ABSTRACT

SOHAIL, MIAN SAJEEL. Enzyme Facilitated Design on Denim. (Under the direction of Dr. Harold S. Freeman).

Trousers made from denim fabric, which are also known as jeans, were invented by Levis Strauss in 1853. Denim fabric was developed for miners but since then it has turned into a worldwide phenomenon. Companies are making jackets, shoes and other accessories using denim fabric. The denim market is growing every year and the fashion options are constantly changing. Denim manufacturers must address the requirements of rapidly changing fashions in textile products and competition from other textiles in order to be sustainable.

While there are numerous mechanical and chemical treatments applied to denim to create different looks, all of the wash down effects are done in haphazard ways and there is a lot of variation in the final product. The wash down can be achieved by decolorizing indigo using agents such as enzymes.

The purpose of this research was to make a design on denim fabric by indigo decolorization using an enzyme. The ultimate goal was to develop a method that is cost effective and repeatable. To achieve this goal cellulase and laccase enzymes were used in the denim wash down process.

Hydrophobic resist materials such as polyethylene, stearic acid and paraffin wax were used to make a design on denim. Designs obtained by applying a polyethylene non-woven film with

a cut-out design were sharpest and provided maximum contrast between decolorized and untreated indigo.

During the course of this research, isatin and anthranilic acids were identified as degradation products of indigo following treatments by laccase enzyme.

Enzyme Facilitated Design on Denim

by
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DEDICATION

I would like to dedicate this work to my parents, Mian Sohail Sarwar and Nasreen Sohail. With them beside me it is possible for me to succeed in everything I attempt.

BIOGRAPHY

Mian Sajeel Sohail was born in Karachi, Pakistan. He completed his Bachelor's degree in Textile Science from Textile Institute of Pakistan located in Karachi, Pakistan in June 2007. He was awarded Fulbright Scholarship in 2007 and has been pursuing his Master's at the College of Textiles since August 2009.

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1. Introduction

When denim was invented by Levis Strauss in the late 1800's for miners, no one would have thought that it will become such a style phenomenon. Jeans, which is a more popular name of denim pants, has evolved into a product that caters to every age. The denim industry is growing so fast every year that there was a 4.6% increase of denim imported to United States during the period 2009 to 2010 (1). It is surviving because of innovative finishes, patterns and easy care.

Usually denim fabric is used for pants and the wash down effects applied to it are usually haphazard. In making patterns on denim, the existing methods are often detrimental to the fiber and are not cost effective.

2. Literature Review

2.1 Enzymes

2.1.1 History of enzymes

The term enzyme was first used by Kuhne in 1878 and comes from the Greek word "enzume" which means "in yeast" (2). In textiles, the earliest use occurred in 1857 when starch sized cloth was soaked with liquor containing barley. Ever since, enzymes are being used in almost all types of textile wet processing.

2.1.2 What are enzymes?

Enzymes are a kind of catalyst. There are two types of catalysts:

1. Chemical catalysts
2. Bio catalysts

The main functions of catalysts are:

- To accelerate chemical reactions
- To lower the activation energy needed for a reaction to occur (i.e. to reduce the magnitude of the energy barrier required to overcome for a substance to be chemically converted into another)

Enzymes can be classified as bio catalysts. They are "high molecular weight proteins produced by living organisms to catalyze the chemical reactions essential for the organism's survival" (3).

2.1.3 Structure of enzymes

Enzymes are proteins that consist of one or more polypeptide chains. Each polypeptide is a chain of amino acid linked together by peptide bonds.

Amino acids are organic molecules that have a central carbon (C), at least one amino group (-NH_2), at least one carboxyl acid group (-COOH) and a side chain (R group) that makes each amino acid unique (4). Figure 2.1 shows the basic structure of an amino acid. In polypeptides, the sequence of amino acids is determined by studying gene code (5) which is different for each polypeptide (6). A three dimensional structure is formed when polypeptide chains are synthesized. They fold up to form a three dimensional structure which is determined by their amino acid sequences. Enzymes functionality and catalytic power is dependent on the three dimensional structure of enzyme and its active site. The enzyme active site is a "three dimensional hole in the protein where the reactant can interact optimally with one of the amino acid side chains" (2). Factors such as pH, heat and chemicals can affect the polypeptide conformation in a negative way. There is a clear dependence of enzyme functionality upon protein structure. Microbial enzymes vary in length from 100 to 500 amino acids and typically have a molecular weight ranging from 25,000 to 50,000 Da.

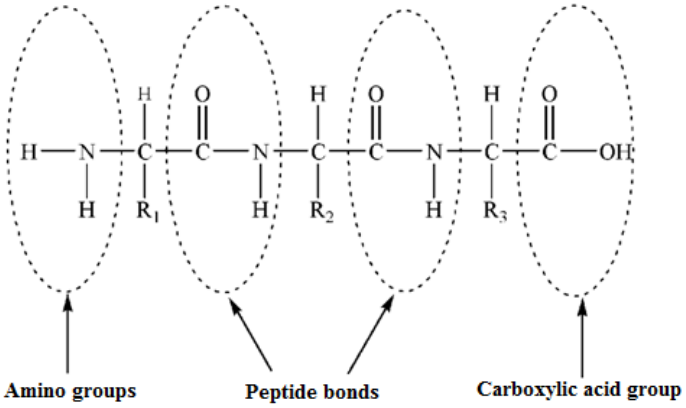


Figure 2.1. Basic structure of amino acids.

2.1.4 Types of enzymes

There are six main groups of enzymes, each having fundamentally different activities (7):

2.1.4.1 Hydrolases

Hydrolases break down proteins, carbohydrates, and fats such as during the process of digestion. They catalyze the cleavage of bonds such as C-O, C-N, C-C. Hydrolases catalyze these reactions by the addition of water (2). The carboxypeptidase enzyme hydrolyzes the first peptide or amide bond at the carboxyl or C-terminal end of proteins and peptides. It has a stronger preference for those amino acids that have aromatic or branched hydrocarbon chains (8).

2.1.4.2 Isomerases

As the name suggests, isomerases catalyze structural or geometrical changes within a molecule. An example of isomerases enzyme is maleate isomerase which catalyzes the

conversion of maleate to fumarate (9) (see Figure 2.2).

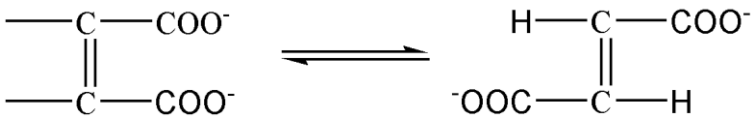


Figure 2.2. Conversion of maleate into fumarate.

2.1.4.3 Ligases

Ligases catalyze the formation of a chemical bond between two large substrate molecules through the use of an energy source (7). Example of ligase is ubiquitin ligase which is a protein that in combination with an E2 ubiquitin-conjugating enzyme causes the attachment of a protein called ubiquitin, to lysine by making an isopeptide bond (10).

2.1.4.4 Lyases

Lyases catalyze the formation of double bonds between atoms by adding or subtracting chemical groups. Lyases are enzymes that cleave C–C, C–O, C–N and other bonds by elimination to form multiple bonds or rings. An example of lyases is carboxy-lyases which catalyzes decarboxylation of organic compounds (11) such as amino acids, alpha and beta keto acids.

2.1.4.5 Oxidoreductases

They catalyze oxidation-reduction reactions i.e. the process by which an atom loses an electron to another atom. An example of an oxidoreductase enzyme is laccase (benzenediol:

oxygen oxidoreductase, EC 1.10.3.2) a blue multicopper oxidase that catalyzes the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water (12).

2.1.4.6 Transferases

Transferases are the enzymes that transfer chemical groups from one molecule to another. An example of transferases is glycosyltransferases. These enzymes act as a catalyst for the transfer of a monosaccharide unit from an activated nucleotide sugar to a glycosyl acceptor molecule, usually an alcohol.

The result of glycosyl transfer can be a carbohydrate, glycoside, oligosaccharide, or a polysaccharide

2.1.5 Enzyme action

Enzyme action can be studied by looking at it from three different points of view: 1) the actual reaction mechanism involved; 2) the molecular geometry of the enzyme; and 3) the changes it brings to the activation energy of the reaction between the molecules.

Although enzymes can change the speed of a chemical reaction, they cannot change its direction, otherwise they could make impossible reactions happen and break the laws of thermodynamics. So an enzyme can just as easily turn a reactant into a product as turn a product into a reactant, depending on the local concentrations. When the active site changes its molecular geometry to accommodate a reactant, the “distortion in shape of the active site” is the associated transition state.

2.1.6 Reaction mechanism

In an enzyme-catalyzed reaction, the substrate first binds to the active site of the enzyme to form an enzyme-substrate (ES) complex, then the substrate is converted into product while attached to the enzyme, and finally the product is released, thus allowing the enzyme transformation to start over (13). Figure 2.3 shows that enzyme sucrase is hydrolyzing sucrose into glucose, by first forming sucrase-sucrose complex (14).

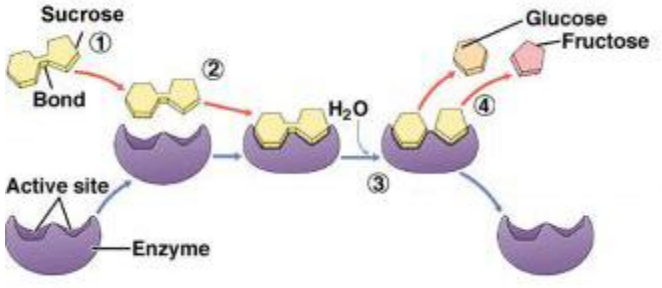


Figure 2.3. Enzyme catalyzed reaction mechanism.

The enzyme action shown in Figure 2.3 can be summarized as follows (14):

1	2	3	4
Substrate (sucrose) consists of glucose and fructose bonded together	Formation of enzyme-substrate complex	The binding of the substrate and enzyme places stress on the glucose-fructose bond the bond breaks	Desorption of the enzyme back into the solution

2.1.7 Molecular Geometry

Active site of enzyme and the substrate both change shape when the enzyme-substrate complex is formed, bending and weakening the target bonds. For example, if a substrate is to be split, a bond might be stretched by the enzyme, making it more likely to break. Alternatively the enzyme can make the local conditions inside the active site quite different from those outside (such as pH, water concentration, charge), so that the reaction is more likely to happen.

There are three types of molecular changes which can happen during enzyme reaction with substrate (15).

2.1.7.1 Lock and Key

In this mechanism (see Figure 2.4), enzyme and substrate are a perfect match for each other.

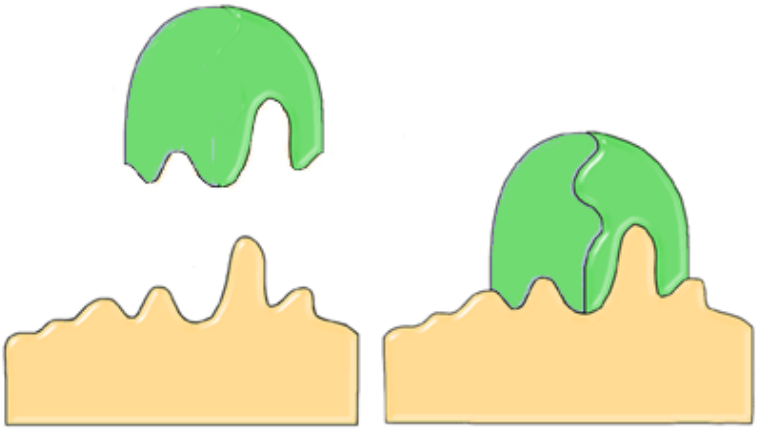


Figure 2.4. Mechanism of lock and key.

2.1.7.2 Induced fit

In this mechanism, enzymes change geometry according to the substrate.

2.1.7.3 Substrate deformation

In this mechanism, substrate changes its molecular geometry according to the enzyme.

2.1.7.4 Energy changes

Energy changes during a chemical reaction is another property of enzyme catalyzed reactions. Before a substrate reacts with enzymes and change into product, the substrate must overcome an "energy barrier" known as activation energy (13). The larger the activation energy, the slower the reaction will be. This is because only a few substrate molecules will have sufficient energy to overcome the activation energy barrier. Most biological reactions have large activation energies, so without enzymes they happen far too slowly to be useful. Enzymes reduce the activation energy of a reaction so that the kinetic energy of most molecules exceeds the activation energy required and so they can react.

For example, for the catalase reaction ($2H_2O_2 \rightarrow 2H_2O + O_2$) the activation energy is 86 kJ mol^{-1} with no catalyst, 62 kJ mol^{-1} with an inorganic catalyst and just 1 kJ mol^{-1} with catalase enzyme (13). Figure 2.5 shows the comparison of “energy changes” between catalyzed and uncatalyzed reaction (15). It can be observed that there is a large difference between the activation energy of enzyme catalyzed and uncatalyzed reactions. The free energy released by enzyme catalyzed reaction is much lower than enzyme uncatalyzed reaction.

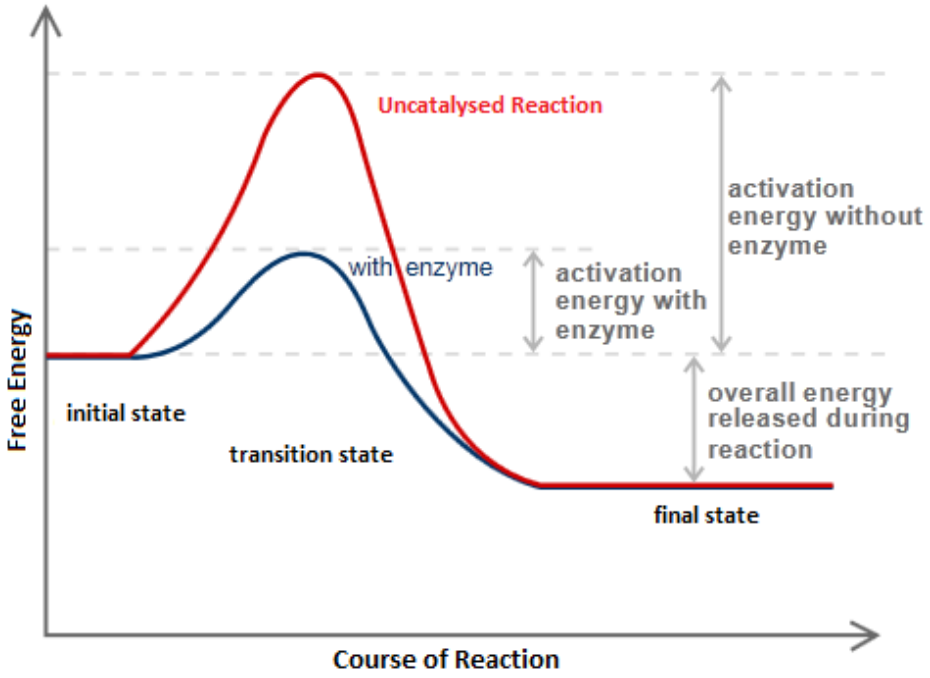


Figure 2.5. Comparison of changes in energy between enzyme catalyzed and uncatalyzed reactions.

2.1.8 Properties of enzymes

2.1.8.1 Faster reactions

An enzyme accelerates a reaction by changing the reaction kinetics, which allows achievement of equilibrium quicker than non-enzyme catalyzed reaction. The explanation of this mechanism is that the reaction of the enzyme with the substrate provides a reaction pathway with lower transition state energy, thereby facilitating a more rapid conversion of substrate to product (16).

2.1.8.2 Specificity

Enzymes are job specific and this is their biggest advantage. Most enzymes have a high level of specificity and will catalyze a reaction with only one or a few substrates. For example, cellulase enzyme will only hydrolyze 1, 4-beta-D-glycosidic linkages in cellulose, lichenin and cereal beta-D-glucans.

2.1.8.3. Mild Operating Conditions

Most enzymes operate under mild temperature and pH conditions. That temperature is actually the temperature at which enzyme is derived, from cell media. For extracellular enzymes of a particular organism or those secreted by a microorganism the optimum temperature may be that of the environment in which the enzyme normally operates (6).

2.1.9 Factors that Affect the Rate of Enzyme Reactions

2.1.9.1. Temperature

Enzymes have an optimum temperature at which they work fastest. Enzyme activity below the optimum range of temperature decreases without damaging the protein structure. The reaction rate increases with increasing temperature until the optimum temperature is reached after which the enzyme activity decreases rapidly. This is the point when the enzymes become permanently deactivated by denaturation.

For example, the optimal temperature for an amylase enzyme manufactured by Novozymes, Aquazyme Ultra 1200 N lies between 50°C to 95 °C. There is a decrease in relative

efficiency if the temperature is lower than 50 °C and slight decrease when the temperature is higher than 95 °C.

Figure 2.6 shows the changes in relative efficiency with respect to temperature of Aquazyme Ultra 1200 N.

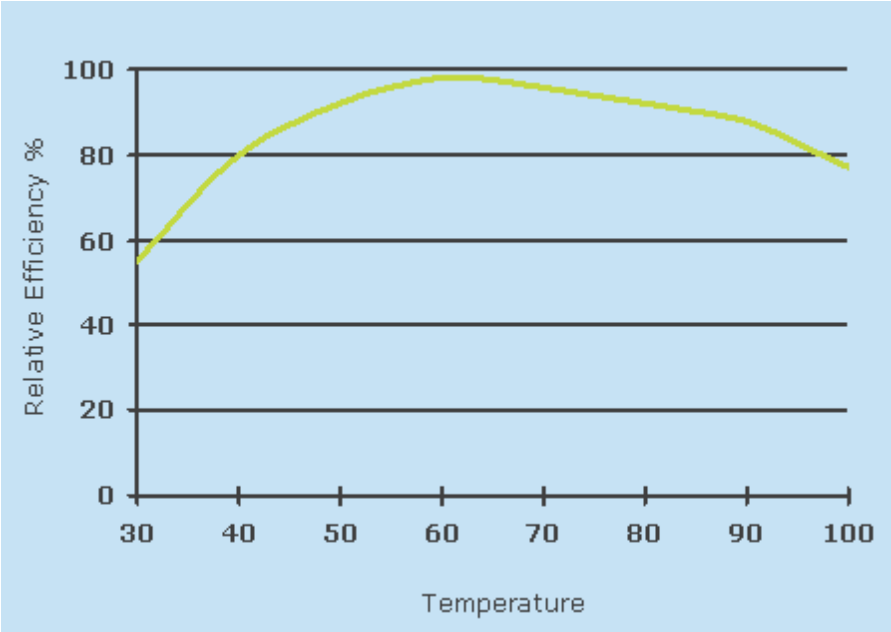


Figure 2.6. Effects of change in temperature on efficiency of Aquazyme 1200 N (16).

2.1.9.2. pH

Enzymes have an optimum pH at which their activity is maximum. The pH affects the charge of the amino acids at the active site, so the properties of the active site change and the substrate can no longer bind. The active site may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization and elsewhere in the

protein ionized side chains may play an essential role in the interactions that maintain protein structure (17). Its activity decreases sharply on both sides of the optimum range. Since they are protein molecules, enzymes can be denatured by extremes of pH.

For example, Amylase enzyme manufactured by Novozymes, Aquazyme Ultra 1200 N relative efficiency is maximum at pH 5-7. There is a decrease in relative efficiency if the pH is lower than 5 and higher than 7.

Figure 2.7 show the changes in relative efficiency of Aquazyme Ultra 1200 N with respect to change in pH.

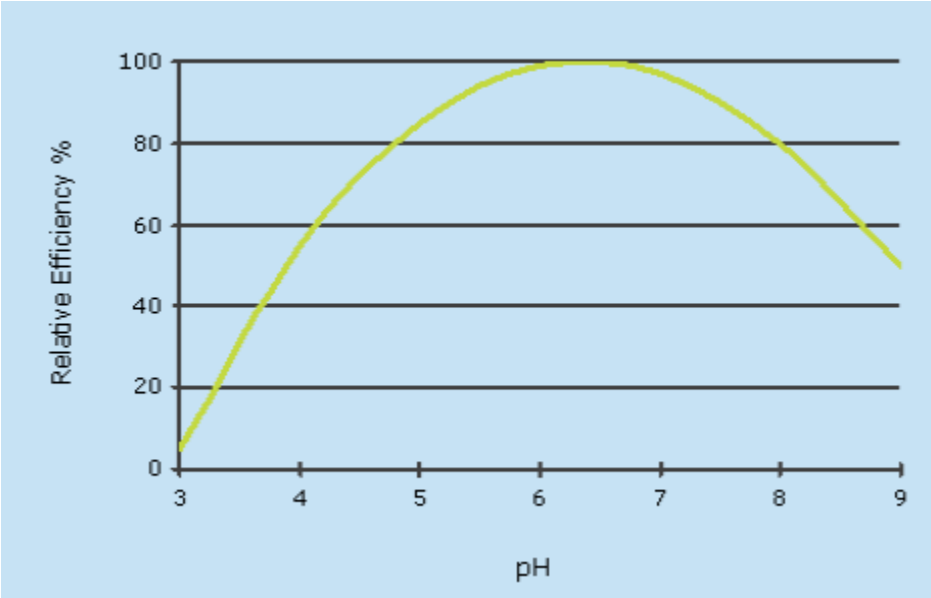


Figure 2.7. Effect of change in pH on Aquazyme 1200 N efficiency.

2.1.9.3. Enzyme concentration

As the enzyme concentration increases the rate of the reaction also increases. More enzymes provide more active sites for substrate to catalyze the reaction therefore more enzyme-substrate complexes are formed.

2.1.9.4. Substrate concentration

Enzyme behavior is also affected by substrate concentration. As the substrate concentration increases, the rate increases because of formation of more enzyme-substrate complexes as more substrate molecules collide with active sites. (13)

The maximum rate at infinite substrate concentration is called v_{max}, and the substrate concentration that gives a rate of half v_{max} is called K_M. These quantities are useful for characterizing an enzyme. A good enzyme has a high v_{max} and a low K_M (18). A low value of K_M means that the reaction is going quickly even at low substrate concentrations (19). Figure 2.8. shows the relation between reaction velocity and concentration of substrate (19). Values of V_{max} and K_M can be determined by plotting a graph between reaction velocity and concentration.

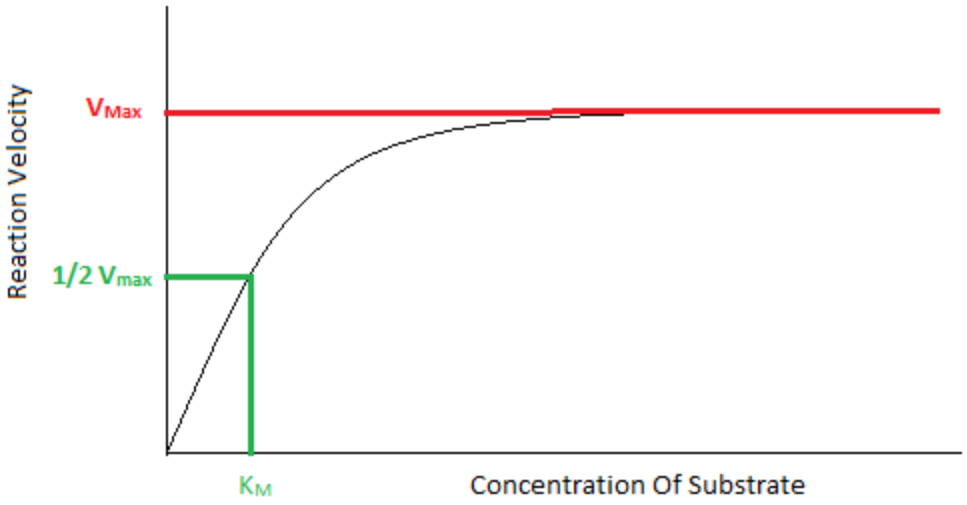


Figure 2.8. Determination of Values of V_{max} and K_M.

