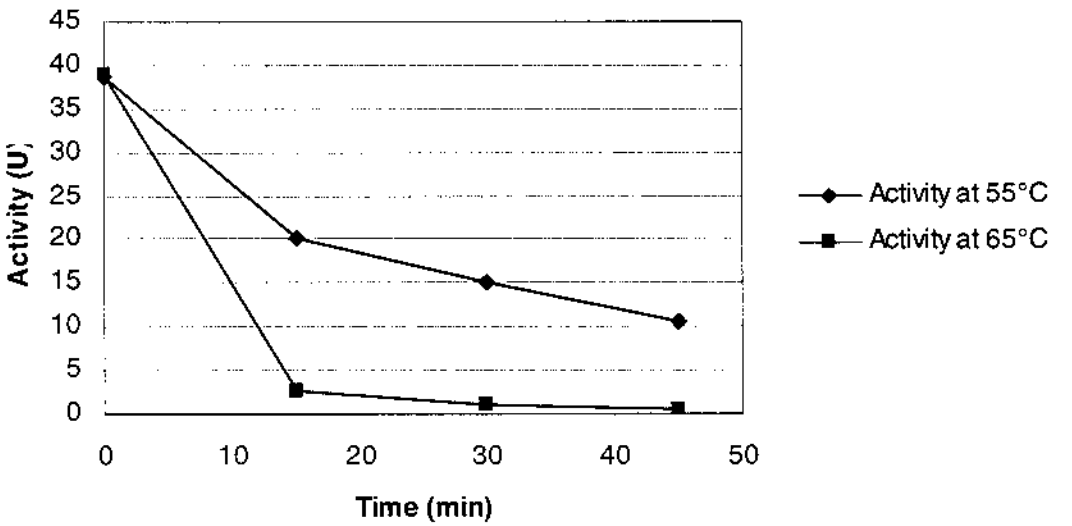


Figure 4.9.4.1. Thermal stability at 55°C and 65°C

4.9.5 Effect of metal ions on α-galactosidase activity

Table 4.9.5.1: Effect of metal ions on α-galactosidase activity

METAL IONS	RESIDUAL ACTIVITY (5 mM) %	RESIDUAL ACTIVITY (10 mM) %
Control	100	100
$CuSO_4$	95.9	94.2
$CdCl_2$	99.5	103
$FeSO_4$	95.9	102
$ZnSO_4$	98.3	103
$MgSO_4$	99	102.6
$NiSO_4$	106.8	114.9
$CuCl_2$	98.7	90.7
$CaCl_2$	106.4	114.9
$FeCl_3$	106	114.4
KCl	106	114
$AgNO_3$	52.2	32.0

It is evident that as Mg^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+} had no effect on α-galactosidase activity, whereas K^{+}, Ca^{2+}, Ni^{2+} and Fe^{3+} slightly enhanced the enzyme activity. However, the enzyme activity was inhibited up to 70% upon the addition of Ag^{2+}.

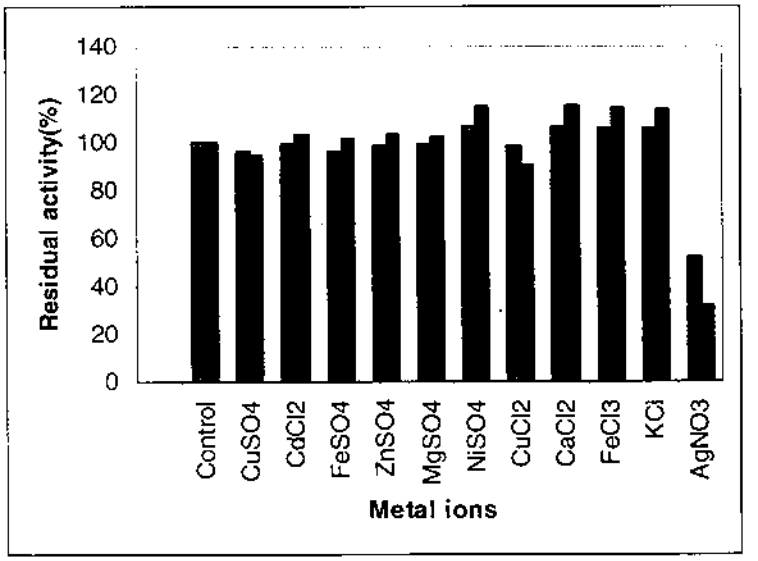


Fig 4.9.5.1: Effect of metal ions on α-galactosidase activity

4.9.6 Effect of sugars on α-galactosidase activity from *P.eous*

Table 4.9.6.1: Effect of sugars on α-galactosidase activity from *P.eous*

SUGARS	RESIDUAL ACTIVITY %
Control	100
Fructose	100
Lactose	100
Sucrose	100
Glucose	98.7
Galactose	95.9
Raffinose	93.0
Ribose	92.6
Xylose	97

