Development of a Novel Wet Process Technique of Cotton Knit Fabric for Textile Applications

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The thesis on "Development of a Novel Wet Process Technique of Cotton Knit Fabric for Textile Applications" submitted by Rasheda Begum Dina, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy, is a new work carried out by her under our joint-supervision. The work has not been submitted elsewhere for the award of any other degrees from the university or any institution. We also certify that she followed the guidelines of the University of Dhaka regarding plagiarism.

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Declaration

I hereby affirm that the writing of this thesis is novel and the content has not been used for any other degree. References have been used in the case of writing this thesis. All the results of the work placed here are solely my work.

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The L value correlates with the level of lightness/darkness of a colour. The a is the red/ green axis; positive and negative a describe redness and greenness, respectively. The b is the yellow/ blue axis; positive and negative b describe yellowness and blueness, respectively. Chroma (C*) and hue (h*) can be extracted from the a* and b*

Abbreviations

Abbreviation	Abbreviation Description
pGLO	A recombinant plasmid that harbors the green fluorescent protein
OD	Optical density
RPM	Rotation per minute
LB medium	Luria-Bertani medium
w/v	Weight/volume
CIE	Commission Internationale de l´Eclairage [French]
	The International Commission on Illumination [English]
CMC	Colour Measurement Committee
DE / ΔE	Colour Difference
FT-IR	Fourier Transform Infrared Spectroscopy
GSM	Gram per Square Meter
SEM	Scanning Electron Microscopy
PAGE	Polyacrylamide gel electrophoresis
PAG	Polyacrylamide gel
SDS	Sodium dodecyl sulfate
GFP	Green Fluorescent Protein

Dedication

This thesis is dedicated to my family

Acknowledgements

Almighty Allah provided me the kindness to perform this critical thesis work in a long merciful way. Without His kindness, it would not be possible to develop this novel technique with new enzymes. During the period of thesis work, supervision was an important factor in bringing success to the whole journey that was taken from my supervisors. First, I would like to show my respect and gratitude to Professor Dr. Papia Haque, my first supervisor, who encouraged, guided, and allowed me to do this critical innovative work with her planning. She encouraged me to attend seminars and conferences, and eventually to write journal articles and to finish the thesis on time. Unless her unconditional support and assistance it was not possible to complete this thesis work. I am thankful for her effort. I should be thankful to Professor Dr. Mizanur Rahman, my joint supervisor, for his facilities in ILET and assistance during my work period. It is not possible to express my feelings to my other supervisor Professor Dr. Zulhash Uddin, Chairman, of the Department of Wet Process Engineering, BUTEX, whose hand was always in my favour to do this thesis work. His direct and undoubtful help assisted me in the completion of the study. I am grateful to another teacher, Professor Dr. Abu. A. Sajib, Chairman, Department of Genetic Engineering and Biotechnology, for his kind eyes and suggestion on the successful establishment of recombinant technology in textile bio scouring and to walk the right track of the work. Without his smartly equipped lab facilities, I couldn't stand on the thesis. I owe to him and his team members. I am indebted to the team members of Orient Chem Tex LTD, the concerns of Micro Fibre Group, and the Department of Wet Process Engineering, BUTEX for the facilities that were received. Because of the help from the department, I could perform mostly lab-scale sample preparations and testing opportunities from the laboratories. I remember Sabrina Samad Shoily, Kaniz Bristy, Tuli, and Anik Biswas, four of the M.Sc. students, for their help in my progress. I cannot avoid the contribution of my colleague Md Motakabbir Tamal for his assistance in the FTIR test work of the thesis. Senior lab technician Rahat helped me a lot in sample preparation, which I cannot reward and forget.

Lastly, my parents and siblings prayed and thought a lot for my successful completion of the PhD. My husband along with my beloved children carried a lot of suffering for this thesis work. I feel sorrow for their sufferings and proud of their support to go through this long journey with them.