Table 2.1 shows some examples of values of K_M (20).

Table 2.1 Values of K_M

Enzyme	**Substrate**	**KM(mM)**
Catalase	H_2O_2	25
Carbonic anhydrase	HCO_3^-	9
b-Galactosidase	D-Lactose	4
Threnine dehyratase	L-Thr	5
Chymotrypsin	Glycyltyrosinylglycine	108

At higher concentrations the enzyme molecules become saturated with substrate, and there are few free active sites, so adding more substrate doesn't make much difference. One example of the effect of substrate concentration is catalase enzyme (21). It is found in food such as potato and liver. It is used for removing hydrogen peroxide from the cells. Catalase speeds up the decomposition of hydrogen peroxide into water and oxygen. In order to study

the optimum concentration of catalase enzyme, the amount of oxygen produced by the reaction between hydrogen peroxide and catalase enzyme is measured. At first, the rate of oxygen produced per second steadily increases when more hydrogen peroxide is added because more of the active sites of the enzyme are being used which results in more reactions so the required amount of oxygen is made more quickly. Once the amount of hydrogen peroxide molecules added exceeds the number of active sites available then the rate of reaction will no longer go up. This is because the maximum number of reactions is being done at once so any extra substrate molecules have to wait until some of the active sites become available.

2.1.9.5. Covalent modification

Enzyme can be modified by modifying the peptide chains. This modification can be carried out in several ways:

- By cleavage of enzyme
- By addition of phosphate or methyl group

The activity of some enzymes is controlled by other enzymes, which modify the protein chain. This modification can turn an inactive enzyme into an active enzyme (or vice versa), and this is used to control many metabolic enzymes and to switch on enzymes in the gut. For example, HCl in stomach → activates pepsin → activates rennin.

2.1.9.6. Enzyme Inhibitors

Inhibitors blocks the activity of enzymes, reducing the rate of their reactions (22). Inhibitor usually reacts with the enzyme itself forming enzyme-inhibitor complexes. Inhibitor is considered reversible if enzyme recovers its activity when the inhibitor is removed. If inhibitor causes permanent loss of activity, it is termed as irreversible. They are found naturally, but are also used artificially as drugs, pesticides and research tools. There are two main kinds of inhibitors (22).

1. Competitive Inhibitors
2. Noncompetitive Inhibitors

Competitive Inhibitors

Competitive inhibitor has a similar structure to the substrate molecule, and so it can fit into the active site of the enzyme. It competes with the substrate for the active site therefore the reaction is slower. Increasing the concentration of substrate restores the reaction rate and the inhibition is usually temporary and reversible. Competitive inhibitors increase K_M for the enzyme, but have no effect on v_{max}, so the rate can approach a normal rate if the substrate concentration is increased high enough.

An example of a competitive inhibitor is strychnine. It acts as an allosteric inhibitor of the glycine receptor in the mammalian spinal cord and brain stem. Glycine is a major post-synaptic inhibitory neurotransmitter with a specific receptor site. Strychnine binds to an

alternate site that reduces the affinity of the glycine receptor for glycine, resulting in convulsions due to lessened inhibition by the glycine (23).

Non-Competitive Inhibitors

A non-competitive inhibitor molecule is quite different in structure from the substrate and does not fit into the active site. It binds to another part of the enzyme molecule, changing the shape of the whole enzyme, including the active site, so that it can no longer bind substrate molecules. Non-competitive inhibitors therefore simply reduce the amount of active enzyme. This is the same as decreasing the enzyme concentration, so they decrease v_{max}, but have no effect on K_M. This kind of inhibitor tends to bind tightly and irreversibly – such as the poisons cyanide and heavy metal ions. Many nerve poisons (insecticides) work in this way too. Some inhibitors work by forming covalent bonds with specific groups of enzymes. For example, lead forms covalent bonds with the sulfhydryl side chains of cysteine in proteins (24). The binding of the heavy metal shows non-competitive inhibition.

2.1.9.7. Feedback Inhibition (Allosteric Effectors)

The activity of some enzymes is controlled binding to a specific regulatory (or allosteric) site on the enzyme, distinct from the active site by inhibitors. Only few enzymes can either inhibit or activate the enzyme, allowing sophisticated control of the rate. They are generally activated by the substrate of the pathway and inhibited by the product of the pathway, thus only turning the pathway on when it is needed. This process is known as feedback inhibition.

2.2. Enzymes used in Denim Garment Washing

There are several types of enzymes used in denim garment washing to impart different finishes and create different looks on denim pants. Two key enzymes are:

- Cellulase
- Laccase

2.2.1 Cellulase

Cellulase enzyme processing is used in textile processing, especially in denim garment washing processes as an alternative to stone washing for years (25). Cellulose is a polysaccharide, which can be hydrolyzed by cellulase enzyme by means of catalytic hydrolysis of 1, 4-β glycoside bonds of the cellulose molecules. Cellulase treatments can produce fabrics with smooth and soft surfaces.

The cellulases used in textiles are of bacterial or fungal origin. The fungal enzymes are extracted from the Trichoderma species and Humicola insolens. The fungal cellulytic systems usually consist of several exo and endoglucanases and one or two β-glucosidases. The number of components secreted depends on the fungus which it is derived from. The cellulolytic system from trichoderma species consists of two exoglucanases, at least four endoglucanases and one β-glucsidase.

Most bacterial systems produce only endoglucanases. Among the bacterial cellulase systems, the most extensively studied system is that of *C.thermaocellum*. This bacterium produces a very active cellulase in the form of complexes termed as cellulosome, which degrades the

crystalline cellulose in the presence of Ca^{2+} and a reducing agent. The cellulase consists of several endoglucanases and at least three exoglucanases. The activity of these systems towards crystalline cellulose is highly dependent on the reducing agent and calcium ions

2.2.1.2 Structure of cellulases

Cellulases are "modular enzymes that are composed of independently folding, structurally and functionally discrete units, referred to as either domains or modules" (26). The fungal and bacterial cellulases usually comprise of two or more structural and functional domains. Cellulases have catalytic domain and a cellulose-binding domain (CBD) linked by a peptide bond (27). The key purpose of the cellulose binding domains (CBDs) is to deliver its resident catalytic domain to the crystalline cellulose substrate. The cellulolytic activity of the catalytic domain is modified by accessory modules that can supplement or alter the overall properties of the enzyme.

CBDs also catalyze the disruption of the noncovalent interactions between the chains of the crystalline substrate (28). Also in the presence of CBDs, the activity towards insoluble cellulose is enhanced greatly. Changing the length of the linker peptide also changed the activity, which implies that these domains act in concert during the hydrolysis of cellulose. It can be summarized that the CBDs are essential for degrading crystalline cellulose as they increase the effective enzyme concentration on the cellulose substrate. Cellulose binding domains of fungal origin are from family I and account for 33-36 amino acids, while bacterial cellulose binding domains from family II are bigger with 105-120 amino acid (14). Family I CBDs are present in textile and detergent applications.

2.2.1.3 Cellulases reaction on cellulose

Cellulases have a four main components which acts on cellulose to hydrolyze (15):

- Endo –Glucanases
- Beta Glucanases
- Cellobiohydrolases
- Cellobiases

Endoglucanases hydrolyze at random locations on polymer chains and converts crystalline cellulose into amorphous form. It can form products of oligosaccharides of different sizes. Figure 2.9 shows that cellulose it converted into small oligosaccharides.

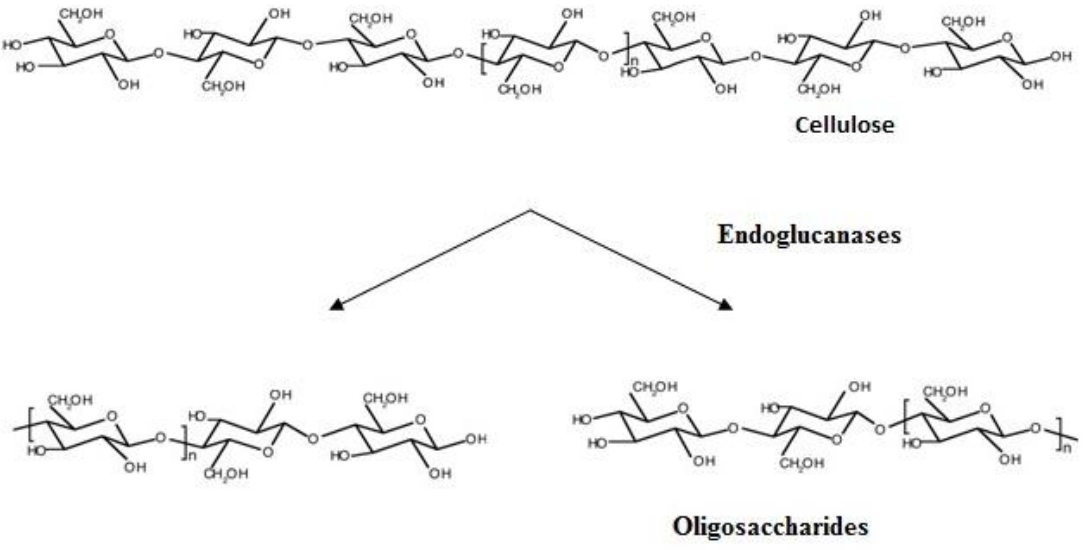


Figure 2.9. Conversion of cellulose into oligosaccharides.

Beta glucanases hydrolyse the cellulose at nonreducing end of polymer chain to produce small polymer chain glucose.

Cellobiohydrolases hydrolyze at non reducing end of polymer chain to produce cellobiose. Figure 2.10 shows the conversion of cellulose into cellobiose.

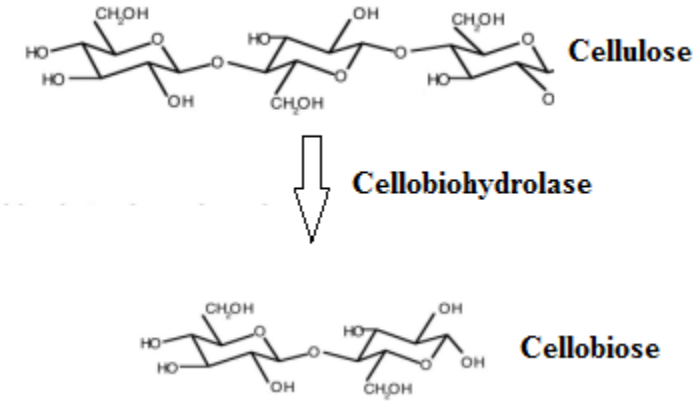


Figure 2.10.Conversion of cellulose in to cellobiose.

Cellobioses hydrolyses cellobiose into glucose.

Extracellular cellulases can degrade both crystalline cellulose (as in cotton) and soluble cellulose derivatives; others can degrade only the latter and are without effect on cotton. Many investigators have considered whether the glucose chains of cellulose are degraded by cellulase in an endo-wise or in an exo-wise mechanism (28).

Synergism

Mutual increase of the efficiency of action of two or more components of a system when they act together in comparison with their additive action when they act separately is called

synergism (29). Synergism between the individual components of a cellulase system acting toward insoluble cellulose adds further complexities to the study of the mechanisms of action of cellulases. The main problem in studying this phenomenon is that the synergistic effect varies depending on which of the multiple forms of the cellulolytic components have been used in the study, on the source of cellulases, and on the amorphous or crystalline cellulose. The efficiency of the cellulase system depends on this concept of synergism which was studied by monitoring the action of purified cellulase components- endoglucanases, cellobiohydralases and β-glucosidases (29).

It can be concluded that action of endoglucanases is necessary for forming chain ends which can be acted upon on by cellobiohydralases. On action of cellobiohydralases, more sites open for subsequent action of endoglucanases. Therefore, these components require each other for efficient degradation of crystalline cellulose. For efficient attack on crystalline cellulose in the form of fiber, at least three enzymes – cellobiohydralases I and II and at least one endoglucanases must be present (30). It has been established that cellobiohydralases and endoglucanases are mainly inhibited by cellobiose, one of the products formed due to action of these enzymes. In this synergistic cooperation, β-glucosidases converts cellobiose to glucose and therefore serves to alleviate the product inhibition of the other two cellulases.

2.2.1.4 Effect of cellulase on properties of cellulose fibers.

Cellulose treated by cellulase display many changes in physical properties such as lowering of the degree of polymerization, disintegration to short separable fibers, loss in tensile strength, increased capacity for moisture uptake and for alkali absorption, and transverse

cracking. The most apparent effects are the improvement in tactile properties, formation of short fibers and the loss in strength (25).

Fragmentation of cellulose fibers

Cellulase cleaves cellulose fibers into small oligosaccharides and into small particles. This activity is called fragmentation (31). Transverse cracks in cotton fibers upon exposure to cellulolytic enzymes leads to fragility of cotton fibers. Degraded fibers show etching of the macro-fibrils of the sheets of secondary wall which results in production of individual micro fibrils (32). Continued enzyme attack produced smaller fragments and hydrocellulose- like particles.

Loss in tensile strength

Cellulase has a biopolishing effect on cellulose (33), which is partial hydrolysis of cellulosic fibers resulting in loss in tensile strength. Transverse cracks on the fiber surface after enzymatic treatment also results in a decrease in tensile strength of the fiber.

Dye uptake

Dye uptake of cellulose fiber is increased after treatment with cellulase enzyme due to decrease in crystallinity of cellulose (34). Reactive dyes showed an increase but not in the same degree as the direct dyes. Direct dyes showed increase uptake with the increase in cellulase treatment. It is also observed that dye uptake of mercerized cellulose fibers increase with cellulase treatment (34).

2.2.1.5 Effect of pH and Temperature

Change in pH and temperature of the conditions, has an effect on the activity of enzyme. This is also the case with cellulase enzyme. Take cellulase from Novoprime B959 as an example. Novoprime B959 is a cellulase based enzyme. It is a good replacement of pumice stone for creating a stone wash effect on denim fabric (35). It can also create different finishes without "back staining" denim fabric and produces high color contrast ratio without any significant effect on the fabric strength. Activity reported by Novozymes is 1500 EGU/g.

The pH and temperature play crucial roles in the efficiency of Novoprime B959. The optimum pH is 4.5-5.5. There is a decrease in relative efficiency if the pH is lower than 4.5 and higher than 5.5 (35).

The optimum temperature for the processing of denim fabric by Novoprime B959 lies between 50°C to 60 °C. There is a decrease in relative efficiency if the temperature is lower than 50 °C and slight decrease when the temperature is higher than 60 °C (35). Figure 2.11 and Figure 2.12 shows the relationship of percentage efficiency of Novoprime B959 according to its pH and temperature, respectively (35).

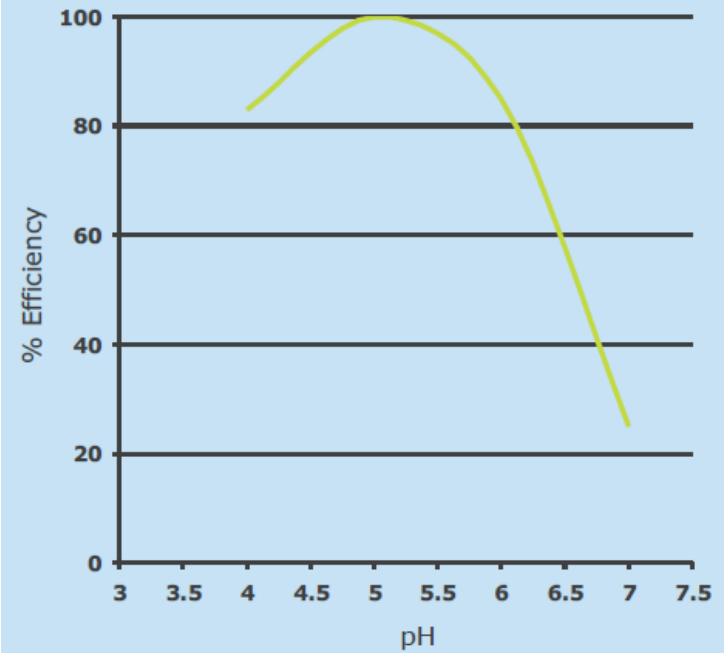


Figure 2.11. Effect of pH on enzyme efficiency.

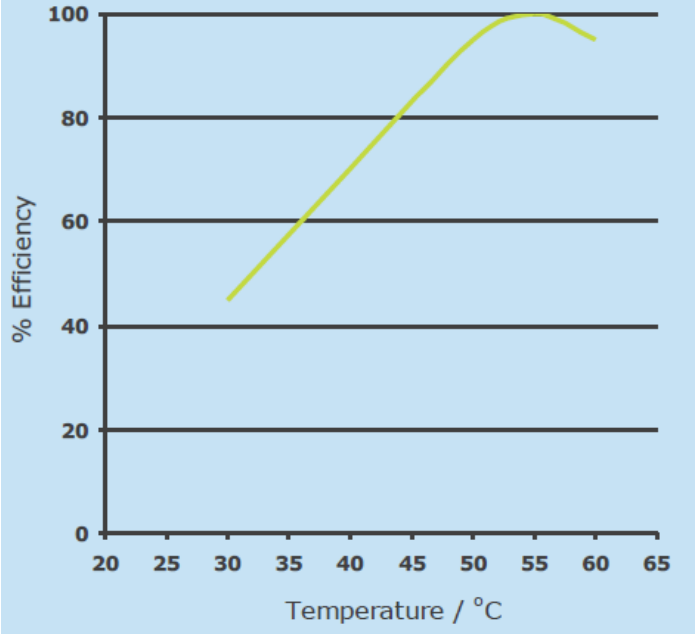


Figure 2.12. Effect of temperature on enzyme efficiency.

2.2.1.6 Application of cellulases in the textile industry

From a practical point of view, the cellulases used in detergents and textile treatment are often differentiated by their origin from fungal and bacterial sources. The fungal enzymes are produced using *Trichoderma* species *(T.reesei, T.longibrachiatum and T.viride*) Or *Humicola insolens*. Suitable bacterial enzymes are from *Bacillus, Cellulomonas* or *Thermomonospora* species. Cellulases from *Trichoderma* species are the most popular in textiles treatment. Enzymatic processing enables the textile industry to reduce production costs, to reduce the environmental impact of the overall process and to improve the quality and functionality of the final products. Enzymes are non-toxic and they operate in mild conditions of temperature and pH without any undesirable side reactions. Today enzymatic treatment of cotton either in denim washing or in biostoning is standard technique in industry. The application of cellulases in the textile industry is described below

Fabric softening

It has been long known that repeated washing of cotton-containing textiles causes harshness in the fabric that was related to physical damage of the cellulosic material. As a result, otherwise highly structured regions of cellulose fibers become amorphous and microfibrillar material protrudes from the smooth fiber. Due to the removal of microfibrils, the texture and the grip of fabrics can be improved. The origin of pilling is based on mechanical damage by wearing and washing a textile. Pilling changes the look of the surface of the fabric and at the

same time can result in a different perception of color, based on different light diffraction. Pilling can be removed using cellulases that attack the attachment site of the pilling to the fabric. The pills become weaker after some cellulase hydrolysis and they are removed by mechanical agitation from the fabric.

Color revival

Cellulose based fabrics, such as cotton, rayon, flax and others often develop a reduction in color brightness. The effect is caused by disordered structures of the cellulose fibrils caused by mechanical and chemical damage by washing and wearing. The brightness of colors can be revived by means of cellulases. The effect can be seen best with dark colored textiles. Cellulases that show this effect are able to remove damaged fibril parts and microfibrils. The effect originally was described for fungal enzymes for *H.insolens, Fusarium oxysporum, Sporotrichum pulverulentum* and *Trichoderma reesei*.

Ageing effect

Ageing effects are obtained mainly with EG or EG rich cellulase mixtures. The dyed yarns on the denim fabric after enzymatic action with sequential or simultaneous mechanical agitation release the “trapped dye” in the bath. This produces areas of high contrast of blue color. The fibrillation produced during the ageing process is a result of the simultaneous action of cellulases and mechanical agitation. Therefore, the ageing look is produced with less aggressive action when compared to previously used pumice stones.

Cleansing effect

Recently published results indicate that enzymes, mainly cellulases and several non-cellulolytic enzymes (lipases, proteases, pectinases) may be used effectively in the cleaning processes of cotton. Raw cotton contains approximately 10% of non-cellulosics depending upon the variety. These impurities are mainly located in the outer layers of the fiber in the cuticle and the primary wall. Traditional cleaning procedure involves scouring with sodium hydroxide solution. Cellulase treatment prior to the alkaline scouring process has been shown to enhance both the removal and degradation of seed coat fragment impurities of cotton fabrics (28).

Yarn twist

Denim fabric made from torque free yarn shows larger color difference as compared to denim fabric made from conventional ring spun yarn after cellulase treatment (36).

The degree of twist or torque in the yarn will affect both yarn and fabric properties. A highly twisted yarn causes fabric "spirality". High-twist yarn will have an effect on both the fabric softness and smoothness in an undesirable way. Conventional methods for reducing the yarn torque include the permanent setting of yarn or the physical balance of yarn by two plying. The yarn structure can be modified by reducing the yarn twist without affecting the strength by having a yarn torque reduction device on the conventional ring-spinning system (37). This torque free spinning method consists of a single step on the ring spinning machine that attaches a mechanical false twister to a conventional ring frame in order to make a false

twist, and then creates twist redistribution in the fiber substrates to achieve a delicately entangled structure of substrates with locally differing twist levels and true twist is formed by the conventional ring-spinning method. Because of the low twist level, the fabrics produced with this torque-free spinning technique were claimed to be softer and smoother and with improved surface appearance compared with fabrics produced from conventional ring-spun yarn. The softly twisted yarn also improves the pilling resistance. Torque-free ring-spinning technology not only improves both yarn and fabric quality, but it can also lower the production costs in yarn manufacturing, which is highly desirable for the textile industry. Other than improvement of the mechanical properties, the torque-free ring-spun yarn can potentially improve the dye uptake property because of its high yarn bulkiness, which reduces the restriction on the dye to access the yarn interior.

2.2.2 Laccase

Laccases (E.C. 1.10.3.2, benzenediol: oxygen oxidoreductase) are either mono or multimeric copper-containing oxidizes that catalyze the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (38):

$$O2+4e- + 4H + \rightarrow 2H2O$$

Laccase belongs to copper containing oxidases, which catalyze reduction of molecular oxygen to water, avoiding the formation of hydrogen peroxide. It belongs to oxido-reductase class of enzyme. Oxidation is the loss of electrons to an increase in oxidation state by molecule, atom or ion. Reduction is the gain of electrons or a decrease in oxidation state by

molecule, atom or ion. Laccase are most common in fungi. They are glycoproteins i.e. they contain oligosaccharide chains covently attached to polypeptide side chains and they have carbohydrate attached to them by a process called glycosylation. These attachment is with hydroxyl (-OH) group or amino group ($-NH_2$). Oligosaccharide is a kind of saccharide i.e. carbohydrate polymer containing small numbers of component sugars (around three to ten).

The amount of carbohydrate in laccase enzyme depends on the origin from which it is extracted. These carbohydrates are responsible for their stability of the globule and protect it from proteolysis i.e. directed degradation of proteins by cellular enzymes. Carbohydrates are also prevents laccase enzyme inactivation by radicals.