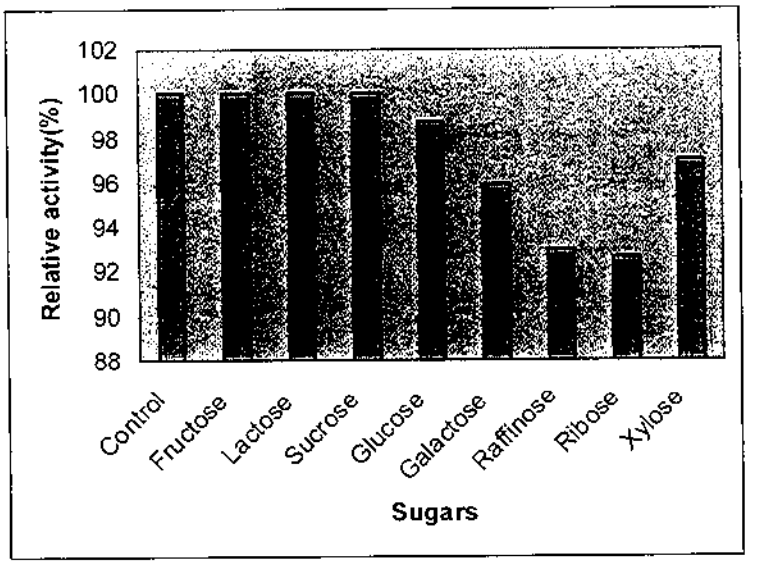


Fig 4.9.6.1: Effect of sugars on α-galactosidase activity from *P.eous*

4.9.7 Effect of reagents on α-galactosidase activity from *P.eous*

Table 4.9.7.1: Effect of reagents on α-galactosidase activity from *P.eous*

Reagent	Activity (U/ml)
EDTA	1.35
Sodium azide	1.37
N-Bromosuccinamide	0.211
1,10-Phenananthroline	1.365
Control	1.347

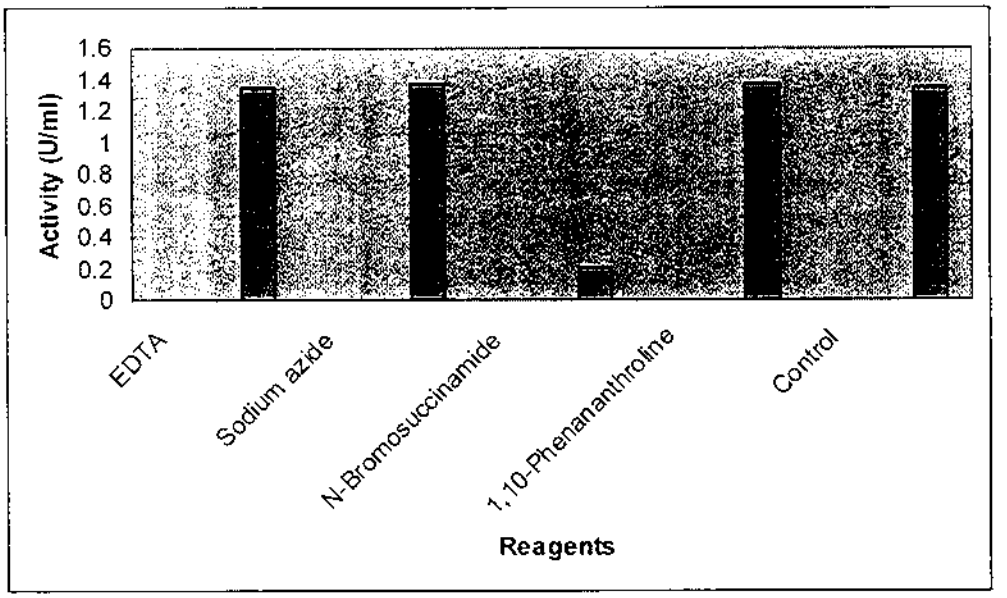


Fig 4.9.7.1: Effect of reagents on α-galactosidase activity from *P.eous*

4.10 Kinetic studies

The Km and V_{max} of α-galactosidase from *P.eous* for p-nitrophenyl-α-D-galactopyranoside was found to be 1.7 mM and 3.8 , respectively.