Author

Abstract

The purpose of this thesis was to develop a novel wet process technique for textile applications, which was accomplished with the production and implementation of natural and recombinant bacterial species having pectinolytic activities, assessed their pectinolytic activity both qualitatively and quantitatively, as well as compared with commercial pectinase enzyme for checking its bio scouring efficiency. Recombinant DNA technology was applied to produce pectinase enzymes and natural pectinase enzymes were produced from the source of chia seeds and soil mixture. Ten synthesized gene fragments were amplified in a PCR machine and ligated gene length was checked by electrophoresis gel run with a ladder to confirm their expected size. Successful transformation of fungal pectinase gene into bacterial plasmid DNA was accomplished to produce recombinant pectinase enzyme with their positive pectinolytic activity test results by iodine and DNS. Plasmid extraction for gene length checking was matched with the gene length of interest which was ~ 1143 bps and protein molecular weight was ~ 38 KDa which was found by PAGE run. Here two types of bacterial species DH5α and BL21 were used for gene transformation. Cotton knit fabric bio-scoured samples with these enzymes' application were checked for wicking test, whiteness index, colour difference, FTIR analysis, SEM, and bursting strength tests. The wicking rate of recombinant DH5α, BL21, and natural enzymes in the cotton knit fabric scoured sample was found 85 mm, 65 mm, and 55 mm after ten minutes, and K/S was 14.5, 14.7, and 14.8 after dyeing with DH5α, BL21 and natural enzyme-treated fabric respectively. The findings disclosed that DH5α had superior scouring performance in cotton knit fabrics among produced enzymes that worked at 37 °C, an energy-saving process, and pectinolytic activity was nearer to commercial enzymes which had recommended a bio-scouring temperature of 60 °C. *Burkholderia cepacia*, a natural bacterial species, showed its better pectinolytic activity comparable with commercially available pectinase enzymes in the textile industry. In the thesis, an attempt was made to increase the whiteness of bio-scoured fabric for oxidative bleaching with hydrogen peroxide and reductive bleaching with sodium borohydride and the values are 61.15, 58.14, 59.72. for CS, BL, and D8 bio-scoured fabric respectively, and compared with chemical-scoured bleached fabric's whiteness which was 64. After dyeing, the chemically scoured bleached dyed sample showed a lightness value of 25.61 for the dark navy and 89.48 for the light-yellow colour. The lightness values were 24.63, 24.75, 26.40 for the dark navy colour, and 88.37, 88.14, and 88.09 for the light-yellow colour of bio-scoured natural, BL21, and DH5α bleached fabric respectively. Thus, light colour, as well as dark colour, can be dyed with low-temperature processed bio-scoured fabric.

Publications of Ph.D. Work

1. The pectinolytic activity of *Burkholderia cepacia* and its application in the bioscouring of cotton knit fabric
Journal of Genetic Engineering and Biotechnology, volume 21, Article number: 136 (2023)

2. Sequential Oxidative and Reductive Bleaching for Improving the Whiteness of Cotton Knit Fabric (OP ICMEC A6125, December 2022). International Conclave on Materials, Energy & Climate, Department of Applied Chemistry and Chemical Engineering, University of Dhaka, Bangladesh.