2.2.2.1 Structure of laccase

Three types of copper can be distinguished. Type 1 copper is responsible for the blue color of the protein at an absorbance of approximately 600 nm, Type 2 copper does not confer color and Type 3 copper consists of a pair of copper atoms in a binuclear conformation that give a weak absorbance in the near UV region.

The Type 1 Cu is usually coordinated to two nitrogens from two histidines and sulphur from cysteine. It is the bond of Type 1 Cu to sulphur that is responsible for the characteristic blue color of typical laccase enzymes. The geometry is described as a distorted trigonal bipyramidal coordination with a vacant axial position where the substrate docks. The coordination is unusual as it is intermediate between the preferred coordination states for Cu (I) and Cu (II) species. A leucine residue is present but is too far away to be directly

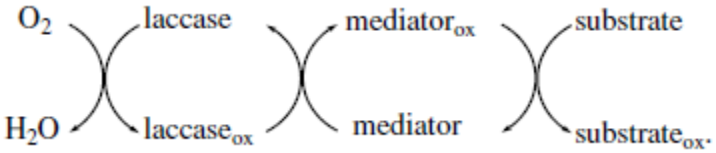
coordinated. The Cu is therefore only coordinated to three atoms. The structure showed that type 2 and type 3 coppers are close together in a trinuclear centre. The copper atoms of the T2/T3 sites are coordinated to eight histidines, which are conserved in four His-X-His motifs. The two T3 atoms are coordinated to six of the histidines, while the T2 atom is coordinated to the remaining two. A hydroxide ligand bridges the pair of T3 atoms, resulting a strong anti- ferromagnetic coupling. The cloned sequences of various laccases also show that the 10 histidine and 1 cysteine residues are copper ligands conserved in all laccase sequences known to date except one from Aspergillus nidulans that has a methionine ligand of type 1 copper. These conserved cysteine and histidine residues serve as a pathway for the transport of electrons from the T1 Cu site where electrons are extracted from phenolic substrates to the trinuclear site that serves as the binding site of dioxygen where the electrons are required for dioxygen reduction (39).

Laccase is regarded as the simplest enzyme that can be used to define the structure-function relations of copper containing proteins. Only laccase presents the possibility to oxidize activated metoxiphenols like syringaldazine (40). Effect of laccase on indigo is discussed in Section 2.3.4.

2.2.2.2 Laccase mediators

Laccase reaction is comparatively slow. In practice, laccase is used with mediators. Mediators are low molecular weight compounds that are easily oxidized by laccase. They produce very unstable and reactive cationic radicals, which can oxidize more complex substrates before returning to their original state.

Properties of ideal mediator should be that it should be a good laccase substrate. It's oxidized and reduction forms should be stable and it should not hinder the enzymatic reaction (41). The ideal mediator should follow the following cycle:



The oxidized mediator form produced by in the course of the enzymatic reaction can non-enzymatically oxidize compounds with ionizing potential exceeding the potentials of laccases (41). The indigo degradation in presence of laccase and mediator was quantified by R. Campos and coworkers (42).The effect indigo degradation in presence of laccase is at least 30% percent faster.

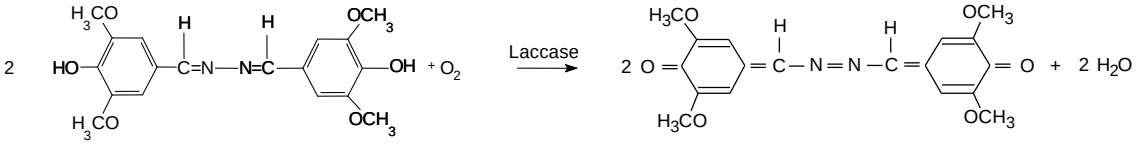
2.2.2.3. Measurement of laccase activity by syringaldazine based assay

Every company makes their own method to measure the activity of laccase. Following method is used by Novozymes Inc. to measure the activity of laccase enzyme

Application

The method is used as a basic method for analyzing the activity of laccase enzymes. The assay can be applied at pH 5.5 or pH 7.5. Note that the dilution medium and buffer used in the assay are different for the two pH values. In our experiment, we will apply assay for pH 7.5.

Principle



The assay is based on the laccase (EC 1.10.3.2) catalyzed oxidation of syringaldazine (4,4´-[azinobis(methanylylidene)]bis(2,6-dimethoxyphenol)) to the corresponding quinone 4,4´-[azobis(methanylylidene])bis(2,6-dimethoxycyclohexa-2,5-dien-1-one), see Figure 1. The reaction is detected by the increase in absorbance at 530 nm.

Definition of unit

One laccase unit is the amount of enzyme which under the given analytical conditions catalyzes the conversion of 1 μmole syringaldazine per minute. For measurements made at pH 5.5 the activity units are labeled LACU, and for measurements made at pH 7.5 the activity units are labeled LAMU.

2.3 Indigo

Indigo is one of the many natural dyes derived from plants (43). Indigo is one of the oldest known textile dyes in the world. The earliest history of its use was probably in India ca. 2600 BC and it is also mentioned in Sanskrit writings from that time. Around 2300 BC, it is said that indigo was imported from India for the dyeing of mummy cloths in ancient Egypt (44).

Indigo dye was the only source of blue dyestuff until the invention of synthetic dyes in 19th century.

The current annual consumption of indigo provides the most common source of blue dye and it is widely used today. The denim industry is based on this dye as it provides easy reduction of color and provides many different shades.

2.3.1 Structure of indigo

Correct nomenclature of indigo dye is 2-(1, 3-dihydro-3-oxo-2H-indazol-2-ylidene)-1, 2-dihydro-3H-indo-3-one (45). It is a kind of vat dye. It is insoluble in water and its has no substantivity for cellulosic fibers in its oxidized form. The structure of indigo was proposed by Von Bayer, although he initially suggested that it had Z-or cis configuration, but X-ray crystal structure determination carried out by Posner in 1936 confirmed that the molecule exists in trans configuration. Figure 2.13. shows the structure of Indigo dye:

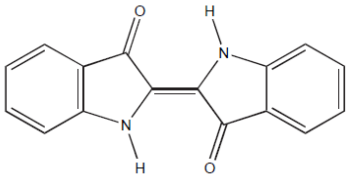


Figure 2. 13. Structure of indigo dye.

The color of indigo is strictly dependent on the processing conditions (46). In the gas phase, the only situation in which a dye will effectively owe its color to single molecules, indigo is red (i.e. λ_{max} 540 nm). In solution, its color is dependent on the nature of the solvent in which

it is dissolved. In polar solvent, it exhibits a far more bathochromic shift as compared to gas phase and non polar solvents. In nonpolar solvents, it is violet (e.g. in CCl_4 λ_{max} =588 nm), while in polar solvents it is blue (e.g. in DMSO λ_{max} =620 nm).

In the solid state and when applied to fabric as a vat dye, usually indigo is blue. Amorphous and crystalline forms of indigo in the solid state differ significantly in their absorption maxima with λ_{max} =650 and 675 nm respectively (46). The reason for this change in λ_{max} is that the crystalline solid state, indigo molecules are highly aggregated by intermolecular hydrogen bonding and this is a major factor in causing the bathochromic shift of color compared to the monomolecular state. This hypothesis was confirmed by X-ray single crystal structural analysis.

The outer benzene rings play a secondary role in determining the color of indigo (47), so general studies are on central core of indigo molecule. One commonly invoked approach proposes that the basic structural unit responsible for the color of indigo is an arrangement consisting of two electron donor groups (NH) and two electron acceptor groups (C=O) ‘‘cross-conjugated’’ through an ethene bridge. This gives rise to the concept of the H-chromophore in Figure 2.14 (48) .

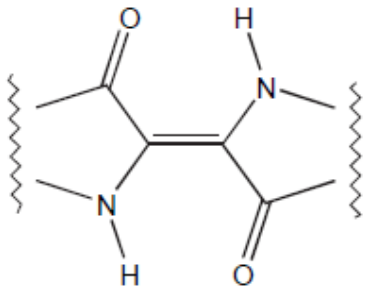


Figure 2.14. H- Chromophore unit of Indigo (48).

An alternative, closely related approach proposes that the important structural arrangement in the indigo molecule consists of two symmetrically coupled polymethine (Figure 2.15):

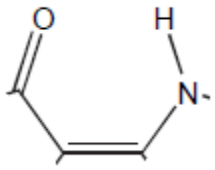


Figure 2.15. Coupled polymethine unit (46).

Another model, conceptually more complex and supported by theoretical calculations, proposes that the basic chromophore may be interpreted as the aza (nitrogen-containing) analogue of two coupled cyclopentadienyl groups (46) (see Figure 2.16). This gives rise to anti-aromatic character of the positively charged five-membered ring structure. Anti-aromaticity refers to the state of ring when it contains 4 pi electrons rather than the 6 pi electrons typical of aromatic compounds such as benzene.

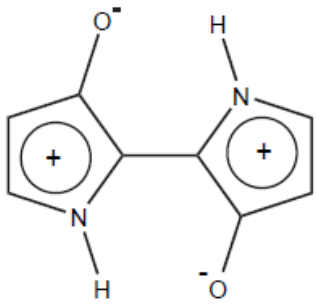


Figure 2. 16. Anti aromatic character of the 5 membered rings of indigo (39).

2.3.2 Sources of indigo

Natural indigo can be derived from a wide range of tropical, sub-tropical and temperate plants from many different species, genera and families. In tropical and subtropical areas, the plants most widely used for indigo production were indifofera spp. of which there are over 350 species (49). In mild climates, the most commonly used species was Isatis tinctoria. Other species, e.g., Polygonum tinctorum, have also been used in Japan, China and Russia for large-scale indigo production (50). These plant sources provided the primary colors from which all colors could be derived, e.g., green was produced by over-dyeing of yellows with indigo. Today the indigo used is mainly synthetic.

2.3.3 Synthesis of indigo

Indigo is not synthesized directly by the plant; it is a product derived from indole glucoside precursors, which are secondary metabolites (51).

The indigo precursor indoxyl, mainly in the form of the glucoside indican (indoxyl-*β*-D-glucoside), is found in most indigo-producing plants. Figure 2.17 shows the structure of indicant (indoxyl-*β*-D-glucoside).

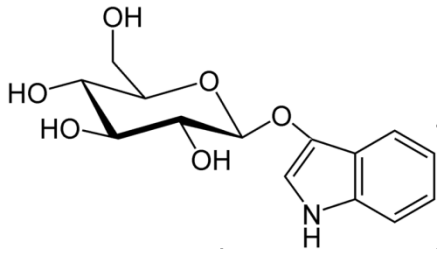


Figure 2.17. indoxyl-β-D-glucoside (52).

However, in *Isatis* (52) the precursor was the ester, isatin B (indoxyl-5- ketogluconate), rather than the glucoside, indican. To form indigo from the precursors, the carbohydrate moiety is cleaved from the indoxyl group and two of the resulting indoxyl molecules combine oxidatively to produce an indigo molecule. In practice, once the molecule has been hydrolyzed, this combination occurs spontaneously in aerobic conditions and indigo precipitates from solution.

In 1881, Professor H.E. Roscoe delivered a lecture at the Royal Institution (53), crediting German scientist Professor Adolf Bayer for the first synthesis of synthetic indigo dye. In that lecture, he reviewed three main processes to obtain indigo dye, based on the principle that isatin, which is a product of oxidation of indigo, can be reduced back to indigo dye. However, one prepared from toluene as a first reactant was considered to be suitable for mass production of indigo. The yields were not good and the product was a mixture containing

mostly indigotin. Indigotin is responsible for color in indigo dye (54) (See Figure 2.13 for its structure).

In 1901, F. Mollwo Perkin, in his article "Indigo and Sugar" (55), mentioned that in 1882, one year after the lecture of Professor Roscoe, Professor Bayer and Professor Drewson figured out another way to synthesize indigo which is still used in laboratories to synthesize small quantities of indigo. They synthesized indigo by condensation of acetone and orthonitrobenzaldehyde in presence of caustic alkali, sodium hydroxide (56).

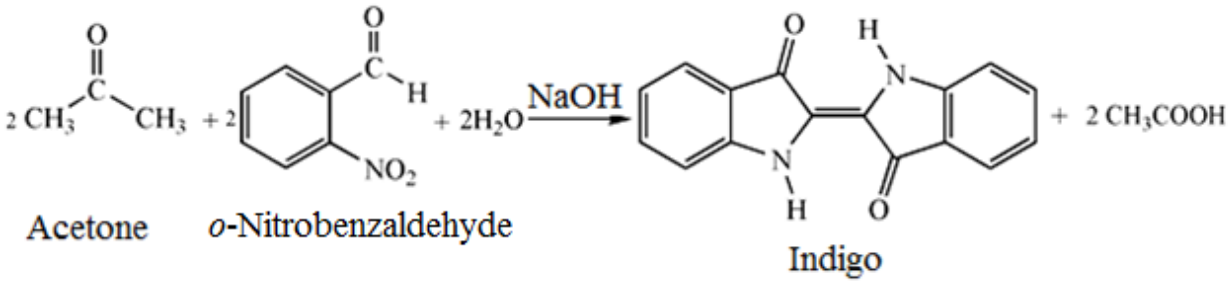


Figure 2.18. Bayer-Drewson indigo synthesis reaction (57).

In 1900, the company by the name of "Badische concern" published a method for the synthesis of synthetic indigo (57) based on the methods of K. Huemaan. They modified the Huemaan method and produced indigo on a mass scale.

Naphthalene is treated with strong sulphuric acid and mercury which results in phthalic anhydride. The product is reacted with ammonia to produce phthalimide. Phthalimide is treated with chlorine and caustic soda to produce anthranilic acid (57). There are two alternate routes by either of which phenylglycine-o-carboxylic acid may be derived. First

method of converting anthranilic acid into phenylglycine-o-carboxylic acid is by reacting anthranilic acid with chloracetic acid. Second method of converting anthranilic acid into phenylglycine-o-carboxylic acid is in assistance with formaldehyde bisulphite acid and potassium cyanide. Indigo is obtained by heating with caustic soda (58).

Figure 2.19 shows the reaction of synthesis of indigo from naphthalene (57, 58).

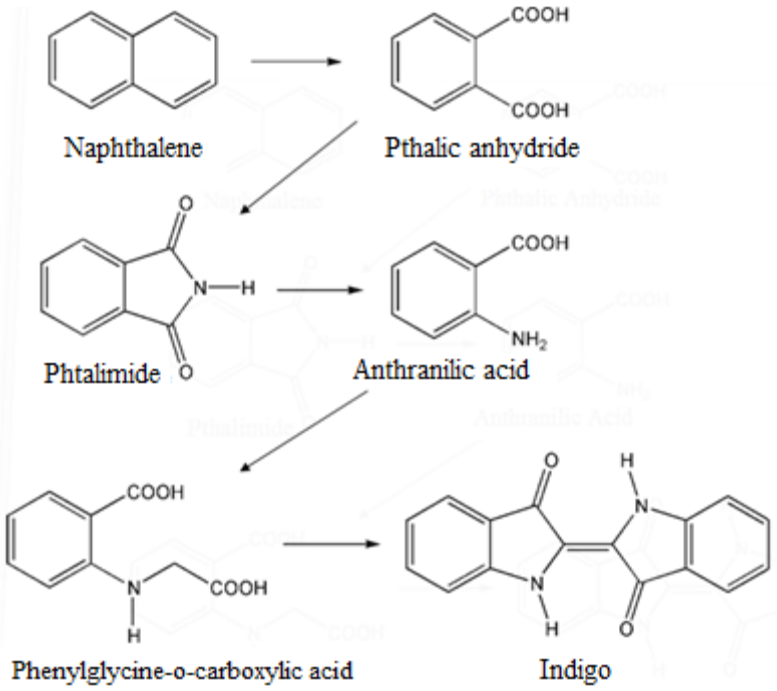


Figure 2. 19. Synthesis of indigo from naphthalene.

Another method to synthesize indigo is from benzene. Benzene is nitrated and reduced to aniline. Aniline is heated with chloroacetic acid and phenylglycine is formed. Phenylglycine is fused with sodamide ($NaNH_2$), to produce indoxyl. This reaction also produces caustic

soda and ammonia is given off during the process. It can be collected and used again for preparing sodamide. The product obtained by this fusion reaction is now dissolved in water. When the air is blown in the system, indoxyl is oxidized and indigo is formed (51, 52). In Figure 2.20, synthesis of indigo is shown from benzene, starting from aniline (51, 52).

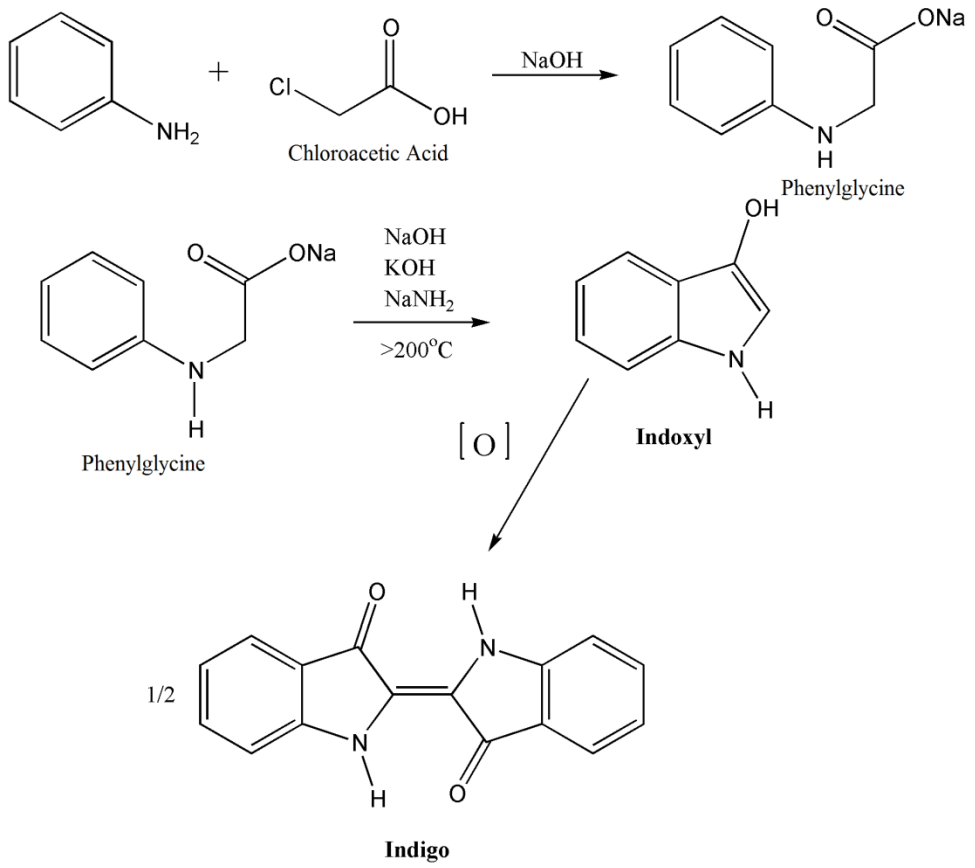


Figure 2.20. Synthesis of indigo from aniline.

Indigo can also be synthesized from aniline. Carbon disulphide is reacted with aniline to produce diphenylthiourea. Then this substance is converted into hydrocyancarbo-diphenylimide with the assistance of potassium cyanide and lead carbonate. This imide is then treated with ammonia sulphide to produce thioamide. Sulphuric acid is then reacted with thioamide to produce alpha- isatinanilide. By reacting ammonium sulphide with alpha-isatinanilide, indigo and aniline are produced.

In 2010, a new novel method of development of indigo dyes was proposed by Yamamoto, et al (59). They developed a novel and highly practical one-pot synthesis of indigo from indole via 3-position selective oxidation of indole and dimerization of the indole moiety. Using 0.1mol% of molybdenum complex and 2.2 equivalents of cumene hydroperoxide in tert-butyl alcohol, the reaction was complete in 7 h and pure indigo was obtained in 81% yield as a deep blue solid just by filtration. Figure 2.21 shows the proposed a mechanism for this reaction.

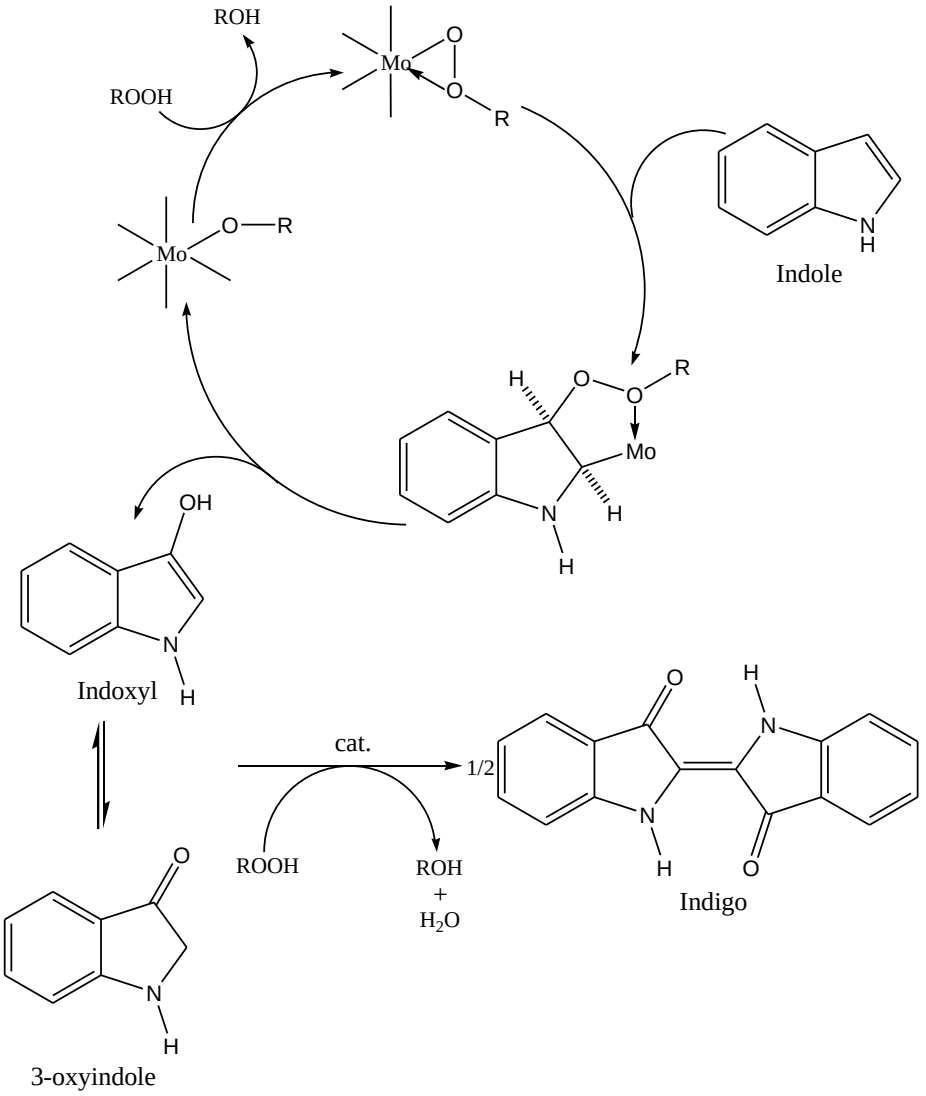


Figure 2.21. Mechanism of conversion of indole to indigo.