Table 4.10.1 Determination of Km and Vmax for α-PNPG

Substrate concentration, S (mM)	Activity (U)	1/S	1/V
0.33	0.023	3.03	42.57
0.66	0.030	1.51	32.26
0.99	0.037	1.06	26.37

1.32	0.043	0.75	22.97
1.65	0.047	0.60	21.15
1.99	0.057	0.5	17.38
2.33	0.062	0.42	16.05
2.66	0.069	0.37	14.304
2.99	0.075	0.33	13.26
3.33	0.085	0.3	11.67

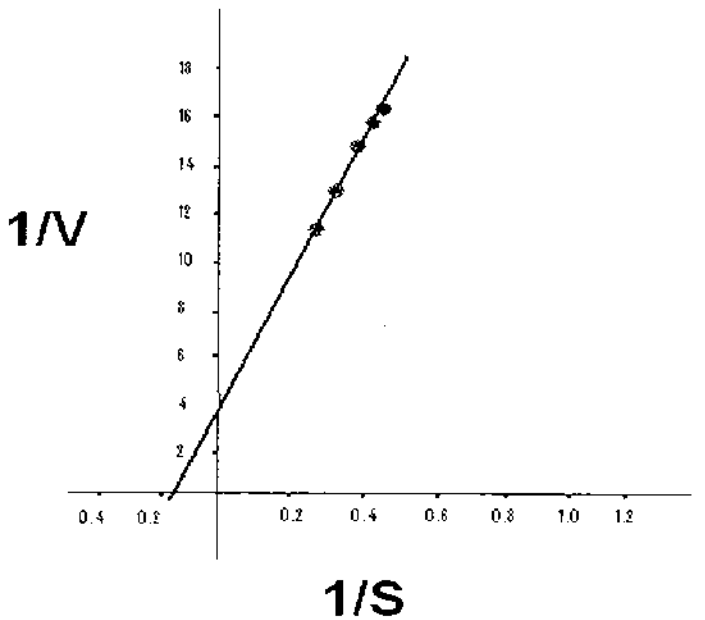


Fig 4.10.1 Determination of Km and Vmax for α-PNPG

4.11 Application:

4.11.1 Treatment of soymilk by α-galactosidase:

By estimating the amount of reducing sugar present in the soymilk at different time intervals by DNS method showed gradual increase in the amount of reducing sugar, thus indicating that raffinose and stachyose present in the soymilk are degraded to reducing sugars by α-galactosidase. The table 4.11.1 shows the increase in amount of reducing sugar.

Table 4.11.1.1 Treatment of soymilk by α-galactosidase

Time(min)	Concentration of Galactose liberated (mg/ml)
0	0.00
5	0.004
10	0.012
15	0.084
20	0.130
25	0.140
30	0.490
60	1.34
90	1.72

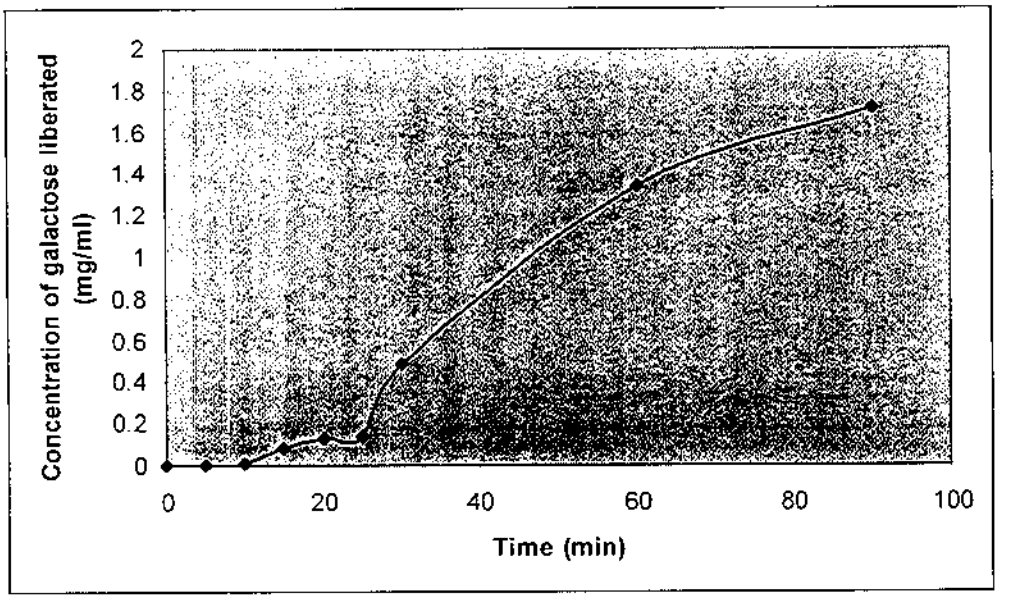


Fig 4.11.1.1 Treatment of soymilk by α-galactosidase

4.11.2 Raffinose hydrolysis

Table 4.11.2.1 Raffinose hydrolysis

Time(min)	Concentration of Galactose liberated (mg/ml)
0	0.00
10	0.03
20	0.07
30	0.11
40	0.14
50	0.14
60	0.14