Chapter 1

Introduction

Chapter 1
Introduction

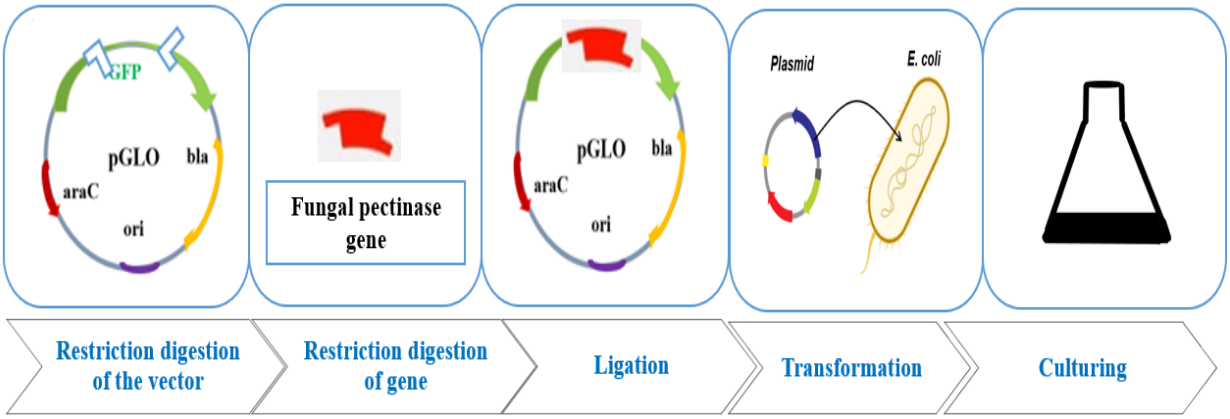
Textiles have been one of the basic needs of humans since the dawn of civilization. People have been trying to decorate their clothing with different colours and accessories since the beginning of advancement. The textile sector has been expanding significantly since the Victorian era, driving up demand with the change in development and contributing to global pollution. Cotton, the most commonly used worldwide fibre, needs wet preparatory processes for colouring and they are of the basic four types: pretreatment, dyeing, printing, and finishing. The research gap has been illustrated initially in cotton wet preparatory treatment and the significance of recombinant technology in pectinase enzyme production for cotton bio-scouring, bleaching with bio-scoured fabric, and reactive dyeing on enzyme-treated fabric have been described in this chapter. Removal of cotton impurities is the prerequisite before coloration and several chemical and biological treatments have been published for the pretreatment process[1]. Cotton fibre has almost 92% cellulose and natural impurities, such as pectins, and proteins, fats, waxes, these impurities make barriers in the penetration of water including colour and chemicals [2][3]. To produce excellently wettable fibres that can be dyed and finished uniformly, oil, wax, fat, pectin, etc. impurities are chemically destroyed and removed by the alkaline scouring process, which involves high processing temperatures like boiling the cotton with hot aqueous NaOH[2]. This alkaline process causes a reduction of fabric strength for cellulose degradation [4]. It was observed, from the previous history of research, that chemical applications had an enormous effect on the environment for cotton pretreatment and ecological problems associated with the conventional scouring of cotton[5].On the other hand, bio-scouring reduces chemical and energy consumption and ecological impact. Therefore, enzyme treatment is an alternative environment-friendly process for removing non-cellulosic impurities from cotton fibre and the hydrophobic cotton fibre surface becomes more hydrophilic fibre with less strength deterioration[2][6]. Pectin, the most complex non-cellulosic constituent in the fibre makes a bridge between the primary wall and the secondary wall, which can be removed by degradation using pectinase enzymes[2]. The progress of industrial biotechnology especially in molecular biology, and protein engineering, enhanced the development of new uses of enzymes in the textile industry [7]. Pectinase enzyme application in bio-scouring leads to a lower processing temperature than the chemical scouring process[8]. Pectinases hydrolyze pectic elements, the most useable enzymes, and are found in bacteria, fungi, and plants, used in the textile industry for the bio-

scouring process. It has significant importance in the biotechnological world. Pectinases are a group of enzymes that catalyze the degradation of pectic substances, such as pectins, propectins, pectinic acid, and pectic acid. Protopectinases, polygalacturonases, lyases, and pectin esterases are the extensively discussed pectinases[9]. Pectic substances, polysaccharides with a long galacturonic acid pillar chain, are the main components of plant middle lamella and primary cell wall[10][11]. Parameter optimization is a considerable factor in pectinase production[12]. The structure and composition of the outer layers of cotton fibre have been established based on a thorough study, which recognizes wax and pectin elimination to be the key steps for a scouring process[13]. In bio-scouring, pectinases break off glycosidic bonds of chains of galacturonic acid in pectic substances and change the polygalacturonic acid into mono-galacturonic acid [11]. Pectinases have attracted the attention of researchers around the world because of their uses as a biological catalyst in various industrial processes, including juice extraction and its clarification, cotton bio scouring, fibre degumming, biomass liquefaction, and wastewater treatment, etc[14]. Microbial pectinases account for 25% of the global food and industrial enzyme scale with market growth occurring regularly [15]. Scientist still take challenges on facing difficulties in finding microbial enzymes, describing their mechanisms of action, and rating up production[11].