Natural indigo is chemically indistinguishable from its synthetic counterpart and it is very difficult to distinguish it even by modern testing equipment. However, Micro-Raman Spectroscopy (60) has helped chemist differentiate between natural and synthetic indigo by using several chemometrics techniques. Among these techniques, principal component

analysis (PCA) was helpful in distinguishing between the synthetic and natural indigo. The best discrimination was obtained by using 2nd derivatives and up to 4 principle components (PCs). By comparing the loading plots, it was possible to identify the spectral features that are signs for the discrimination between natural and synthetic indigo species. In these loading plots, Raman bands at 1582 cm^{-1} and 1571 cm^{-1} contribute to the discrimination between natural and synthetic indigo. Figure 2.22 shows the loading plots which are used to distinguish between synthetic and natural indigo dye (60).

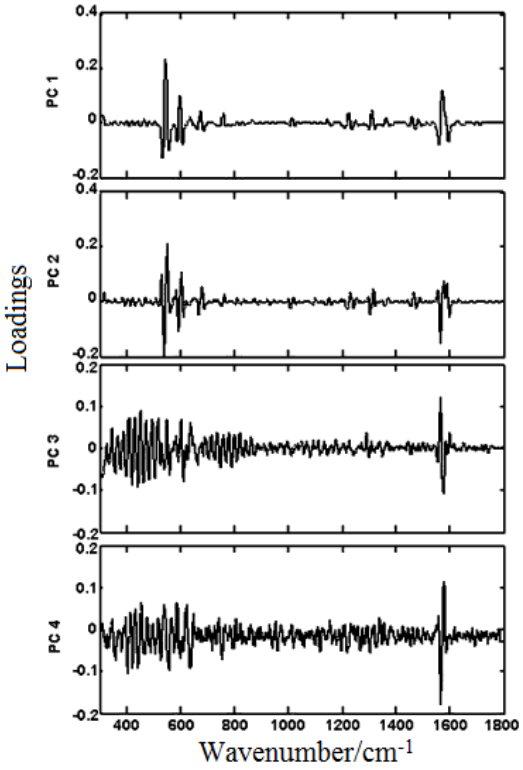


Figure 2.22. Loading plots for the first 4 principal components of synthetic and natural indigo.

2.3.4. Effect of laccase on indigo

Laccase enzyme degrade indigo and reduces its particle size (42)**.** Laccase enzyme extracted from Trametes hirusa (TH) and Sclerotium rolfsii (SR**)** has an effect on particle size of indigo. It seems to reduce the size of indigo dye particle**.**

An important observation in Figure 2.23 and Figure 2.24 (42) is the effect of time in reduction of particle size. It can be observed that exposure of indigo to laccase for long periods of time will further reduce the particle size.

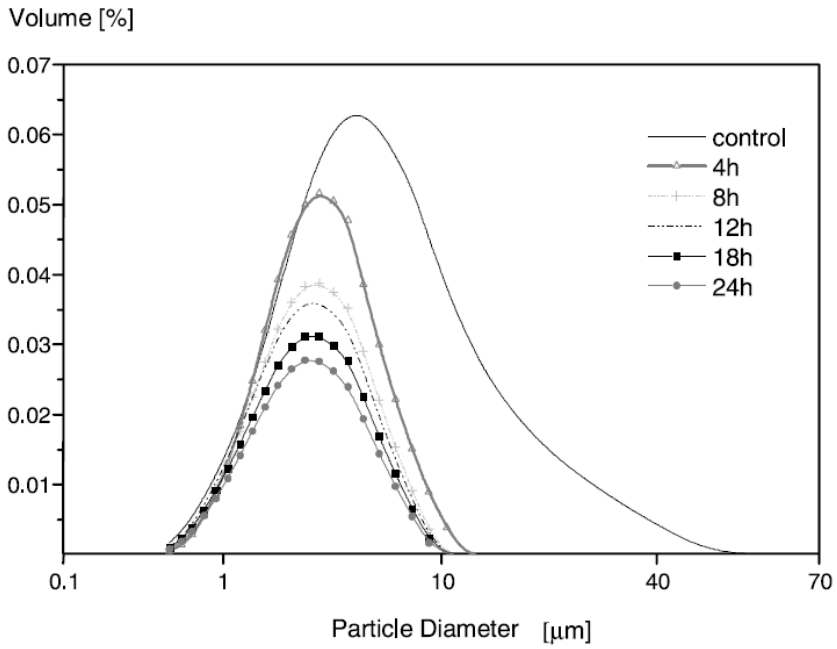


Figure 2.23. Indigo dye particle size reduction by TH Laccase.

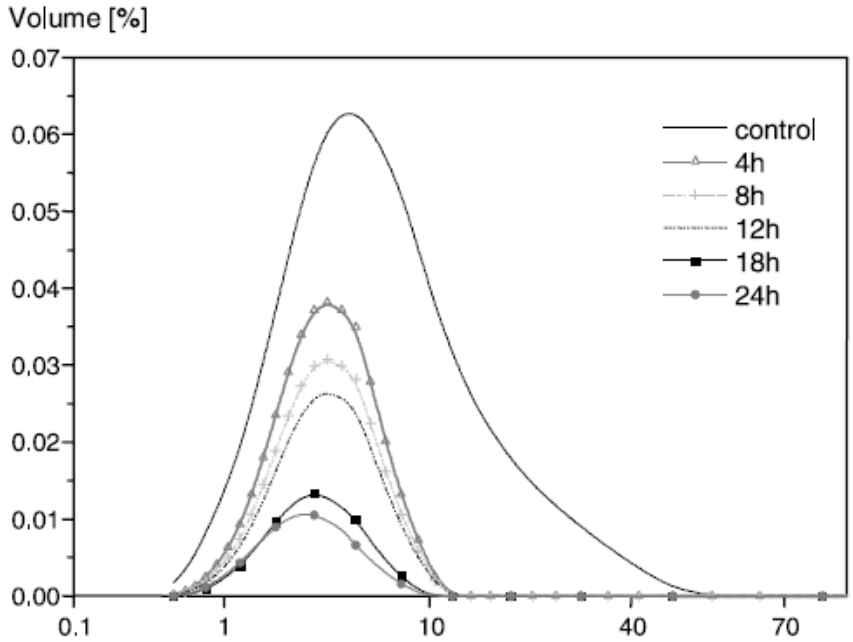


Figure 2.24. Indigo dye particle size reduction by SR.

The mechanism of indigo degradation by laccase was proposed by R Campos (42) and coworkers. Anthranilic acid and isatin were found after the degradation of indigo by enzyme extracted from TH and SR.

There is step wise abstraction of four electrons from the substrate. In the first step, there is an oxidation of indigo to dehydroindigo. This dehydroindigo is oxidized to isatic acid. Isatic acid is not stable, therefore it will decompose by decarboxylation to produce anthranilic acid as a final product. There are several factors which may effect this degradation such as adsorption, transport limitations, accessibility and redox potential of the laccase and the substrate.

Figure 2.25 shows the mechanism of reduction of indigo to anthranilic acid in the presence of laccase enzyme (42).

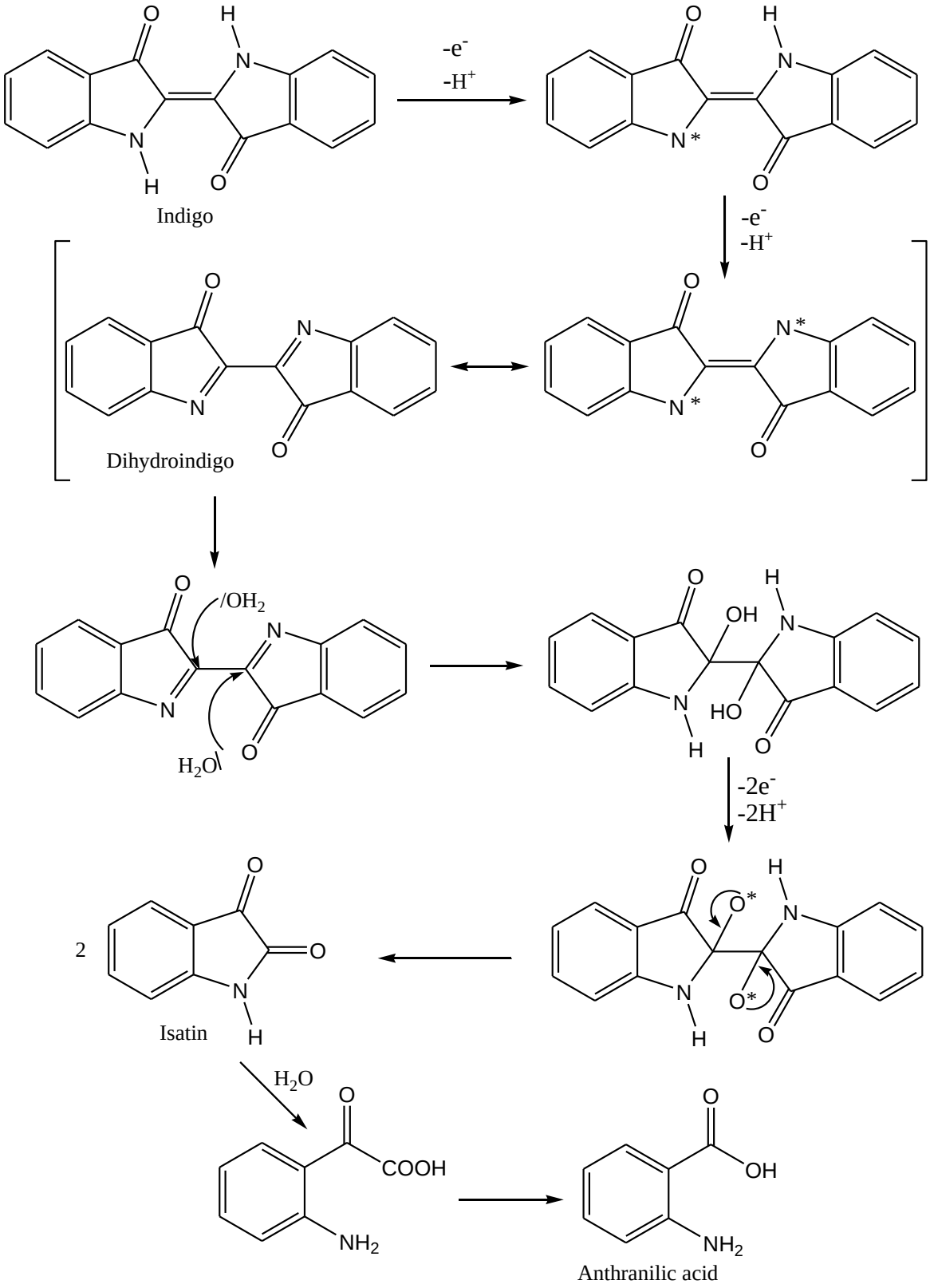

Indigo
-e-
-H+
Dihydroindigo
/OH2
H2O
-2e-
-2H+
2
Isatin
H2O
COOH
NH2
OH
NH2
Anthranilic acid

Figure 2. 25. Degradation of indigo by laccase.

In degradation of indigo by laccase, exposure time plays an important role in the degradation of indigo. In Figure 2.26, it can be observed that more the exposure time, more degradation products are formed.

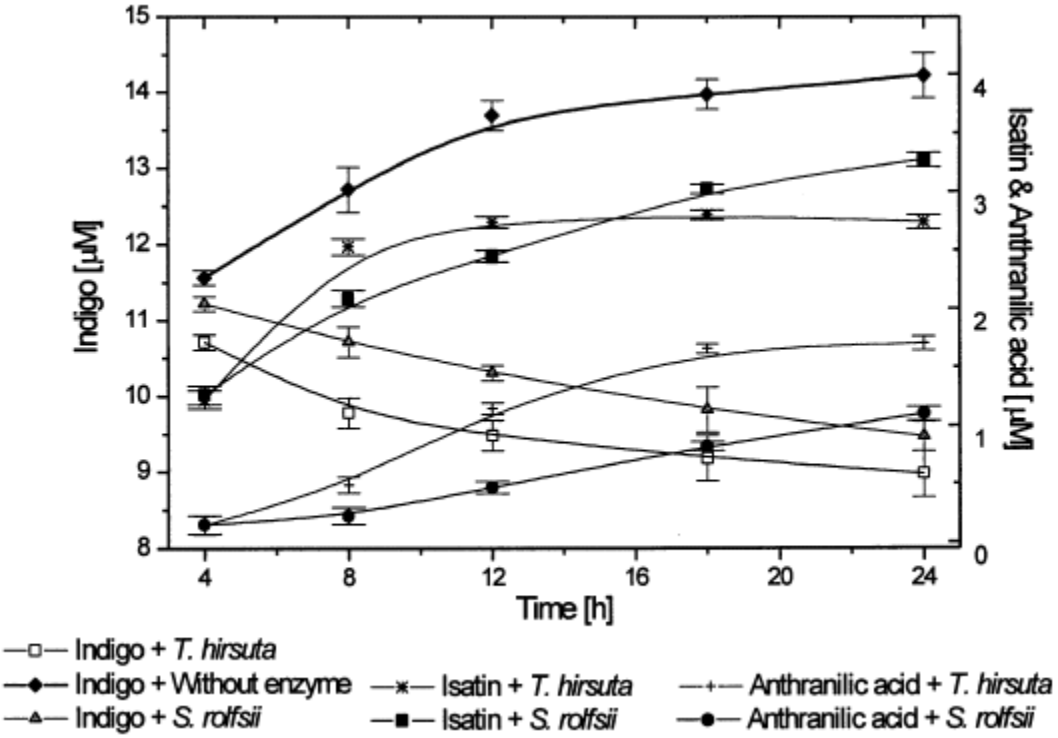


Figure 2.26. Degradation products formed from indigo stained fabrics treated with enzyme preparation from T.hisuta (TH) and S. rolfsii.

In denim processing, indigo is exposed to laccase enzyme for only about 15 – 60 min. So, it will be interesting to see if anthranilic acid or isatin is produced in such a small time and how it impacts the particle size of the indigo.

2.3.5. Effect of pH and temperature

Laccase enzyme is affected by the change in pH and temperature. Laccase enzyme Novoprime Base 268 from Novozymes is the perfect example to study the effect of pH and Temperature.

Novoprime Base 268 is laccase based enzyme which is used for decolorization of indigo in denim processing. It can enhance the effect of stone wash finish and create a different shade from Novoprime B959. It is used with mediator, Novoprime F258, to shorten the processing time. Like Novoprime B959, it can be used in denim washing without back staining, maximum strength retention and improved garment contrast. The reported activity of Novoprime Base 268 is 800 LAMU/g (61).

Effect of pH and temperature on Novoprime Base 268

The pH and temperature plays a crucial role in the efficiency of Novoprime B268. The optimum pH is 4.0-5.5. There is a decrease in relative efficiency if the pH is lower than 4 and higher than 5.5 (61).

The optimum temperature for the processing of denim fabric by Novoprime B268 lies between 60°C to 70 °C. There is a decrease in relative efficiency if the temperature is lower than 60 °C and slight decrease when the temperature is higher than 70 °C (61).

Figure 2.27 and Figure 2.28 (61) show the effect of pH and temperature on Novoprime Base 268.

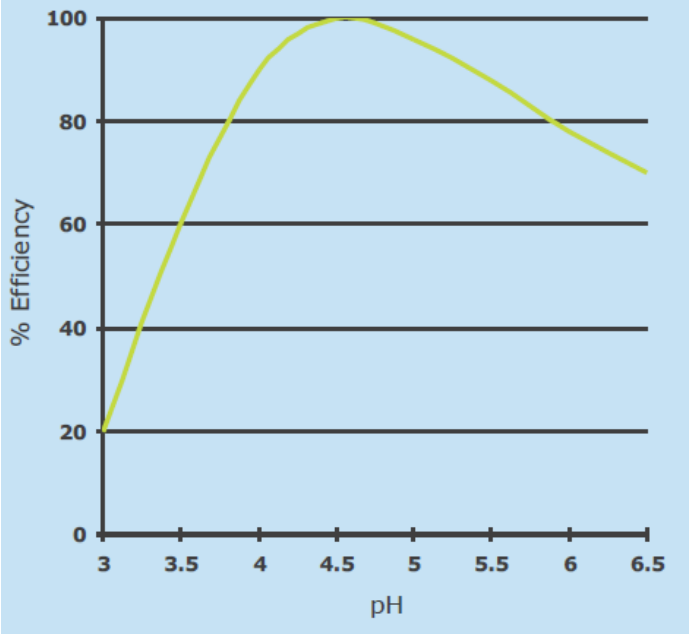


Figure 2.27. Effect of pH on efficiency of Novoprime B268.

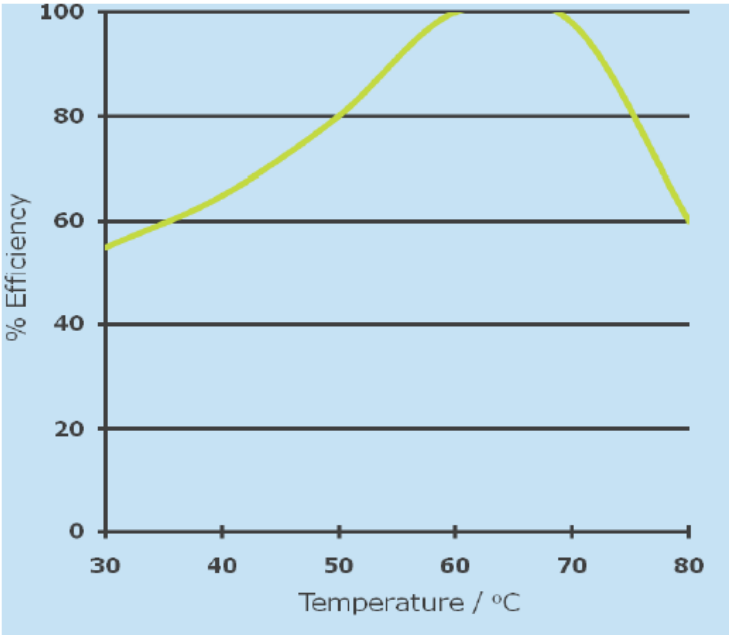


Figure 2.28. Effect of temperature on efficiency of Novoprime B268.

Novoprime Base 268 works best with Novoprime F258, which function as a mediator. The combine action of the Novoprime 268 and Novoprime F 258 can be used in denim processing applications specifically to decolorize indigo dyes and create unique finishes on denim fabric. Novoprime F 258 is based on methyl syringate (i.e. methyl 3,5-dimethoxy-4-hydroxybenzoate), which is low molecular weight organic compound. Novoprime F 258 has 12% wax, which is used to prevent dusting.

2.3.6. Application of indigo dye on denim

Indigo dye is insoluble in water but can be reduced to acid leuco indigo, an off white solid. A mixture of hydrosulfite and alkali converts indigo to a readily water soluble form that has modest substantivity to cellulose. Once it is adsorbed to the cellulose it is oxidized back to its indigo form. Figure 2.29 shows the conversion of indigo in to water soluble leuco indigo and converting back to indigo after oxidation.

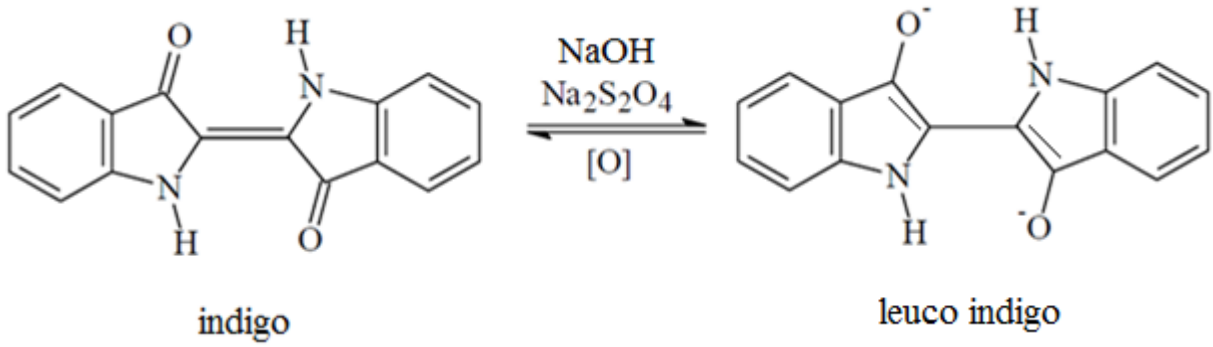


Figure 2.29.The reaction shows the conversion of insoluble indigo into soluble leuco indigo.

In the textile industry, indigo is used to dye warp yarns for denim fabric. It is a common practice in industry to use pre-reduced indigo for the convenience of the dyer. After yarn is converted to denim fabric and the fabric is cut into patterns, it is washed in the presence of different materials such a pumice stones and chemicals to impart a finished look. During this process there is loss of indigo dye and in some cases there is a reduction of indigo.

2.4 Denim

2.4.1 History

Denim is one of the few fabrics which are famous in all socio economic groups. Denim started when Levi Strauss invented jeans in 1853. Miners in San Francisco complained about easily torn britches and pockets that split right out, spilling the ore that miners stored inside. When Levi Strauss learned about it, he came up with the idea of making rugged overalls from the canvas he originally bought for making tents. It turned out be very popular among the miners. The design of jeans was patented in 1873 and full production of jeans started in 1876 (62).

2.4.2 Denim specification

The warp yarn is traditionally dyed with indigo dye. In a twill weave (used in denim), the fabric is constructed by interlacing warp and filling yarns in a progressive alternation which creates a diagonal effect on the face, or right side, of the fabric and has a surface of diagonal parallel ridges. In some twill weave fabrics, the diagonal effect may also be seen clearly on the back side of the fabric. Due to the denim's right-hand twill construction, one color

predominates on the fabric surface. Also, because of this way of weaving the threads to make the fabric, the fabric is very strong and durable. Classic denim fabric is 2x1 twill.

2.4.3. Innovation in denim fabric

Denim is a fashion driven market which means that there is a rapid change in trends and styles in denim industry. In order to cope with this change suppliers had to innovate in processing of denim and introduced different blends and finishes.

2.4.3.1 Topping and bottoming

Sulfur bottoming is a process in which warp yarns are pretreated with sulfur dye prior to being dipped into indigo. This promotes a quicker wash down (the sulfur protects the yarn's core from the indigo), and can change the cast or hue of the denim to yellow or gray for a vintage look. Sulphur bottoms can be regular, heavy or extra heavy, depending on the desired effect. It is also used to give a darker look on the denims without using extra indigo (1).

Sulfur topping is a process in which yarns are sulfur dyed after they have been indigo-dyed. This adds depth to color and is sometimes used to create novelty looks.

2.4.3.2 Blends

Blending of different fibers with cotton is a common practice in home textile industry and other consumer markets but blending other fibers in denim fabric is comparatively a new trend. Polyester blends, nylon, bamboo, lycra and viscose rayon are regular fibers present in denim jeans nowadays.

2.4.3.3 Novel finishes

Novel finishes like antimicrobial finishes, resin finishes etc are applied on denim which were unheard of a decade ago.

2.4.4 Finishes in denim

Denim finishing is one of the most unpredictable processes in the textile industry. Two fabrics of the same weight and similar concentration of chemical put together in a same machine can result in different end products.

Finishing of denim garment is usually referred as denim washing. There are two kinds of processes:

1. Dry process
2. Wet process

2.4.4.1 Dry process

Dry processes are finishes applied mechanically on the denim garment fabric. There is no use of water or any other chemical in these kind of processes. Some of them are:

Brushing

This process is usually carried out to after the fabric is stitched in to a garment. Emery paper is used to brush away indigo from the surface of denim fabric. The garment is mounted on a air blown mannequin and then garment is scratched by emery paper to remove dye from the

fabric, which expose the white surface of the yarn and creates a very interesting “contrasting” and “ageing” effect.