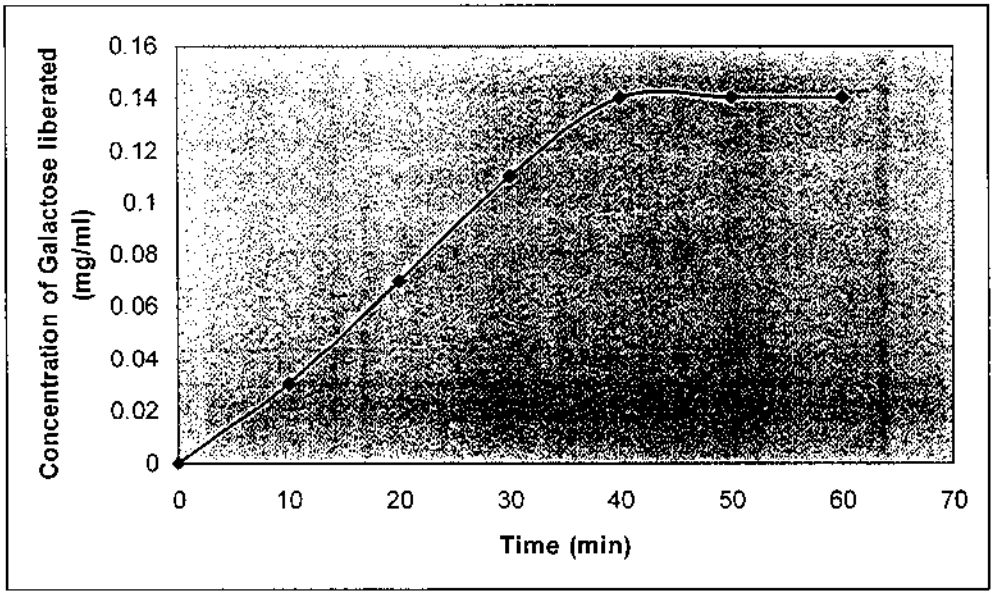


Fig 4.11.2.1 Raffinose hydrolysis

CONCLUSION

5. Conclusion

Indiscriminate disposal of industrial effluents into water bodies posses a major threat to the environment. Triphenyl methane dyes are the largest and most versatile class of dyes, which share more than 50% of the dyes produced annually. The most often used processes for the color removal are based on chemical or physical-chemical treatments. The two physico chemical methods used are 1. To reduce dye concentration in the textile effluent using activated powdered and granular carbon and 2. To check the ability of polyelectrolyte to reduce the dye concentration in the textile effluent.

The α-galactosidase from *P. eous was* partially purified by ammonium sulphate precipitation and dialysis. The properties of partially purified enzyme such as optimum pH, optimum temperature, pH and thermal stability, effect of metal ions, sugars and reagents were studied. The enzyme has the ability to degrade raffinose-family sugars in soymilk. The presence of raffinose-family sugars and the beany-flavour makes soymilk unfit to human consumption. The soluble form of α-galactosidase from *P.eous* was found to degrade the raffinose-family sugars. Since *P.eous* is a GRAS (generally regarded as safe) state organism, it will be more suitable for human consumption. Thus, α-galactosidase from *P. eous* can be used in various food processing industries to improve the nutritive value of food products..

APPENDICES

APPENDIX I

Acetate Buffer

Stock Solutions

A: 0.2M solution of acetic acid (11.55ml in 1000ml)

B: 0.2 M solution of sodium acetate (16.4g in 1000 ml)

"x" ml of solution A and "y" ml of B is diluted to 100ml with distilled water.

X	Y	pH
48.2	1.8	3.2
46.3	3.7	3.6
44	6	3.8
41	9	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20	30	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6
3.6	45.4	5.8
2.4	47.6	6.0

Phosphate Buffer

Stock Solutions

A: 0.2M solution of monobasic sodium phosphate (27.8g in 1000ml)

B: 0.2M solution of dibasic sodium phosphate (53.65g of disodium hydrogen phosphate in 1000ml)

X ml of A, Yml of B diluted to a total of 200ml.

X	Y	pH
87.7	12.3	6.0
68.5	31.5	6.5
39	61	7.0

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