Identifying, characterizing, and manipulating genes in biotechnology has an impact on environmental pollution[16]. To reduce environmental pollution, recombinant technology has been applied, and a recombinant enzyme is used to manipulate the characteristics in DNA to get desired properties from the bacteria that produce the enzyme. Recombinant enzymes had been expressed in bacteria (e.g., Escherichia coli, Bacillus, and lactic acid bacteria), filamentous fungi (e.g., Aspergillus), and yeasts (e.g., Pichia pastoris) [17]. The optimum conditions for pectinase production from fungi(*Aspergillus flavus*) were an incubation temperature of 50 ℃ and an incubation time of 96 h[18]. Fungi have a comparatively lower production rate than bacteria[19][20], which is why it was focused on recombining the DNA of fungi with bacterial DNA. To minimize the bio-scouring temperature, the ten fragmented fungal genes have been codon optimized for the novel development of the recombinant pectinase enzyme. Recombinant DNA technology, gene cloning, or molecular cloning is used to isolate genes and to transfer genes from one organism to another [21]. A perfect vector should be selected for inserting the desired gene[22], and plasmids could act as vectors for carrying cloned genes[23]. Recombinant DNA technology is applied in different fields like gene therapy, HIV treatment, clinical diagnosis, genetically modified organisms, insulin, etc[24]. In the textile bio-scouring field,

recombinant DNA technology is a novel approach for producing pectinase enzyme from the ligation of a fungal gene in plasmid DNA to change the usual higher bio-scouring temperature from 60 °C to a lower temperature of 37 °C as well as to get higher production capacity of bacteria than fungus. It will be also bacterial culture at low temperatures of 37 °C for pectinase enzyme from the natural source.

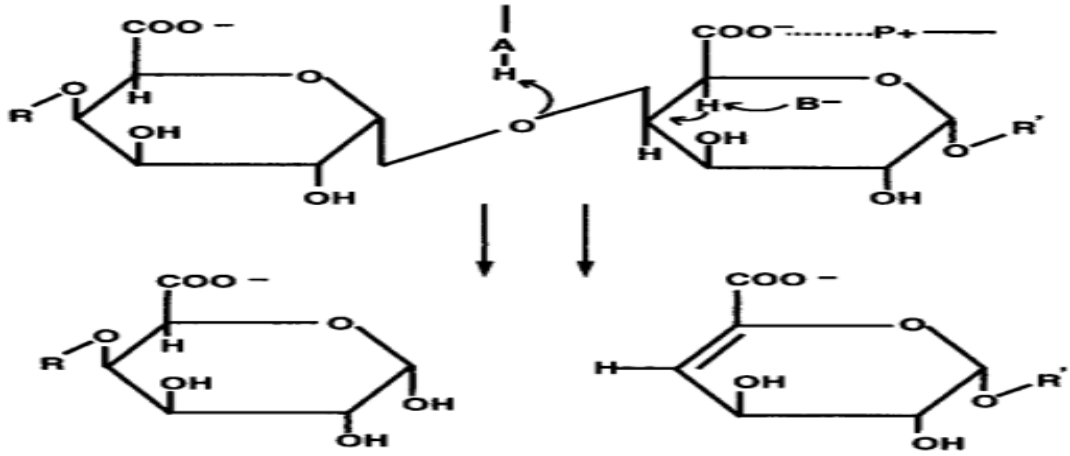
A fungal pectinase gene will be designed to transfer bacterial plasmid DNA to produce recombinant pectinase enzyme and compare with pectinase enzyme produced from the natural source. The schematic design of the recombinant pectinase enzyme is shown in Scheme 1.1.