Whiskers

Whiskers are the horizontal wrinkled lines are produced on the fabric (63). The effects is similar to whiskers on animals i.e. the hair around nostrils of animals, such as cat, fox etc. Whiskers are created manually with help of sharp edge Emery paper rolled on fine wood stick or pasted on plastic material. Before starting execution placements and pattern must be marked on garments, this will help operator to execute the pattern right to match the aesthetics of garment (64).

Destruction of denim

“Destruction” is an art which make denim look unique and used. To create a used look a “pen” type tool is used during the wash process on desired areas of the denim garment. It can also be achieved by cutting it through knife the warp yarns and keep the weft yarn as is to show white thread. Holes also can be made by cutting weft & warp yarns. These are all manual processes and every garment will look unique and different than others (64).

Grinding

The grinding tools will chip away at the fabric and break the yarn to get small tears and nicks. This abrasive damage to the fabric gives color loss at seams and is most commonly used at the bottom hem and pocket edges.

Laser etching

This comparatively new technique applied to denim fabric. The pattern or any other fading effect can be made on denim fabric using CO_2 and Nd:YAG laser machines (65). But studies have shown that it has an effect on structure of textile fibers. (65).

Other dry finishes

They are many mechanical processes available which can abrade denim fabric and create different looks including tagging, clipping, skate board wheels rubbing and pigment painting.

2.4.4.2 Wet process

There is great versatility in wet processing finishing of denim fabric. Before applying any finish on the fabric, pretreatment is very important because it has an immense effect on the quality of end product. In pretreatment, fabric is cleared of any unwanted chemical or material like size, stains and dust. After pretreatment, chemical finishes can be applied on fabric or abrade the indigo dye to create different looks.

Denim wet process can be classified in to two types:

- Chemical
- Mechanical

Chemical washes

In chemical wash treatments, the fabric color is reduced by the help of chemicals such as bleach, enzymes and acids.

Tinting and over dyeing is also carried out to give a different tone and hue to the fabric. Applying a light yellow direct dye on denim can create a dirty look on the fabric.

Mechanical finishes

These finishes are the oldest type of denim finishes. The effects obtained by chemical finishes were inspired by the results obtained from mechanical finishes such as stone wash and sand blasting.

Stone washing involves the use of pumice stone. The stones are inserted into the washing machine with water and other auxiliaries to create a worn look in denim fabric.

Sand blasting is now banned by many companies such as “H N M”, because it has various health hazards (66).

2.5 Inkjet Printing

Ink jet is a technology that enables the delivery of liquid ink to a medium whereby only the ink drops make contact with the medium. It is therefore a non- impact printing method. Lord Raleigh was instrumental in developing the elementary hypothesis behind the ink jet technology but the development of the technology itself did not take root until the early 1960s (67). The first inkjet printing device was patented by Siemens in 1951, which led to the introduction of one of the first inkjet chart recorders.

The continuous inkjet printer technology was developed later by IBM in the 1970s. The goal continuous inkjet technology basis is to deflect and control a continuous inkjet droplet stream

direction onto the printed media or into a gutter for recirculation by applying an electric field to previously charged inkjet droplets.

The drop-on-demand inkjet printer technology was led to the market in 1977 when Siemens introduced the PT-80 serial character printer. The drop-on-demand printer ejects ink droplets only when they are needed to print on the media. This method eliminates the complexity of the hardware required for the continuous inkjet printing technology. In these first inkjet printers ink drops are ejected by a pressure wave created by the mechanical motion of the piezoelectric ceramic.

Companies such as Hewlett Packard (HP) are producing printers which specializes printing on fabric but the fabric needs special paper backing in order to get print on it.

Laccase was printed on indigo dyed fabric by inkjet printer (68) but the fabric used was not denim. Several poplin fabrics were dyed by soaking in indigo dye solution for 5 minutes and some for 2 hrs. Indigo was not converted in leuco indigo during this dyeing process and dyeing was followed by detergent wash. Laccase enzyme was filled in inkjet printer cartridge and a block pattern was printed on the fabric. Some of the printed fabrics were steamed and some were left for drying without steaming. Fabric was left to dry for 7 days and reading of L* were taken after two, three, four and seven days

The L* value of standard was mentioned as 41.28 and the maximum increase in L* value was observed in the fabric which was steamed and left for seven days, which was 46.98.

2.6 Project Proposal

Most denim finishing processes involve fading of color in a haphazard way to create a worn out look. However, only a few techniques can be used to make a pattern on a fabric, examples of which are:

- Laser Etching
- Bleaching
- Emery Brushing

All of the aforementioned processes affect the textile fiber and none of them are suitable for mass production of denim.

The aims of this research are: 1) to develop a cost effective way to produce a pattern on denim fabric without having a destructive effect on fabric properties; 2) to make a fabric design using enzymes because enzymes are environmental friendly and minimally destructive on the textile fibers.

The following steps will be used in this research:

1. Identify a suitable enzyme for making a pattern on denim
2. Identify a hydrophobic compound that can resist enzyme penetration into the fabric
3. Identify a hydrophobic compound that can withstand the vigorous conditions in washing machine
4. Design a new process for making a pattern that is repeatable.

Experiments were carried out in order to determine:

1. Best enzyme for pattern making on indigo dyed denim
2. Optimum concentration of chosen enzyme
3. Best technique for pattern making
4. Best technique for repeatable results
5. Compounds formed by degradation of indigo by laccase

3. Experimental Work

3.1 General

The desizing enzyme (Aquazyme Ultra 1200 N), cellulase enzyme (Novoprime B959) and laccase enzyme (Novoprime B268) were obtained from Novozymes, Denmark. Mediator (Novoprime F 258) was obtained from Novozymes, Denmark. Another laccase enzyme (Genencor® PrimaGreen® EcoFade LT100) was obtained from Genencor, Denmark. Sodium dihydrogen phosphate (SDHP), 2-amino-9-hydroxymethy)-1,3-propanediol, MES hydrate, syringaldazine 19µM, poly(ethylene glycol), sodium acetate, paraffin wax and stearic acid were obtained from Sigma Aldrich, St. Louis, Missouri. Non-woven poly(ethylene) sheets were obtained from the Non wovens Laboratory, North Carolina State University.

Inkjet cartridges were purchased from Ningbo Yadi Import and Export Company Limited. HP Deskjet D1000 and HP deskjet 3847 printers were purchased from Hewlett-Packard, U.S.A.

Reynolds Freezer paper for fabric paper backing was purchased from Walmart Store. Filter paper used was “Whatman type 2”.

The fabric used in all these experiments was denim cotton fabric. The construction was 2 x 1 twill. The warp yarn was indigo dyed and weft yarn was undyed. The fabric was woven at the Weaving Laboratory, North Carolina State University. The yarn was bought from Cotton

Incorporated, Cary, North Carolina. The yarn count was 10/1, color description was 250 indigo and mix number was 324.

3.2 Enzymatic treatments

3.2.1 Sample preparation for enzyme treatments

Denim fabrics (280 g) were cut into rectangular shapes of approximately 4 inches x 6 inches. These rectangular fabrics were cut into half and the ends of each one stitched in such a way that they formed a tubular legging.

3.2.2 Fabric treatments

Aquazym Ultra 1200 N

Denim fabric was desized by using the recipe and the conditions in Table 3.1.

Table 3.1. Desize recipe and conditions.

Weight of fabric	280 g
Liquor Ratio	1:15
Water	5 L
Aquazym	5g/L
Acetic acid	0.2g/L
pH	4.7
Machine	Wernes Mathis AG. Jumbo Jet
Temperature	70°C
Time	30 min

Novoprime Base 268

Denim fabric (21.7 g) was treated with Novoprime Base 268 using recipe and conditions in Table 3.2.

Table 3.2. Recipe for Novoprime B268 enzyme treatment conditions.

Weight of Fabric	21.7 g
Liquor Ratio	1:10
Water	250 mL
Novoprime B268	0.225g/250 mL
Novoprime F258	0.105g / 250 mL
Acetic acid	0.585g
SDHP	0.585g
pH	5
Machine	Ahiba Nuance Eco
Temperature	60°C
Time	25 min

Genencor® PrimaGreen® EcoFade LT100

Denim fabric (21.7 g) was treated with Genencor® PrimaGreen® EcoFade LT100 by using recipe and conditions in Table 3.3.

Table 3.3. Recipe for treatment with Genencor® PrimaGreen® EcoFade LT100.

Weight of Fabric	21.7 g
Liquor Ratio	1:10
Water	250 mL
LT100	0.225g/250 mL
pH	5
Machine	Ahiba Nuance Eco
Temperature	25 °C
Time	25 min

Novoprime B959

Denim fabric (60 g) was treated with Novoprime B959 by using recipe and conditions in Table 3.4.

Table 3.4. Recipe for Novoprime B959 washing.

Weight of Fabric	60 g
Liquor Ratio	1:10
Water	600 mL
Novoprime B959	2.5 g/600 mL
Acetic acid	0.2g/L
pH	4.5
Machine	Ahiba Nuance Eco
Temperature	55°C
Time	55 min

After the Novoprime B959 enzyme wash, the fabric was treated with 1 g/L soda ash dissolved in 1L of water at 80 °C to deactivate enzyme.

3.3 Combination enzyme treatments

Desized denim fabric resulting from recipe and conditions using Table 3.1 was treated with Novoprime B959. In this experiment, sodium dihydrogen phosphate (SDHP) was replaced with sodium acetate as a buffer. The ratio of acetic acid to sodium acetate was 1:4 to maintain the pH at 4.5-4.6. The 48 g fabric sample was cut into four squares. These squares were cut in half and the edges of each one stitched in such a way that they formed a tubular legging. Eight tabular leggings were made. The recipe and conditions used are given Table 3.5.

Table 3.5. Novoprime B959 treatment with sodium acetate as buffer.

Weight of fabric	48 g
Liquor Ratio	1:10
Water	480 mL
Novoprime B959	4 g
Acetic acid	0.54g/480 mL
Sodium acetate	2.16 g/480 mL
Machine	Ahiba Nuance Eco
Temperature	55°C
Time	55 min
pH	4.5

After the Novoprime B959 enzyme wash, the fabric was treated with 1 g/L soda ash dissolved in 1L of water at 80 °C to deactivate enzyme.

Four leggings obtained from the Novoprime B959 wash were reused in this experiment. Novoprime B959 treated fabric was washed with Novoprime B26* according to the formulations and conditions given in Table 3.6.

Table 3.6. Novoprime B268 wash with SDHP as buffer.

Weight of the fabric	24g
Liquor Ratio	1:7
Water	250 mL
Novoprime B268	0.15g/250 mL
Novoprime F258	0.08g / 250 mL
Acetic acid	To adjust pH
SDHP	0.2 g
pH	5
Machine	Ahiba Nuance Eco
Temperature	70°C
Time	35 min

The remaining four leggings obtained from Novoprime B959 treatment were washed with Genencor® PrimaGreen® EcoFade LT100 by using the recipe shown in Table 3.7.

Table 3.7. Recipe and conditions for Genencor® PrimaGreen® EcoFade LT100.

Weight of Fabric	24 g
Liquor Ratio	1:7
Water	250 mL
LT100	0.15g/250 mL
pH	5
Machine	Ahiba Nuance Eco
Temperature	25 ℃
Time	25 min

3.4 Determination of optimum number of washes for enzymatic treatment

Denim fabric was desized using the method and recipe in section 3.2.2. Desized fabric (100 g) was washed five times with Novoprime B268 using recipe and conditions in Table 3.8 for each wash cycle.

Table 3.8. Recipe for Novoprime B268 for five wash cycles.

Weight of fabric	100g
Water	1000 mL
B268	15.12 g
F258	7.01 g
Sodium acetate	44.28 g
Acetic acid	19.45 g
pH	4.8
Temperature	70 ℃
Time	35 min

Desized fabric (100g) was washed four times with Genencor® PrimaGreen® EcoFade LT100 using recipe and conditions in Table 3.9 for each wash cycle.

Table 3.9. Recipe for Genencor® PrimaGreen® EcoFade LT100 for four wash cycles.

Weight of fabric	100 g
Water	1000 mL
LT 100	15.12 g
pH	4.8
Temperature	70 °C
Time	35 min

3.5 Measurement of laccase activity by a syringaldazine based assay

The purpose of this experiment was to measure enzyme activity. This procedure was provided by Novozymes Inc.

3.5.1 Syringaldazine stock solution

A stock solution was prepared by dissolving syringaldazine of 19µM (10 mg) in 50 mL ethanol (96%) for 3h. The storage stability of the stock solution was 5 days when kept in the dark at 4°C.

3.5.2 Syringaldazine solution for analysis

Syringaldazine solution (0.28 mM) was prepared by mixing 25 mL of the stock solution with 25 mL deionized water. The stability of syringaldazine solution was checked by preparing a solution containing 300 µL of 0.28 mM syringaldazine, 5 mL ethanol (96%) and 4.7 mL deionized water and the absorbance was measured at 360 nm in a 1 cm cuvette. The absorbance reading should be 0.3 +/- 0.015 for stable syringaldazine solution. The storage stability of this analytical solution is 4 h at room temperature.

3.5.3 Procedure for measuring laccase activity

Laccase enzymes was diluted with the dilution medium (polyethylene glycol PEG 6000 = 50 g/L; Triton X-10 =10 g/L) to an expected activity of 0.18 units/mL. Each weighed sample was analyzed in duplicate.

Buffer of pH 7.5 was prepared by mixing 25 mL of 1.0 M Tris (tris (2-amino-2-(hydroxymethyl) propane-1,3-diol)) solution with 5 mL of 1.0 M maleic acid and deionized water up to 1 L. The pH for this solution was checked and was found to be 7.50 +/- 0.05. Buffer (4 mL) was preheated for 10 min at 30°C.

A 100 μL diluted laccase sample was added and the solution was stirred. The temperature was maintained at 30°C. Syringaldazine solution (300 μL of 0.28 mM) was added (with mixing) whereby the reaction begins. The sample was placed in the spectrophotometer using a cuvette and the change in absorbance was measured at 530 nm every 15 s up to 90 s. Between the analysis of each sample the cuvette was rinsed with a 6% aqueous ethanol solution. The following specifications should be used for this procedure:

Specifications

Recommended enzyme concentration in sample	:	0.18 units/mL
Lower limit of detection in sample	:	0.007 units/mL
Lower limit of quantification in sample	:	0.07 units/mL
Upper limit of quantification in sample	:	0.28 units/mL
Wavelength	:	530 nm

Absorbance range : 0.1 - 0.4 ΔABS/min

3.5.4. LAMU calculation

LAMU activity of the sample was calculated using the following formula.

Activity = ((Δ ABS x 4.4 mL x 10^{-3}) / (0.065 x 0.1 mL)) x D

Which is simplified to,

Activity = ΔABS x 0.677 x D

Where,

Δ ABS: Change in absorbance per minute

4.4 : Total volume (mL)

0.1 : Sample volume (mL)

0.065 : μ molar absorption coefficient

D : further dilution factor (of the sample)

3.6 Fabric preparation for Ink Jet Printing

Two methods were developed for this study.

3.6.1 Method 1 (Figure 3.1)

1) A solution of 2.5 g of Novoprime B 268 (Laccase enzyme), 1.25 g of Novoprime F258 (mediator), 6 g of sodium dihydrogen phosphate and a few drops of glacial acetic acid (to set pH to 4.8) in 100 mL of water was prepared. This enzyme solution was padded on denim fabric at 70% wet pickup. The fabric was stored at 70°C for 35

min. Fabric was washed in hot water and containing 1g/L solution of non-ionic detergent.

2) Novoprime B 959 (18 g), sodium dihydrogen phosphate (6 g) and a few drops of glacial acetic acid (to set pH to 4.8) were dissolved in 500 mL of water. This enzyme solution was padded on denim fabric at 70% wet pickup. The fabric was stored at 70°C for 35 min. The fabric was washed in hot water containing 1g/L solution of non-ionic detergent.

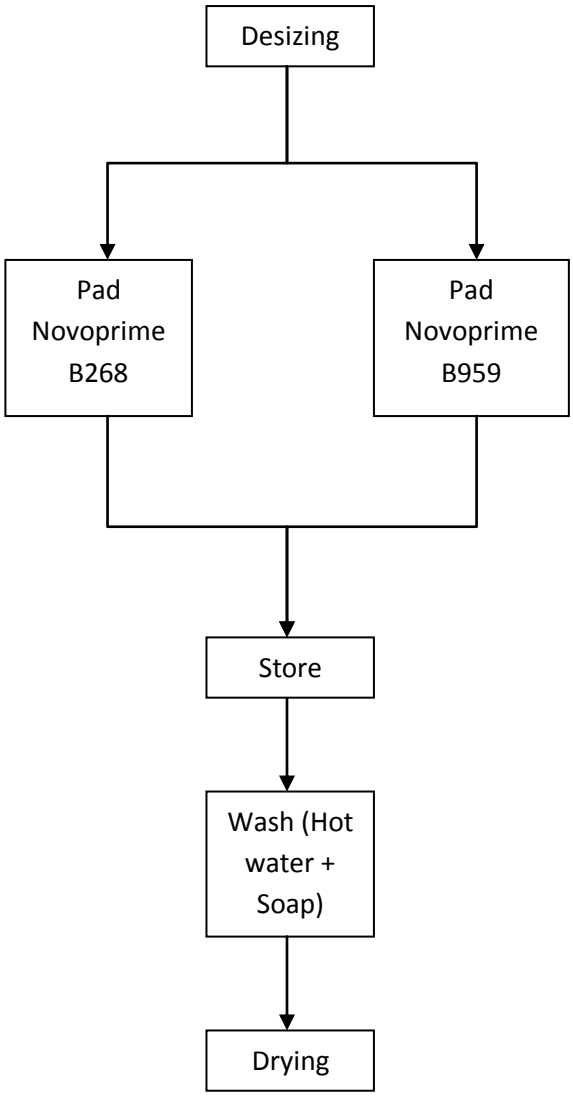


Figure 3.1. Denim fabric preparation by method 1.

3.6.2 Method 2 (Figure 3.2)

1) The desized denim fabric was padded with a diluted solution of glacial acetic acid and buffer (sodium acetate) of pH 4.5. After that 9 g Novoprime F258 (mediator) was dissolved in 500 mL water and this solution was padded on the desized fabric. Next a solution of 18 g Novoprime B 268 (laccase) in 500 mL water was padded on the fabric, using padder wet pickup of 70%. This fabric was stored at 70°C for 24 h and then fabric was washed in hot water containing 1g/L non-ionic detergent.

2) Genencor® PrimaGreen® EcoFade LT100 (18 g) was dissolved in 500 mL of water. This solution was padded on desized fabric and the fabric was stored for 24 h in a plastic bag at room temperature. Fabric was washed with hot water and dried.

3) Novoprime B 959 (18 g), 6 g of sodium dihydrogen phosphate (buffer) and a few drops of glacial acetic acid (to adjust the pH to 4.8) were dissolved in 500 mL water. This enzyme solution was padded on the fabric at 70% wet pickup. The fabric was stored at 70°C for 24 h. Fabric was washed by hot water and 1g/L non-ionic detergent.

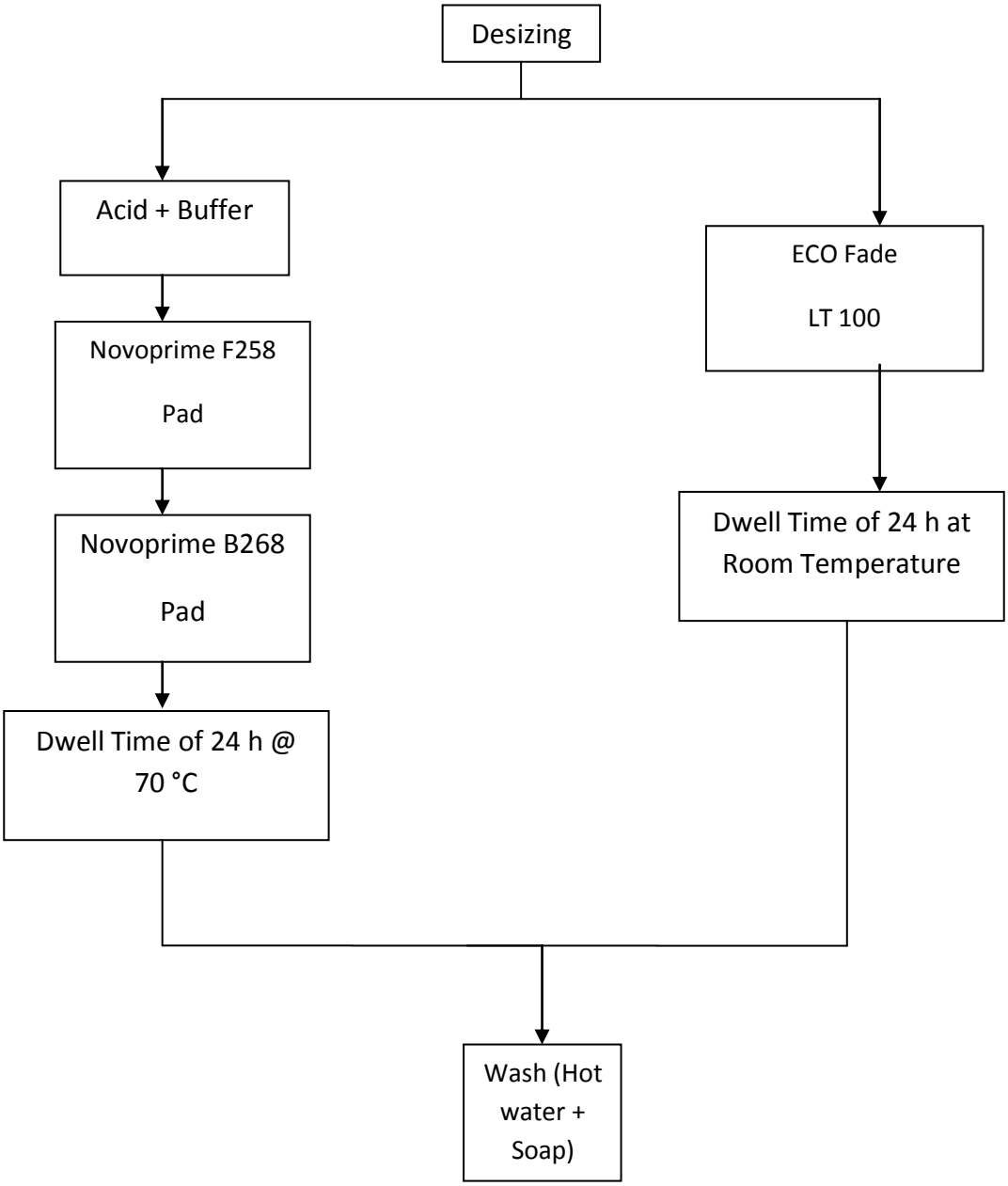


Figure 3.2. Denim fabric preparation by method 2.

3.7 Ink jet Printing

3.7.1 Fabric sample preparation

Desized fabric was coated with freezer paper on the back side with the help of a Black and Decker steam iron and then was cut into A4 size (i.e. 8.27 in × 11.69 in) using a Lectra cutting machine.

3.7.2 Enzyme solution preparation

Genencor® PrimaGreen® EcoFade LT100 (5 g) was dissolved in 50 mL of deionized water. This solution was filtered through Whatman Type 2 filter paper and injected into empty inkjet cartridges using a syringe. The filled cartridges were then installed into the inkjet printer.

3.7.3. Inkjet printing of enzyme solution

Using "Microsoft Paint" a pattern was printed on the fabric and stored for 24 h and 48 h at room temperature. L*, a* and b* values were recorded using a Datacolor instrument (parameters mentioned in section 3.10.5).

3.8. Screen printing

The desized denim fabric was cut in to pieces (7 in x 4 in). Novoprime B268 print paste was made using recipe in Table 3.10 and applied through a screen, which had a design on it. After printing, the fabric was stored for 30 min at 65 °C.

Table 3.10 Recipe for laccase print paste.

Water	600 mL
Solvitose C-5	100 g
B268	5 g
F258	2.5 g
SDHP	3.9 g
Acetic acid	3.9 g
pH	4.8

3.9. Pattern formation using resist processes

3.9.1. Wax resist application

Fabric involving wax application was prepared by following method:

1. A circular object was placed on the top of the fabric
2. Wax was melted and a film layer was applied to the fabric using a glass beaker.
3. The circular object was removed leaving an uncoated area.

The fabric prepared by the above method was washed using the recipe illustrated in Table 3.11 was used for enzyme washing:

Table 3.11. Recipe of cellulase enzyme for wax resist experiment.

Fabric weight	24 g
Liquor ratio	1:20
Water	480 mL
Novoprime B959	6 g
Acetic acid	0.2 g
SDHP	0.2 g
pH	4.5
Temperature	55°C
Time	55 min

3.9.2. Stearic acid resist application

Fabric involving stearic acid application was prepared by following method:

1. A star shaped object was placed on top of the fabric.
2. Stearic acid was melted and a film layer applied on the fabric using a star shape utensil.
3. The star object was removed leaving the uncoated area on fabric.

This fabric was washed with Genencor Eco Fade LT100 using the recipe shown in Table 3.12.

Table 3.12. Recipe for Genencor® PrimaGreen® EcoFade LT100 solution for stearic acid resist experiment.

Fabric weight	24 g
Water	480 mL
LT 100	2 g
pH	5
Temperature	25℃
Time	30 min

3.9.3. Polyethylene resist application

Two small swatches (2 in x 3 in) desized denim fabric were prepared. A small square pattern was cut from a polyethylene nonwoven sheet. Denim swatches were placed between polyethylene non woven sheets and hot-pressed at 90 ° C for 5 sec. After attaching the polyethylene nonwoven sheet to the fabric, the coated fabric was washed by Genencor®

PrimaGreen® EcoFade LT100 enzyme in an Ahiba Nuance machine using the formulation shown in Table 3.13.

Table 3.13. Recipe for washing of denim sandwiched between polyethylene nonwoven sheet.

Fabric weight	8 g
Liquor Ratio	1:10
Water	80 mL
Eco fade LT 100	1 g
pH	4.5
Temperature	25℃
Time	25 min

3.10 Analytical Methods

3.10.1 FT-IR

For FTIR, a Nicolet Nexus 470 instrument was used with following conditions

- Frequency Range: 400-5000 cm^{-1}
- Spectral Resolution: 0.125 cm^{-1}
- Beam splitters: KBr (375 - 7000 cm^{-1})
- Detector: DTGS (Deuterated triglycine sulphate)
- Source: IR (Globar)
- Location: Beamline 1.4
- Accessory: Single bounce attenuated total reflectance (ATR) with Germanium crystal
- Minimum resolution = 0.5

3.10.2. DSC

Perkin Elmer diamond Differential Scanning colorimeter (DSC) was used to measure the melting point of Polyethylene sheet. Liquid nitrogen gas was used to prevent ice formation. Samples were placed in non-volatile sample pans. Maximum heating temperature of the instrument was 720 °C. the temperature range is 25 °C to 300 °C.

3.10.3. Liquid chromatography–mass spectrometry (LC-MS)

Liquid chromatography–mass spectrometry (LC-MS) was carried out using an Agilent 1260 LC- MS QTOF instrument. The mobile phase was a degassed mixture of 95% H_2O containing 0.1% formaldehyde and 5 % methanol containing 0.1 % formaldehyde. The spectral region used for this experiment was 300 nm to 780 nm.

The mass spectrometer was equipped with a Dual EDI ion source. The gas temperature was 350 °C, with gas flow 12 l/min and Nebulizer set at 35 psi. Ion polarity was kept as positive.

Solutions (1mL) from afterwash treatments involving Novoprime B268, Novoprime B959 and Genencor® PrimaGreen® EcoFade LT100 were filtered and placed in sample cells.

3.10.4. TOF-SIMS

Time of Flight Secondary Ion Mass Spectrometer (TOF-SIMS) experiments were carried out on denim fabrics that were treated by Novoprime B268 and Genencor® PrimaGreen® EcoFade LT100 enzymes.

TOF-SIMS was also carried out on the indigo and laccase reduced indigo. Genencor® PrimaGreen® EcoFade LT100 (2 g) was dissolved in 100 mL of deionized water and indigo

(1 g) was added. An aliquot (1 mL) of this solution was dried on a silicon wafer and a spectrum was taken after 24 h reduction time and second after 96 h of exposure of indigo to laccase.

Machine used was “ION TOF” with following parameters

- Ion Beam Energy (ev) = 25000
- FoV (µm x µm) = 100 x 100
- Polarity = Negative
- Cycle Time = 100µs

3.10.5 Color assessment

Lightness (L*), a* and b* values were measured by Datacolor SX 650 instrument. Fabric was folded twice and presented to the spectrophotometer. Software used was “color icontrol” by Xrite with following conditions:

- UV energy = out/UV Inc
- R/T mode = reflectance
- Specular Condition = Included
- SAV = 9 mm
- Observer = 10°

3.11 Microscopic image

Indigo dyed yarn was removed from each fabric sample. These yarns were rolled in the nylon fibers and inserted in to a soft cork with a help of needle. A blade was used to cut this cork in

such a way that the cross-section of this yarn was exposed. Microscopic images of magnification 100 µm and 500 µm of sized, desized, and cellulase and laccase treated denim fabrics were captured using a Nikon eclipse microscope with a DS-Fi1 camera.

4. Results and Discussion

4.1. Comparison of enzymes

In order to determine which enzyme was best suited for achieving the goals of this research, three enzymes were evaluated with respect to their ability to increase the Lightness (L*) value of indigo dyed denim fabric.

Results from a single wash cycle using Novoprime B959, Genencor® PrimaGreen® EcoFade LT100 and Novoprime B268 are summarized in Table 4.1.

Table 4.1. Comparison of enzyme effectiveness on wash down of denim fabric shade.

Fabric Treatments	**L***	**a***	**b***
Amylase Desized	25.44	0.96	-13.93
Novoprime B959	34.81	-1.54	-16.43
Genencor LT100	31.54	-1.41	-17.04
Novoprime B268	26.89	-0.19	-11.1

It can be observed that there is a greater increase in Lightness (L*) value for denim fabric following the Novoprime B959 (cellulase) and the Genencor LT100 (laccase) washes as compared to Novoprime B268 (laccase) wash. Results in Table 4.2 indicate that Novoprime B268 (laccase) has minimal effects on indigo. Three to four washes are required to reach the L* values produced by a single wash involving the other two enzymes. Also, Figure 4.1. shows a graph for change in L* values as function of number of washes.

In order to show that laccase enzyme is capable of decolorizing indigo to such an extent that it removes dye all the way down to the cellulosic white core fiber, several washes were carried out. Spectrophotometer readings of this fabric were made and the results are summarized in Table 4.2. It can also be observed that there is a sharp L* increase after wash 5. This shows that Novoprime B268 has the ability to reduce layer after layer of indigo on the denim fabric.

Table 4.2. Effects of multiple Novoprime B268 washes on denim shade.

Number of Wash	**L***	**a***	**b***
Desized fabric	25.44	0.96	-13.93
1	26.89	-0.19	-11.1
2	28.51	-1.16	-11.11
3	31.78	-3.29	-7.87
4	33.72	-4.05	-7.8
5	43.04	-4.89	-3.26

Figure 4.1 shows the comparison of L* values with desized fabric.

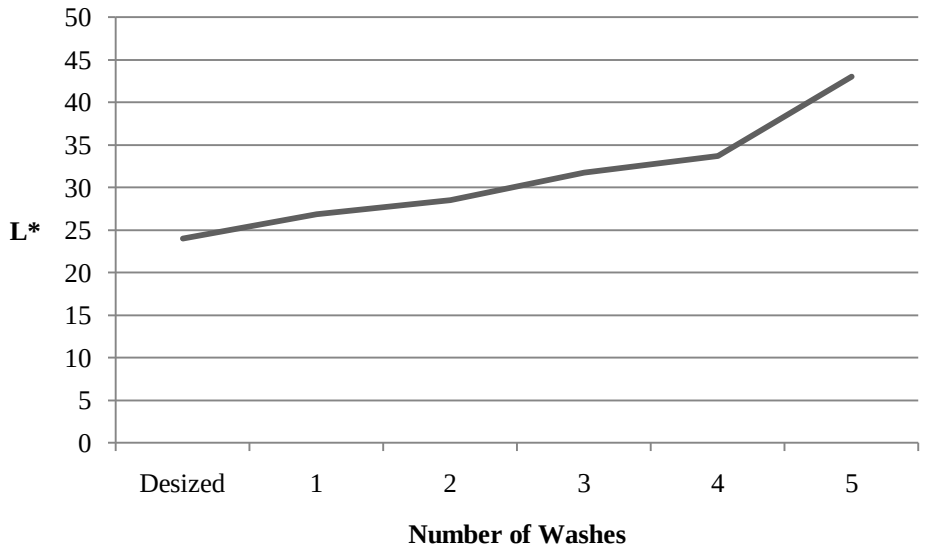


Figure 4.1. Effect of Novoprime B268 washes on denim.

A similar study was carried out using Genencor® PrimaGreen® EcoFade LT100 (laccase). The results showed step wise increase in L* value after each wash (see table 4.3). Figure 4.2 shows a graphical comparison of L* values versus number of washes.

Table 4.3. Effects of Genencor® PrimaGreen® EcoFade LT100 washes on denim fabric.

Number of Washes	L*	a*	b*
Desized fabric	25.44	0.96	-13.93
1	31.28	-2.02	-16.86
2	37.07	-2.71	-18.72
3	43.83	-4.18	-17
4	46.03	-4.64	-14.24

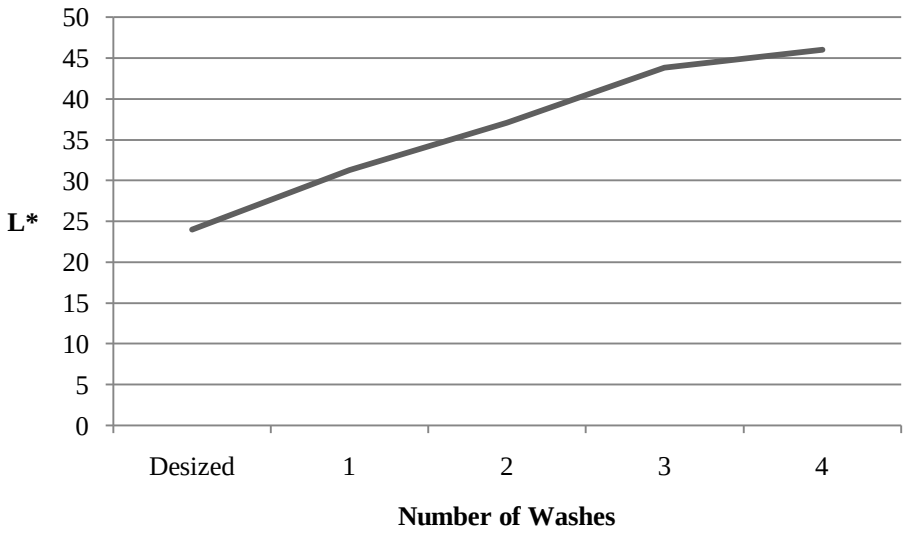


Figure 4.2. Effects of Genencor® PrimaGreen® EcoFade LT100 washes on denim shade.

It can be observed from Table 4.2 and Table 4.3 that Genencore LT 100 has more decolorization power than Novoprime B268 as it takes less wash cycles to achieve same L* values.

Microscopic images taken using a "Nikon eclipse microscope", show that indigo dye does not penetrate inside the core of the yarn. Figure 4.3 and Figure 4.4 show the 100 µm microscopic and 500µm microscopic images of sized indigo dyed yarn respectively. It can be observed that the fibers in the core of the yarn are undyed.

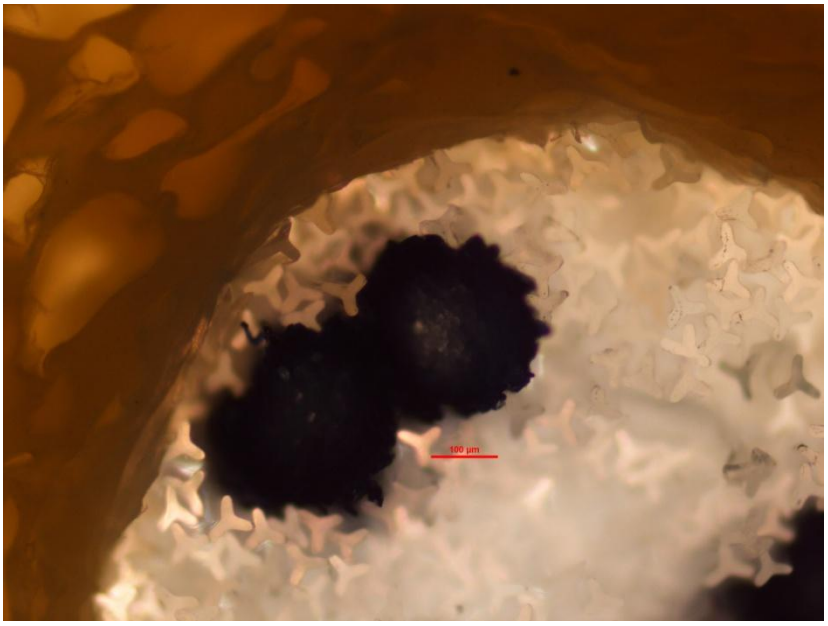


Figure 4.3. Microscopic image (100 µm) image of sized indigo dyed yarn.

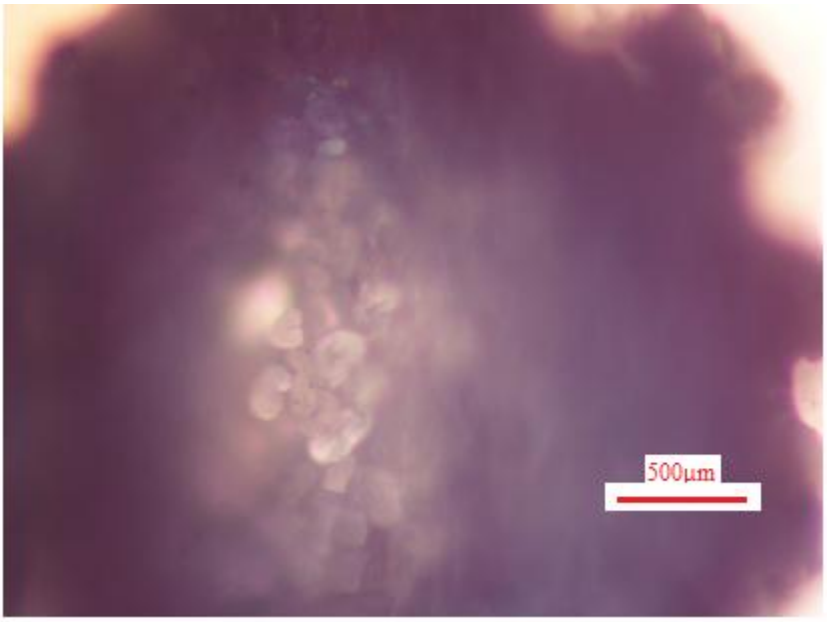


Figure 4.4. Microscopic image (500 µm) of sized indigo dyed yarn.

After washing the fabric with enzymes such as cellulase and laccase, there is decolorization of indigo but microscopic images showed that indigo tends to penetrate inside the core of the yarn after cellulase wash and after laccase treatment indigo remained on the outer core of the yarn. Figure 4.5, Figure 4.6 and Figure 4.7 show the 100 µm microscopic images of desized, cellulase treated and laccase treated indigo dyed yarns.

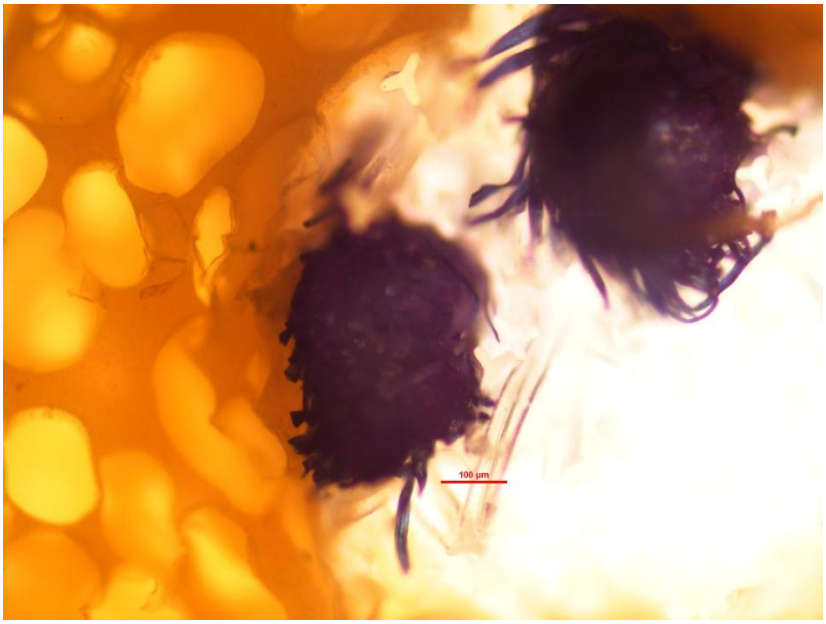


Figure 4.5. Microscopic image (100 µm) of desized indigo dyed yarn.

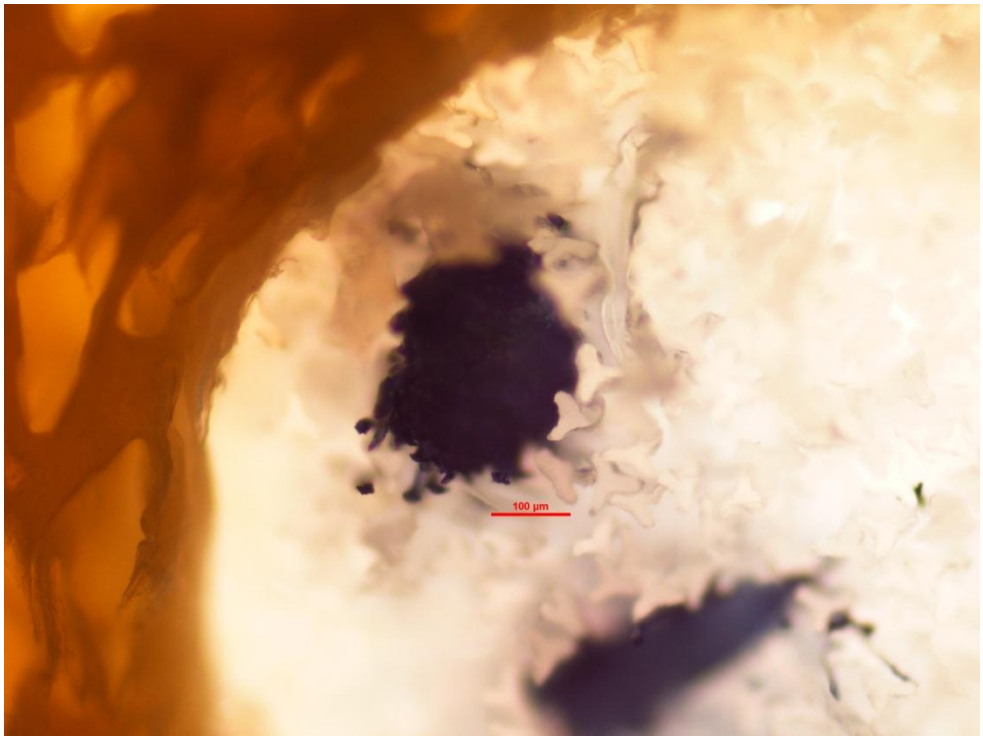


Figure 4.6. Microscopic image (100 µm) of cellulase treated indigo yarn.

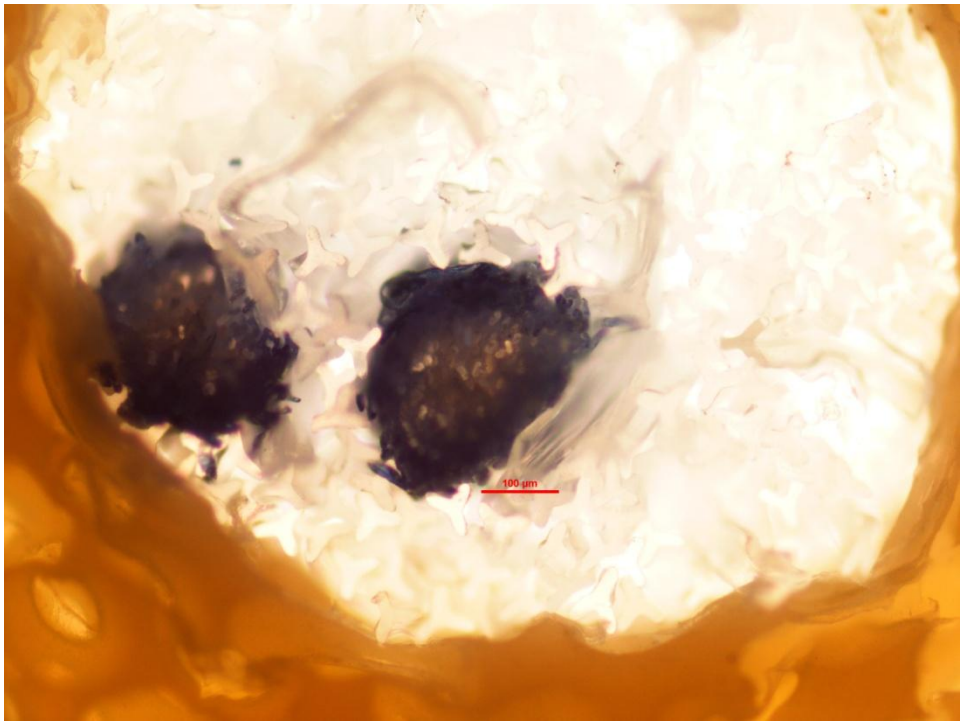


Figure 4.7.Microscopic image (100 µm) of laccase treated indigo dyed yarn.

It is the common practice in industry for laccase washing to be carried out on cellulase washed fabric (63). Therefore this experiment was carried out to determine the combined effects of cellulase and laccase. Denim fabric was treated with laccase after cellulase treatment. The resulting L*, a* and b* values are recorded in Table 4.4.

Table 4.4. Cellulase wash followed by laccase enzyme wash.

Fabric	**L***	**a***	**b***
Cellulase Wash+ Novoprime B268	51.44	-4.02	-13.8
Cellulase Wash+ LT100	54	-2.02	-17.08

The results in Table 4.4 show that laccase washing after a cellulase wash is a good way to reduce the number of washes, as the L* values are much higher as compared to five washes involving Novoprime B268 and Genencor® PrimaGreen® EcoFade LT100.