Scheme: 1.1 Schematic presentation of the transformation of fungal pectinase gene into bacterial plasmid DNA

In the site of environmental issues bio scouring showed an extraordinary achievement instead of alkaline scouring, minimizing chemical degradation of cellulose, having a lower impact on the environmental pollution issue[25]. Pectin substances, present in the plant cell wall, are depolymerized with recombinant pectinases like polygalacturonase, and pectate lyase[18]. The current developing demand in the textile industry for more comparable economical and environmentally liable approaches is responsible for the recent development of the bio-scouring process. Pectinase enzymes are potentially suitable elements in these aspects because of skipping the use of the amount of toxic sodium hydroxide and other chemicals in this process. More than 40 novel microbial pectate lyases were discovered by applying high throughput checking or screening of complex DNA libraries, characterization was done for those enzymes. Some enzymes were suitable that had specific pectinolytic activities on pectic substances in cotton fibres with their application in the bio-scouring process [26].

Pectinase catalyzes the cleavage of α-1,4-glycosidic linkages of polygalacturonic acid by ß-elimination reaction shown in Scheme 1.2 [27] . Several types of enzymes, like pectinases, cellulases, proteases [28] lipases/cutinases have been studied for cotton bio-scouring. Using recombinant DNA technology, first fungal gene was cloned and expressed in E. coli in 1976 [29].The most common fungal sources of enzymes are *A. fumigatus, Penicillium, Fusarium* , *Aspergillus niger, Rhizopus* , and *P. frequetans* . Pectinases produced from *Sclerotium,Paecilomyces* , *Coniotryrium diplodiella, Fusarium solanipisi,* and *A. niger* have been showed for their application in bio scouring of cotton fabrics. In this thesis, DNA from *Aspergillus steynii* has been used for recombination.



Scheme: 1.2 Schematic diagram of α-1,4 polyglacturonic acid cleavage by ß-elimination reaction [27].

The ability to target and edit specific genes has revolutionized genetic engineering, providing more control over the genetic makeup of organisms. This can lead to the development of novel traits, improved characteristics, or the removal of undesirable features. These advancements have the potential to address the limitations associated with traditional breeding methods, offering a more targeted and efficient approach to genetic modification[21]. Genetic engineering allows for the creation of entirely new enzymes or the improvement of existing ones. This has led to the development of an expanded portfolio of enzymes with novel functionalities and improved performance characteristics. Recombinant enzymes can be designed to be more environmentally friendly. For instance, enzymes used with detergents can be engineered for better performance at lower temperatures, reducing the energy required for bio-scouring processes. The impact of recombinant DNA technology on the enzyme market is substantial,

with over 50% of the enzyme market supported by recombinant enzymes. This technology continues to play a crucial role in advancing enzyme production and application across various industries[30].

Now is a details discussion of recombinant DNA. The invention of recombinant DNA (rDNA) technology was due to the discovery of endonucleases to cut DNA. In 1970, it was discovered that bacteria contained nucleases that had short nucleotide sequences within DNA duplex and cleaved the DNA backbone at specific sites on both strands, called restriction enzymes [29]. Recombinant DNA technology, one of the fast-growing technologies, is used to combine DNA segments, a recombinant DNA molecule is formed from fragments of two or more different DNA molecules [24]. This technology is used to isolate genes and to transfer them from one organism to another, began in 1973. In Cohen's method plasmid (small circular DNA molecules) is transferred into bacterial cells. A specific segment of DNA is cut by a restriction enzyme and transferred into a plasmid. After that, the recombinant plasmid is inserted into a host known bacterium. The transformation is a process to introduce of DNA into a bacterial host cell. Restriction endonucleases and DNA ligases are the two categories of enzymes required for isolation of DNA and preparation of recombinant DNA technique[31].The restriction enzymes assist in cutting, the polymerases assist in synthesizing and the ligases- assist in binding. The restriction enzymes used in determining the location at which the desired gene is inserted into the vector genome, are two types, namely endonucleases(cut within the DNA strand) and exonucleases(remove the nucleotides from the ends of the strands)[16]. The genes and the vectors are cut separately by the same restriction enzymes so that ligases can appropriately bind the gene of interest to the target vector[16][24].

DNA is the term to express as keeper or holder of all the information needed to recreate an organism. All DNA consists of sugar, phosphate, and one nitrogen base. A DNA molecule consists of double polynucleotide chains, DNA chains, or DNA strands. Each stand is made of four types of nucleotides, each nucleotide is composed of a five-carbon sugar to which are attached one or more phosphate groups and a nitrogenous base. In DNA nucleotides, the sugar is a deoxyribose-base attached to a single phosphate group. So, the main parts of nucleotides are sugar, a phosphate group, and a nitrogenous base. The central dogma describes the flow of genetic information within a biological system. In simple terms, it states that DNA is transcribed into messenger RNA (mRNA), and mRNA is then translated into proteins. Proteins are essential for the structure and function of cells and, ultimately, the entire organism. By changing the DNA

sequence, protein formation can be changed, leading to either a different protein. A new strand of DNA can be created by combining two or more different strands of DNA. The DNA of two different organisms is commonly combined for the recombinant process[32].

The basic principle of recombinant DNA technology is to digest a DNA molecule from the organism of interest in a separate tube with the restriction enzyme and also a vector (e.g., plasmids) or viral DNA with the same restriction enzyme which cuts both DNA at specific sites. The two digested DNAs are then mixed and joined by DNA ligase enzyme, to produce double-stranded DNA molecule. Then the vehicle-like plasmid containing the ligated DNA transfers into the host organism by transformation or transfection. After the multiplication of the DNA, it is necessary to ensure that the signals involved in the expression (transcription and translation) of the new gene are compatible with the expression machinery of the host. This new ligated DNA molecule will be isolated for bio-scouring purposes. This DNA will be sequenced and used to generate protein from E. coli etc [29].

The vectors assist in carrying the gene of interest and transfer it to the host organism. In rDNA technology, plasmids and bacteriophages are the most common vehicles. Plasmid is a circular and extrachromosomal DNA that has the capability of independent replication. Plasmids are double-stranded DNA molecules that range in size from 1 kb to more than 200 kb and are the most useful tools in genetic engineering. They are the most widely used as vectors to carry foreign DNA [33].

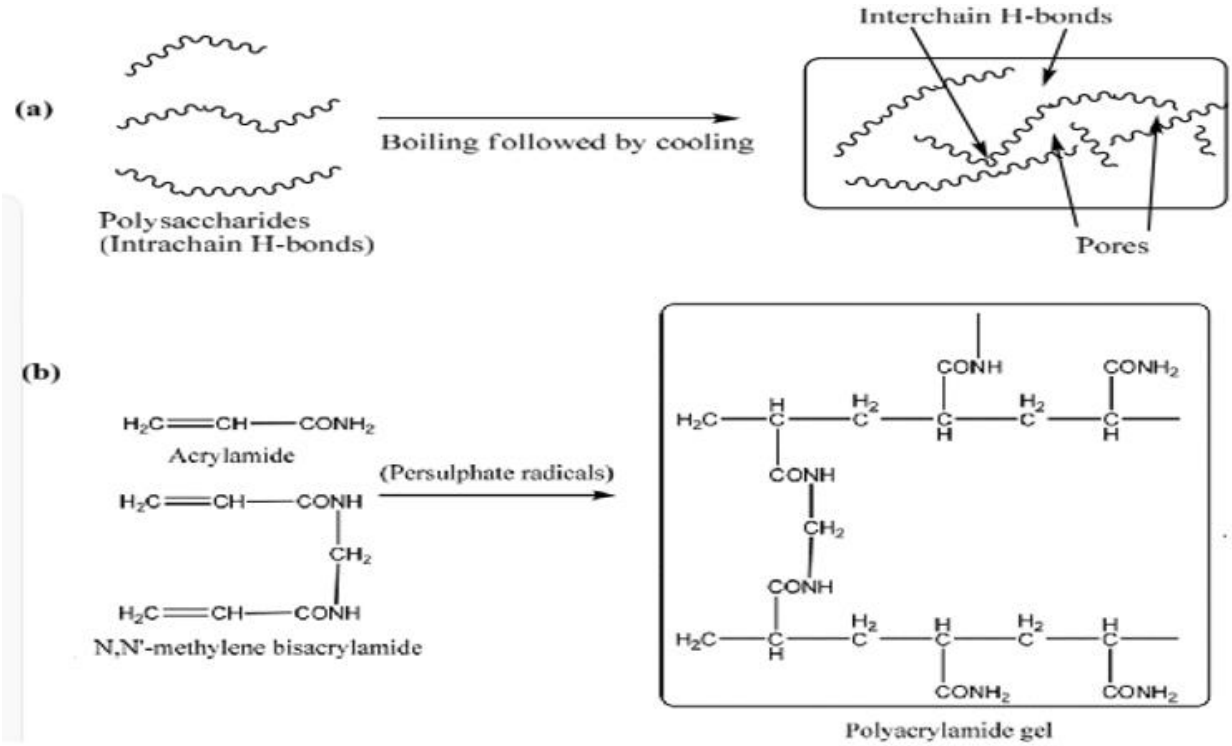
Transcription means that the genetic information stored in double-stranded DNA is copied or printed in the form of a single-stranded RNA molecule like mRNA, tRNA, rRNA. Electron-dense particles in a cytoplasmic region of a bacteria are known as ribosomes. Ribonucleic acid has three types such as —messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) and ribosomes are composed of this RNA[34]. Translation means that genetic information copied into RNA with transcription is converted to a protein or polypeptide chain. In other words, it is the expression of genetic information in the form of protein molecules[35]. In a DNA molecule finding and determining the order of nucleotides is commonly known as DNA sequencing. Each DNA sequence that contains instructions to make a protein is known as a gene. The automated DNA sequencing principle is the same as Sanger's method but the way of detection method is different. The primer or the ddNTPs are denoted by using a fluorescent dye and a laser in the machine is used to read it [36].