It is important to point out that denim fabric has multiple shade variations. Therefore, L*, a* and b* values were taken from multiple points on the denim fabric and the average was recorded as a given data point. Figure 4.8 illustrates the shade variations on washed denim fabric.

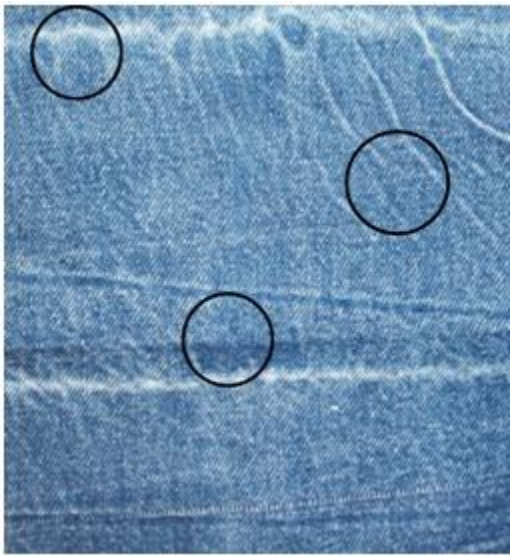


Figure 4.8. Shade variations on denim fabric.

4.2. Laccase Enzyme Activity

Activity of Novoprime B268 (laccase) was measured using a syringaldazine based assay and the LAMU unit was calculated. Change in absorbance was recorded over 15 s to 90 s using the "Kinetic" software of the spectrophotometer. It can be observed in Table 4.5 and Figure

4.9 that there was a linear increase in absorbance, which indicated that laccase was actively oxidizing syringaldazine. The absorbance was measured at λ_{max} = 530 nm.

Table 4.5. Change in absorbance after 15 s interval.

Time (Sec)	Change in Absorbance
0.02	0.135
15.02	0.172
30	0.204
45	0.236
60	0.270
75	0.300
90	0.331

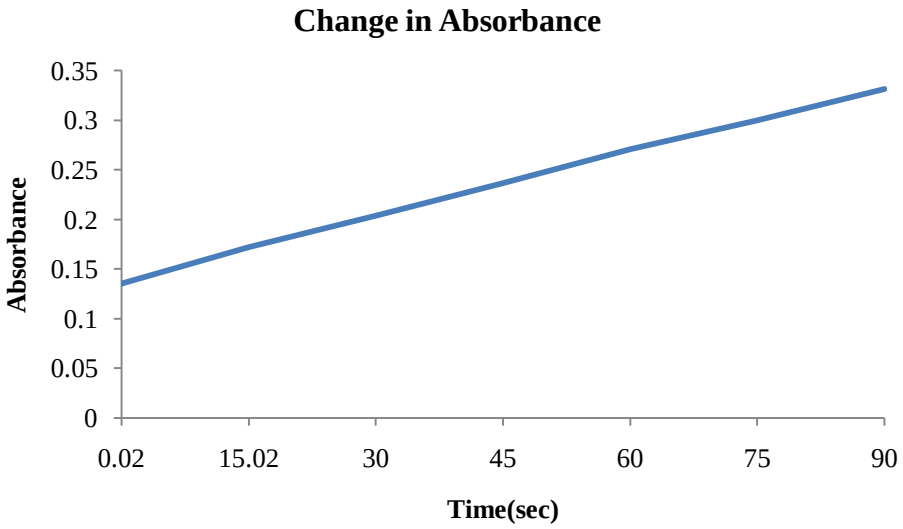


Figure 4.9. Change in absorbance with time.

If dilution factor D = 1000 and change in absorbance = 0.13 then, activity = 893 LAMU. This LAMU value is very close to the value of 800 LAMU reported in application data sheet of Novoprime B268 (61).

4.3 Ink Jet Printing

4.3.1 Approach

After the evaluation of enzymes, this investigation was directed towards the determination of the best method for producing patterns on indigo dyed denim.

In this aspect of the study, laccase was applied to the fabric using a pad and cure method. There were several reasons to conduct these experiments.

1. To find out whether the indigo can be decolorized without putting the dyed fabric in the drum extension of the Ahiba machine (i.e. to determine the effects of color decolorization by friction between two fabrics in the drum and other forces like centrifugal forces on the color decolorization).

2. To determine whether the laccase added to the fabric by applying it from a padder would result in color loss.

3. To use the laccase enzyme like an ink in an inkjet printer to make a variety of designs on denim.

Two routes methods were developed for continuous enzyme treatment of indigo dyed denim fabric. Table 4.6 shows L*, a* and b* results in comparison with desized fabric.

Table 4.6. L*, a* and b* values for enzyme padded denim fabrics.

Fabric	**L***	**a***	**b***
Desized	23.99	1.28	-13.55
Method 1 Novoprime B959 inkjet	23.53	1.26	-16.06
Method 2 Novoprime B959 inkjet	26.03	-0.25	-15.02
Method 1 Novoprime B268	25.4	1.27	-14.05
Method 2 Novoprime B268	26.05	1.33	-15.06
Genencor® PrimaGreen® EcoFade LT100	31.05	-0.97	-16.74

Based on results in Table 4.6, the application of Novoprime B959 (cellulase) enzyme to desized fabric using method 1 resulted in no significant loss of color. This is consistent with results from the laccase wash carried out in the Ahiba machine, which did not show significant loss of color.

Results in Table 4.6 also indicate that Genencor® PrimaGreen® EcoFade LT100 gave better results than the other two enzymes. Therefore, this enzyme was used for inkjet printing, especially since it also works at room temperature.

4.3.2 Trials

A4 size cellulase washed denim fabric was printed with Genencor® PrimaGreen® EcoFade LT100 (laccase) enzyme. After printing, the fabric was dried for 24 h, washed with water and dried. The target designs are shown in Figure 4.10.

Figure 4.10. Target designs printed on fabrics using an inkjet printer.

Table 4.7 shows the L*,a* and b* values following inkjet printing of laccase on cellulase washed denim fabric. Surprisingly, there was no noticeable change in the L* values and the target patterns were not visible. The reason is that inkjet printers print on fabric by dispensing small droplets either by charging or thermally heating. To decolorize fabric one needs an enzyme or other chemical which has higher indigo reducing activity as compared to the enzymes used in these experiments, or a mechanism for placing much higher laccase levels on the fabric.

Table 4.7. L*,a* and b* values following inkjet printing of laccase on cellulase washed denim.

Fabric	**L***	**a***	**b***
Cellulase washed	34.81	-1.54	-16.43
Genencor® PrimaGreen® EcoFade LT100 printed fabric	35.8	-1.49	-17.86

4.4 Screen Printing

The purpose of these experiments was to develop a laccase based print paste that could be applied through a screen to make designs on indigo dyed denim. Novoprime B268 (laccase) was applied from a flat bed screen. However, the target pattern did not appear after removing the print paste by washing. Likely reasons for the failure of this experiment were:

- The viscosity of the paste was too high
- The concentration of laccase in paste was too low
- Thickener blocked the flow of oxygen to the laccase, which is necessary for its activation
- Not enough dwell time

4.5 Resist Methods for Pattern Formation

The goal of these experiments was to produce a design on indigo dyed denim using enzymes, by first applying wax or stearic acid on the fabric in a way that leaves open areas in the form of basic patterns forming circles or squares. Figure 4.11 shows the schematic for this method. This was followed by washing the fabric with laccase enzyme solution.

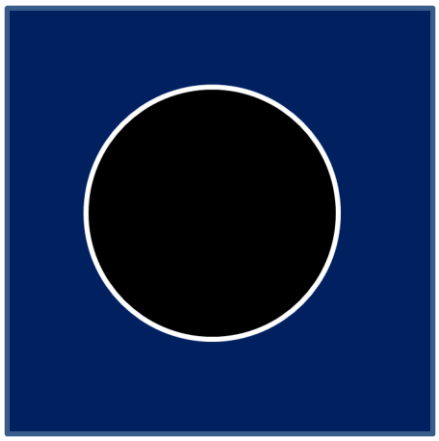


Figure 4.11. Schematic of wax film on fabric surface (blue) that forms a circular pattern (black).

Figure 4.12 illustrates how wax protects a fabric surface from penetration of an aqueous enzyme solution.

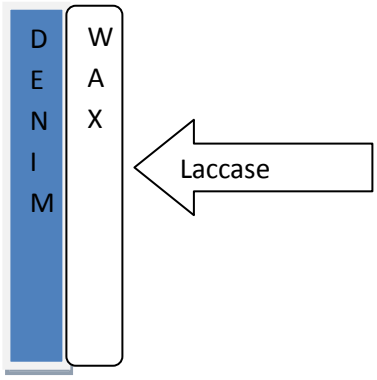


Figure 4. 12. Illustration of wax protection on fabric surface.

4.5.1 Paraffin wax resist fabric

After washing the paraffin wax coated fabric with Novoprime B959 (cellulase) enzyme, it was observed that this method was only a partial success. Although, the wax was effective in preventing enzyme solution from accessing the fabric surface, during washing there was spreading of wax in a manner that led to a low quality pattern.

It can be observed in Figure 4.13 that there are areas of decolorization on the fabric. However, since the decolorization is not in a precise pattern, the result of this experiment provide only partial demonstration of the concept.

It was also found that paraffin wax tended to crack during the enzyme washing step and it could not be used at temperatures higher than 38°C. It also stuck to the surface of the machine requiring frequent clean up.

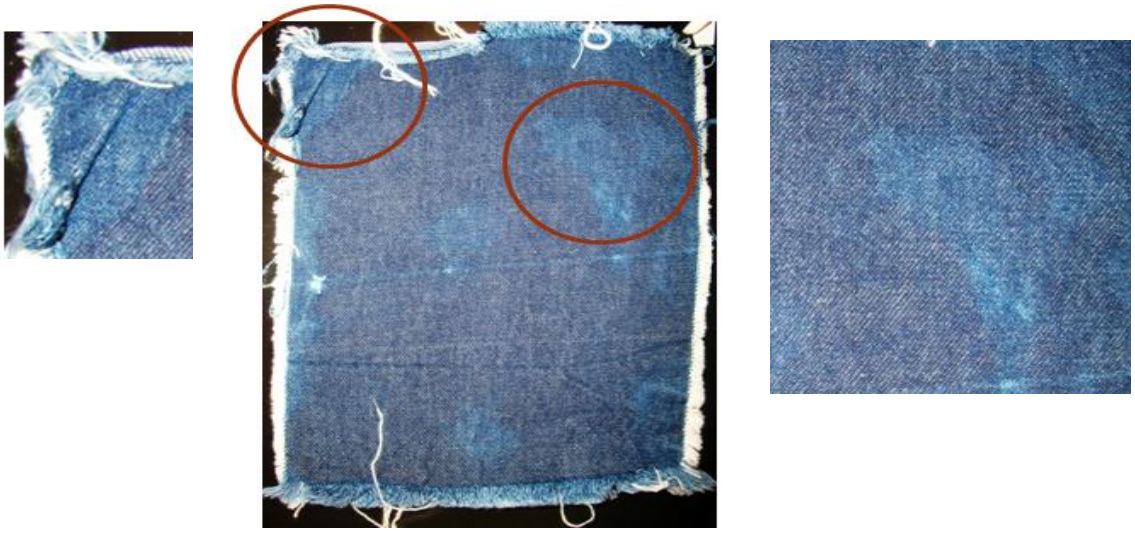


Figure 4.13. Uneven decolorization of denim fabric using a paraffin wax resist and cellulase enzyme.

4.5.2 Stearic acid resist fabric

The melting point of the paraffin wax and stearic acid were determined using a Mel-Temp instrument. Table 4.8 shows the results of these measurements.

Table 4.8. Melting points of hydrophobic materials.

Hydrophobe	Melting Point
Paraffin wax	37°C
Stearic acid	67°C

In view of these results, stearic acid was used in lieu of paraffin wax as a resist.

A "star" pattern was made on denim fabric using stearic acid. Figure 4.14 shows the resulting fabric.

Figure 4.14. Stearic acid star on denim fabric.

As can be seen in Figure 4.15, the stearic acid experiment was a success. Stearic acid survived the friction and other adverse forces during the washing process, producing the desired design.

Figure 4.15. Star pattern produced using stearic acid resist and laccase enzyme wash down.

Stearic acid offers advantages over paraffin wax, such as:

- Stability to cracking
- Processability at higher temperatures
- Less machine fouling

- No penetration into the fabric and easy to control during design application

4.5.3 Polyethylene nonwoven resist sheet

This substrate was considered because it did not require heating to the melt in order to apply it to the fabric surface. Like the previous two substrates it provides a very hydrophobic film in denim. During the early stages of this work, it was understood that the film received was a polyurethane (Pu) product. DSC analysis (Figure 4.16), however, indicated that it was polyethylene (mp = 108.48° C). Fourier Transform Infrared (FTIR) spectrum was also recorded and the results further confirmed the presence of polyethylene. Figure 4.17 shows the FTIR spectrum of this material.

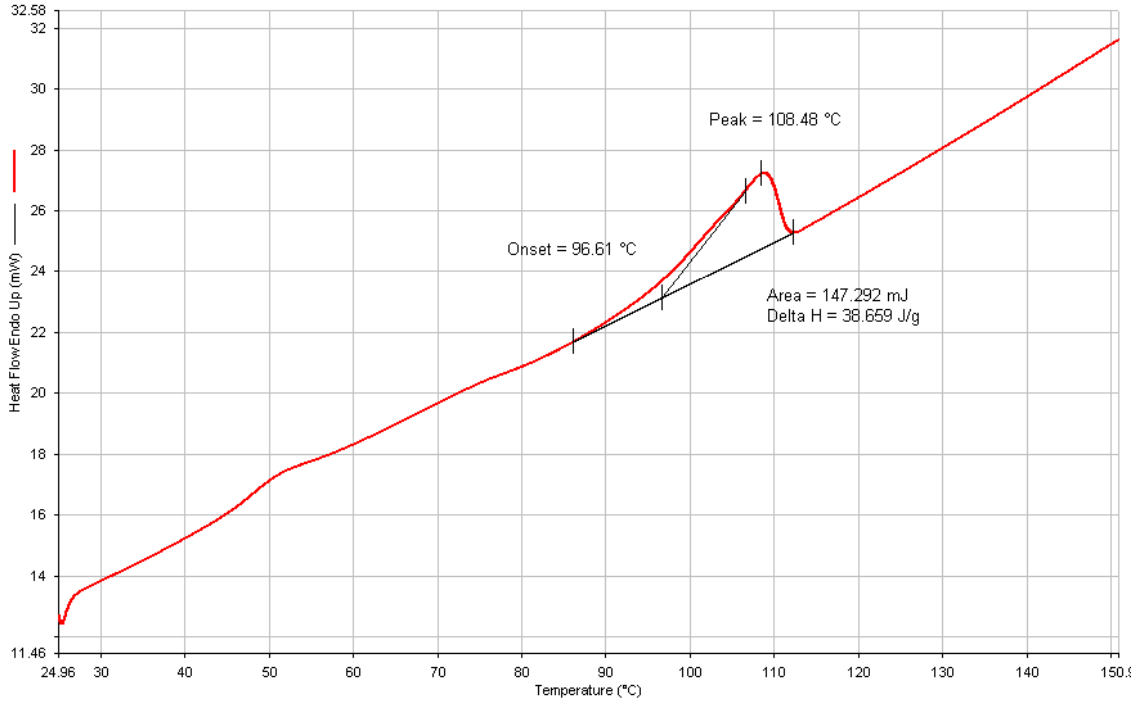


Figure 4.16. DSC thermogram of polyethylene sheet used in this study.

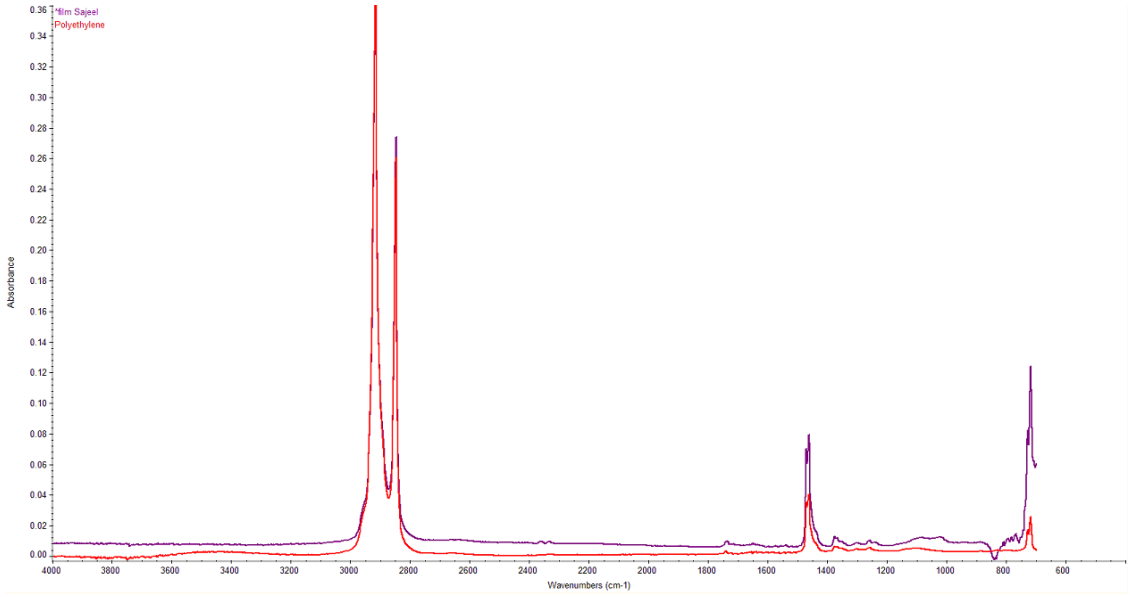


Figure 4.17. FTIR of polyethylene sheet.

The objective of this experiment was to make a design on the side of the polyethylene sheet that covers the front of denim fabric, keeping the design area exposed to enzyme solution during the wash step. Figure 4.18 shows denim fabric sandwiched between polyethylene nonwoven sheets.

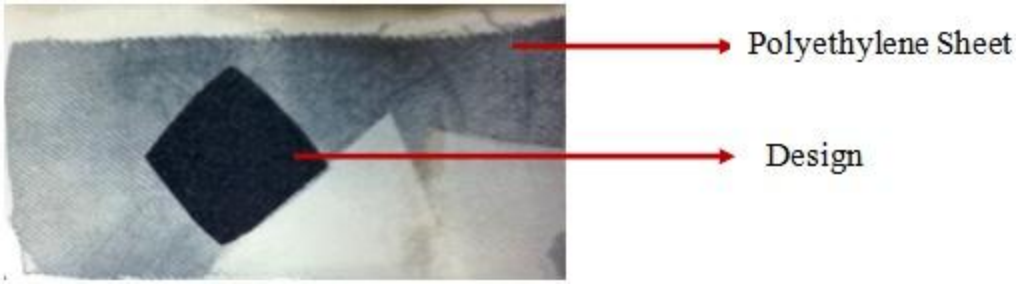


Figure 4.18. Denim fabric sandwiched between polyethylene non-woven sheets containing a cut-out section for the design.

Figure 4.19 shows the laccase enzyme treated sample of the denim fabric in Figure 4.18. Polyethylene sheet is partially peeled off this sample to show the contrast between the covered denim and design area formed by wash down. It can be observed that a sharp square design is made.

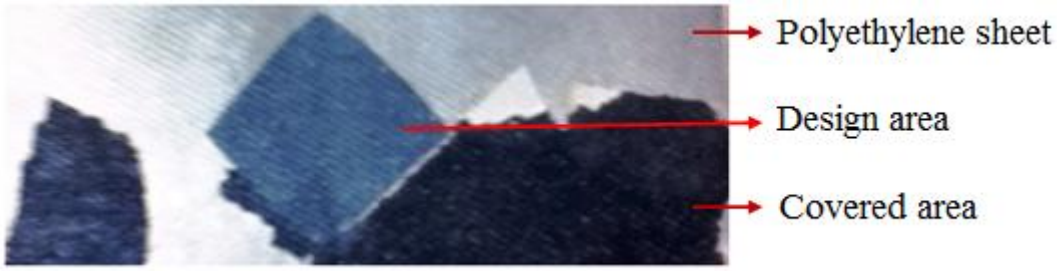


Figure 4.19. Laccase enzyme treated polyethylene coated denim fabric.

The polyethylene coating survived four Genencor® PrimaGreen® EcoFade LT100 (laccase) wash cycles in the Ahiba machine without cracking or other damage. Table 4.9 shows the L*,

a* and b* values of design area following four washes. Other designs such as the striped pattern shown in Figure 4.20 were achieved.

Table 4.9. L*, a* and b* values for the laccase treated and untreated areas of polyethylene coated fabric.

Fabric	**L***	**a***	**b***
Desized fabric	25.44	0.96	-13.93
Design area	46.03	-4.64	-14.24

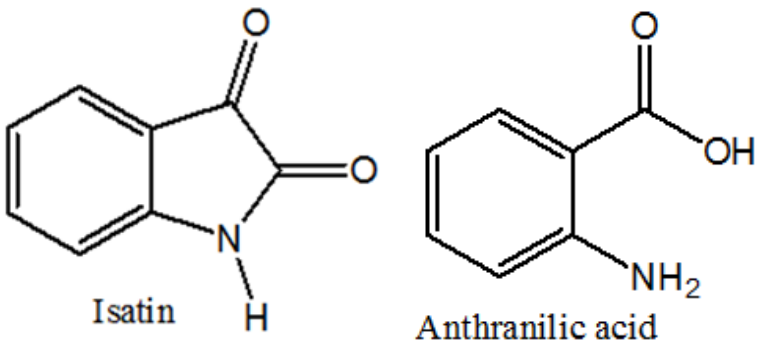
Figure 4.20. Striped design using polyethylene sheet method and laccase enzyme treatment.

"Pattern" making on denim fabric by using a polyethylene non-woven resist sheet has many advantages:

- The film can withstand higher processing temperatures than stearic acid or paraffin wax.
- The film can survive multiple washes without being damaged. Therefore, high contrast patterns can be made on the fabric.
- The film is easy to remove from the fabric.
- The film can potentially be recycled, making it cost effective.
- The film is very easy to apply to the fabric. A simple home iron can also be used to fuse it to the fabric surface.
- The process causes no harm or damage to the washing machine.
- The process can be used to make logos, stripes or any other designs.

4.6. Laccase-induced degradation products

According to previous literature, isatin and anthranilic acid were the products formed by the chemical degradation of indigo (42). In order to identify products formed by reduction of indigo using laccase, LC-MS and TOF SIMS were used. The mass to charge ratio (m/z) for isatin and anthranilic acid are 147 and 137.



LC-MS results from the analysis of laccase afterwash solutions showed the presence of isatin but there was no indication of anthranilic acid. Figure 4.21 shows the mass spectrum of anthranilic acid solution.

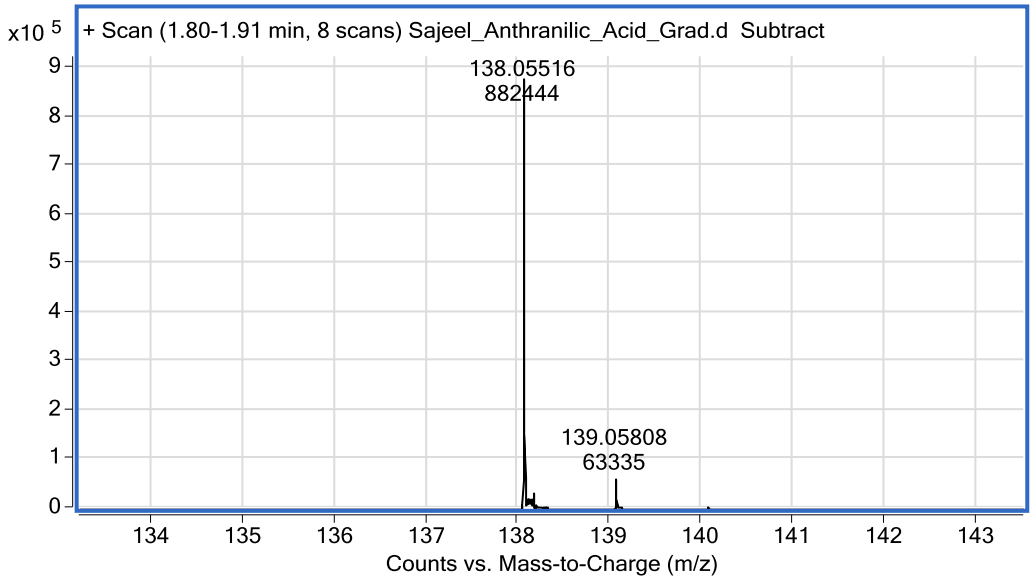


Figure 4.21. ESI mass spectrum of anthranilic acid solution.

Figure 4.22 shows the mass spectrum of the solution collected after Genencor® PrimaGreen® EcoFade LT100 enzyme treatment on denim. The peaks at m/z 169 and m/z 129 are derived from the enzyme used.

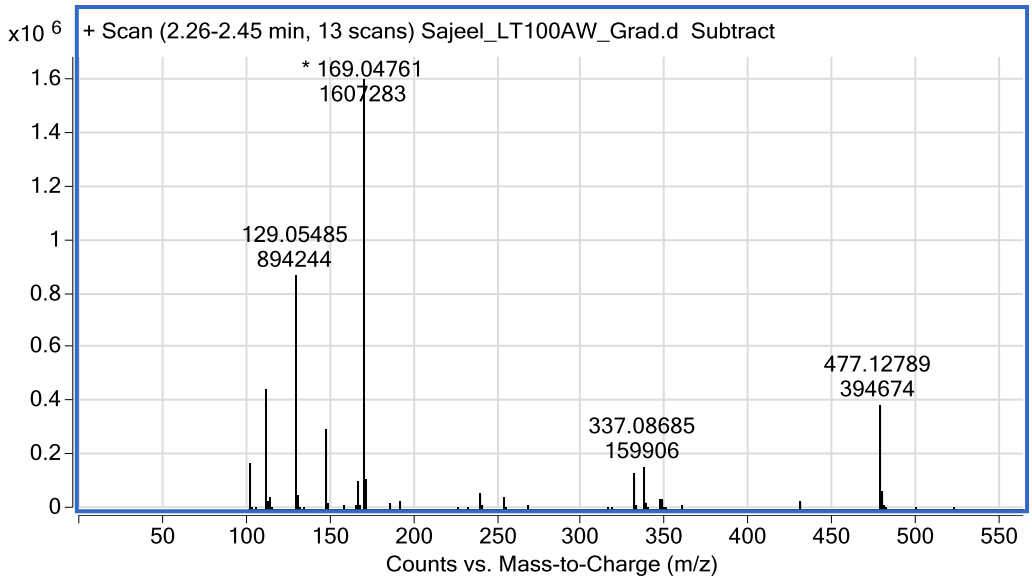


Figure 4.22. ESI mass spectrum of Genencor® PrimaGreen® EcoFade LT100 afterwash solution.

Figure 4.23 shows the mass spectrum of the solution collected after Genencor® PrimaGreen® EcoFade LT100 enzyme treatment on denim. The spectrum shows a peak at m/z 170.02, which corresponds to the $[M+Na]^+$ peak for isatin.

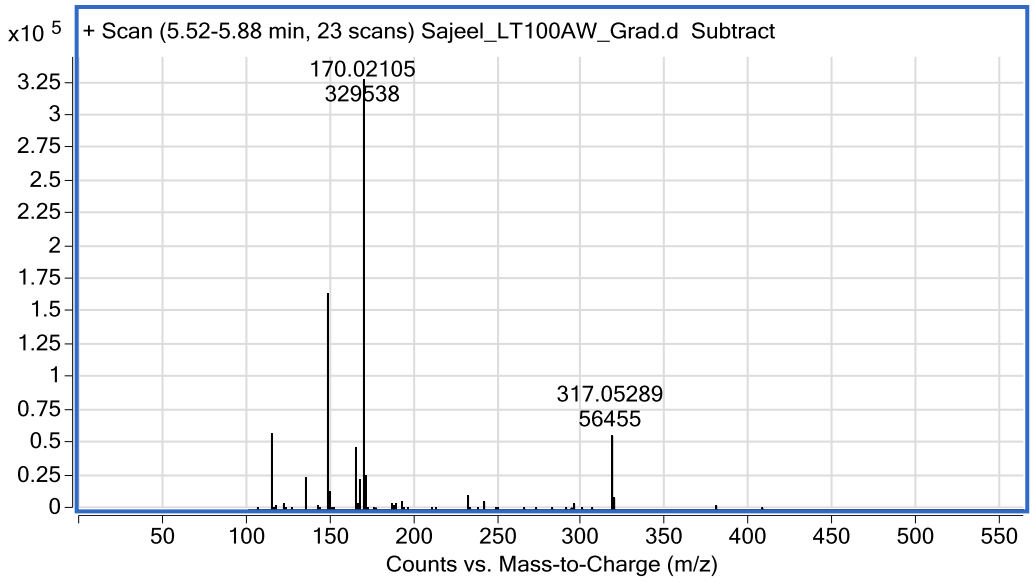


Figure 4.23. ESI mass spectrum of Genencor® PrimaGreen® EcoFade LT100 afterwash solution.

Figure 4.24 shows the mass spectrum of the solution collected after Novoprime B268 enzyme treatment of denim fabric. It shows a small peak of isatin at m/z = 148 (M+H), but there is no indication of anthranilic acid.

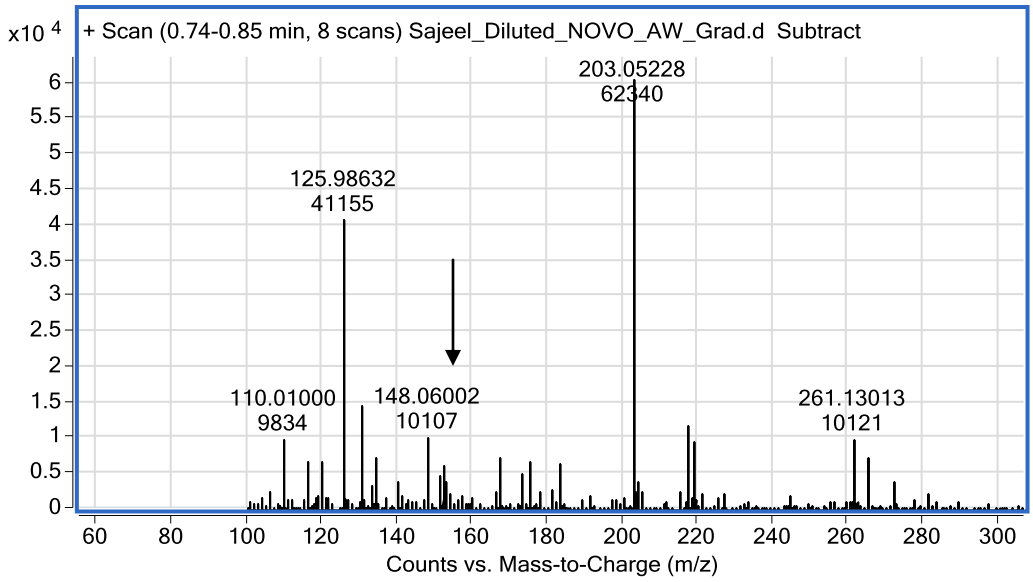


Figure 4.24. ESI mass spectrum of solution from Novoprime B268 treated denim fabric.

Time of flight secondary ion mass spectrometry (TOF-SIMS) analysis of the desized, Novoprime B959 (cellulase) enzyme treated, and Genencor® PrimaGreen® EcoFade LT100 (laccase) treated denim fabric was performed in order to characterize any indigo degradation products on the fabric surface. The results showed the presence of isatin and anthranilic acid. Mass spectra in Figures 4.25 and 4.26 provide comparisons of desized, laccase treated and cellulase treated fabric. The results show that desized fabric and cellulase treated fabric gave comparable results but on laccase treated fabric there was evidence for the presence of anthranilic acid (m/z 137).

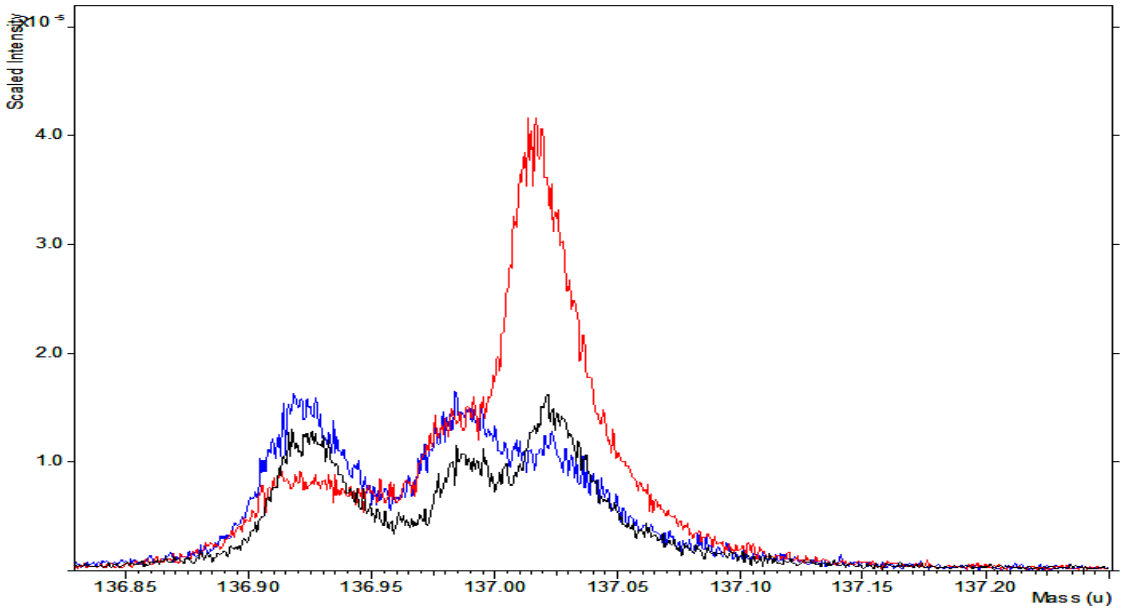


Figure 4.25. TOF SIMS mass spectrum at m/z 137.

Laccase Reduced Desized Fabric Cellulase Wash

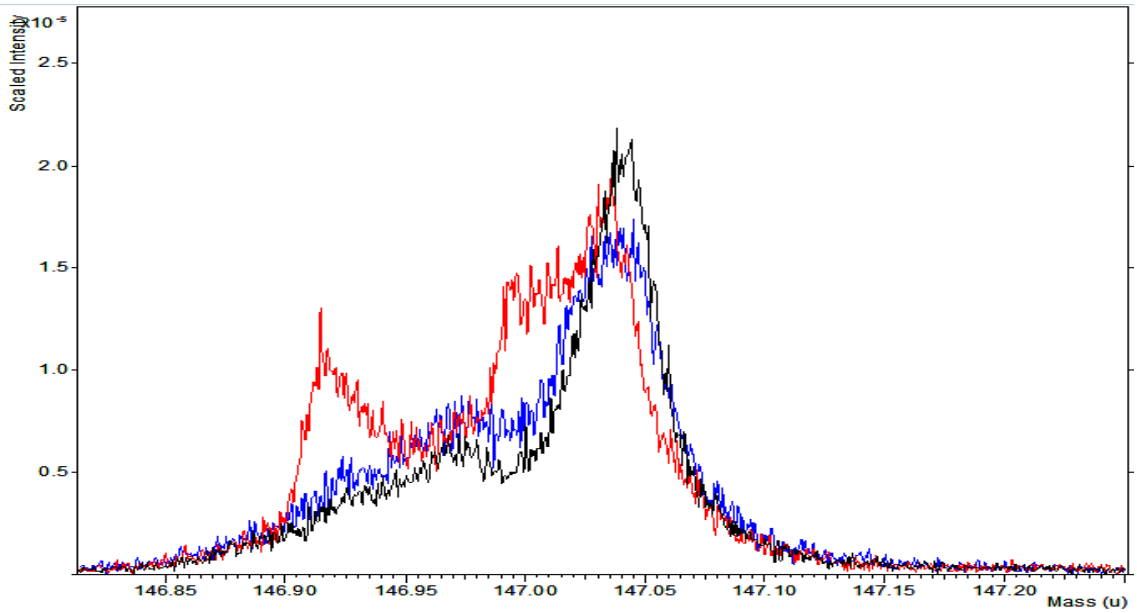


Figure 4.26. TOF SIMS mass spectrum at m/z 147.

TOF-SIMS analysis of indigo reduced by laccase enzyme was conducted to confirm that isatin and anthranilic acid were the major products of this treatment. Mass spectra of laccase reduced indigo were recorded after 24 h and 48 h. Figure 4.27 and Figure 4.28 show the mass spectrum at m/z = 137 and m/z = 147, respectively. In Figure 4.28, the peak intensity of reduced indigo after 24 h and 48 h of exposure to laccase enzyme are very high as compared to indigo dye. Similarly, in Figure 4.28, peak intensity at m/z = 148 for reduced indigo after 24 h of exposure and 48 h of exposure to laccase enzyme is much higher than the peak for indigo.

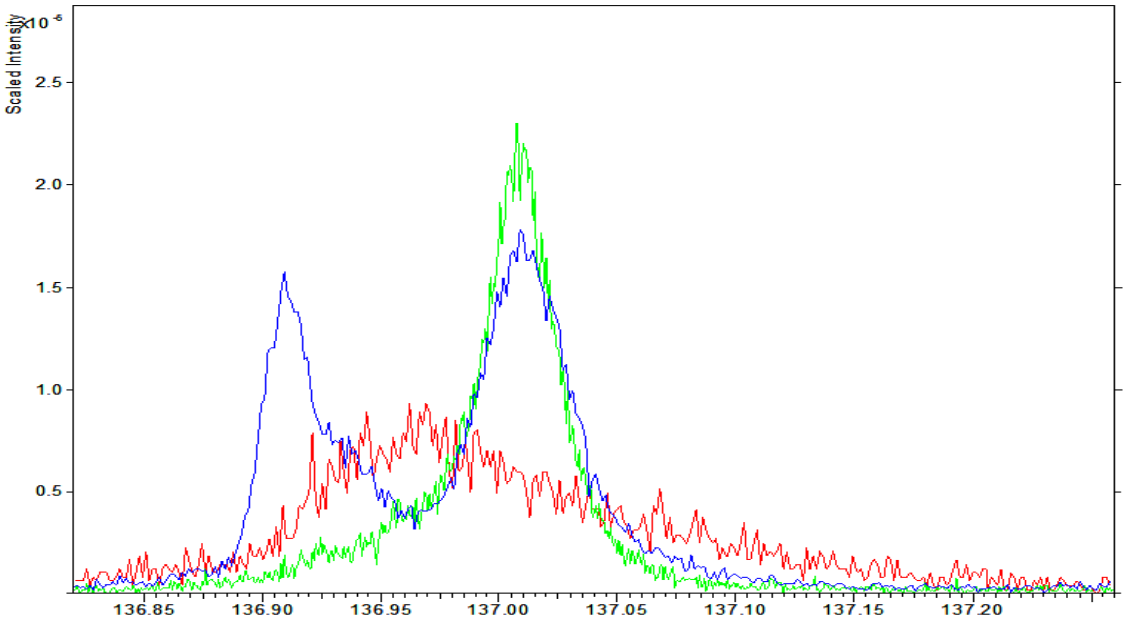


Figure 4.27. TOF SIMS of indigo treated with laccase (m/z=137).

Indigo Indigo Reduced after 24 h Indigo Reduced after 96h

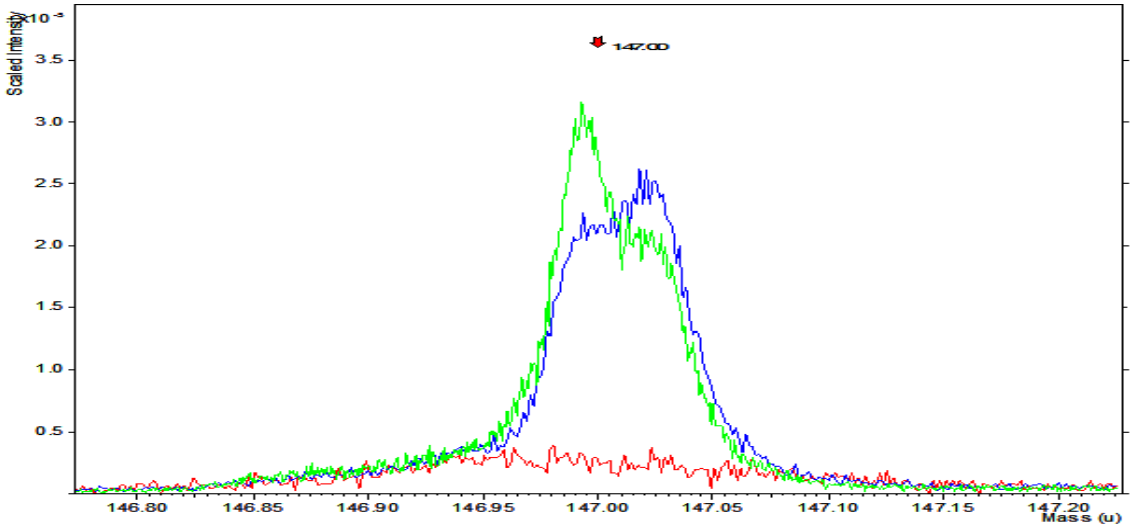


Figure 4.28. TOF SIMS of indigo treated with laccase (m/z = 147).

The general mechanism of indigo reduced by laccase is given in previous literature (42), but the detailed mechanism for the conversion of isatic acid to anthranilic acid was not mentioned. The Figure 4.29 mechanism is suggested for this reduction process in acidic media (70).

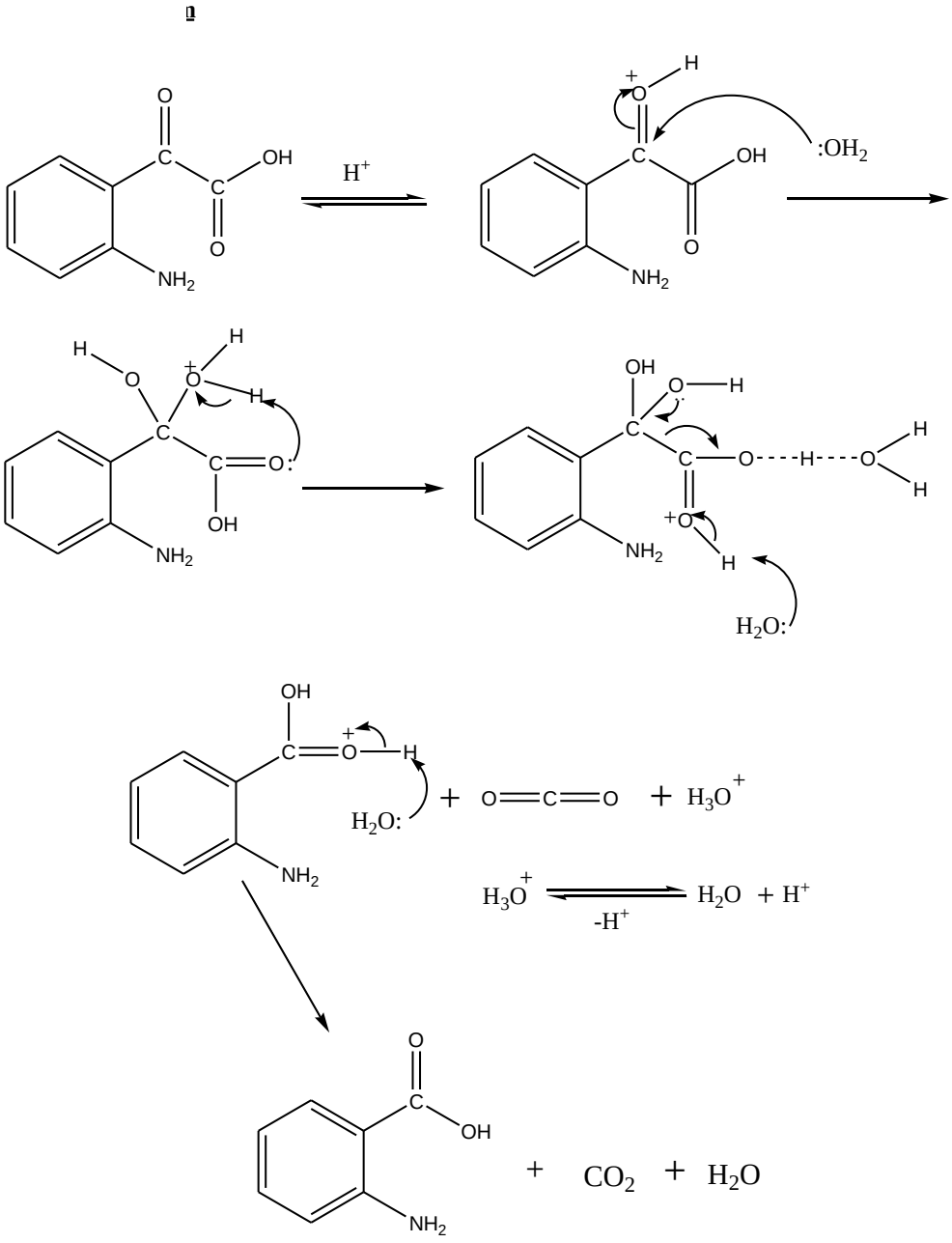


Figure 4.29. Proposed mechanism for anthranilic acid formation from isatic acid in acid media.

5. Conclusion

It was found that laccase treatment of denim fabric after a cellulase treatment led to a greater increase in lightness (L*) values as compared to a laccase only treatment. Regarding laccase enzymes, Genencor® PrimaGreen® EcoFade LT100 was more effective in this study than Novoprime B268 for making a design on denim, as it was effective at room temperature, did not require acid and buffer, and required fewer wash cycles to achieve the same the wash down properties as Novoprime B268.

Making a design on denim by using a polyethylene (PE) non-woven sheet applied to the denim surface gave advantages over using stearic acid or paraffin wax as the resist material. It was found that PE can work at higher temperatures, can withstand several wash cycles, and make sharper designs. Stearic acid experiments demonstrated that a design on denim can be made using a solid that is easy to melt. In this regard, paraffin wax was less suitable because of its tendency to spread on the denim surface during the wash down process.

Inkjet printing has the potential to become a good method for making a design on denim but either the use of an enzyme with very high activity or an approach to delivering high enzyme levels in the droplets is needed to make this method effective.

Using LC- MS and TOF SIMS analytical methods, isatin and anthranilic acid were identified as laccase enzyme reduction products of indigo. Also a mechanism for the conversion of isatic acid to anthranilic acid was proposed.

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