Bacteria or fungi are cultured or grown in the solid, liquid, or semisolid medium after the sterilization process. There are different types of media to grow different types of cells [37]. Different types of cell media such as cultural media, minimal media, selective media, differential media, transport media, indicator media, etc are used for different purposes. Nutrient broths and agar plates are the most common growth media for microorganisms; sometimes specialized media are required for microorganism and cell culture growth[38] . Culture media contain a carbon source, water, various salts, and a source of amino acids and nitrogen that most bacteria need for growth. Minimal media is a defined medium that has just enough elements to support the growth of cells. Selective media are used for the growth of only selected microorganisms. If a microorganism is resistant to a certain antibiotic, such as ampicillin, then ampicillin should be added to the medium to prevent other cells from growing. The gene insert contains a selectable marker which used for the detection of recombinant DNA. An antibiotic marker is applied so a host cell without a vector dies when exposed to a known antibiotic, and the bacterial host with the plasmid vector will survive because it is resistant. The vector is inserted into a host cell differently like a process called transformation. E. coli. is a common example of host cells that should be carefully prepared to receive the desired DNA. To distinguish transformed bacteria from untransformed bacteria, selectable markers should show properties like antibiotic resistance, and colour changes under light. Different vectors are used for different applications according to their characteristics [24].Some pectinase enzymes that work at high temperatures of 60 °C and appropriate pH conditions can be of benefit at high temperatures[39]. Growth temperature exerts a specific control upon the activity of certain enzymes[40]. Normal growth was observed after 24 hours at 37°C for Escherichia coli bacteria in Luria-Bertani (LB) agar media[41].

PCR is a technique for the amplification of DNA, which generates thousands to millions of copies of a particular DNA sequence by denaturation, annealing, and extension steps. A DNA molecule is set in a thermocycler for the doubling process which is accomplished by polymerases (specific proteins) and polymerases require the nucleotides consisting of the four bases. The primer (small DNA), attaches with the building blocks as well as a longer DNA molecule to serve as a template for building the new strand. The PCR product can be run for digestion with restriction enzymes and sequencing for the next step [42].

Biomolecules are associated with positive or negative electrical charges, in the placement in an electric field, charged biomolecules run to the electrode of opposite charge due to the characteristics of electrostatic attraction. Electrophoresis is the process of separation of charged molecule samples in a provided electric field. Gel electrophoresis is a general technique for resolving proteins or nucleic acid size. A sample (like protein, DNA, or RNA) is usually placed in a well at or near the end of a gel matrix. After sample loading, an electric field is set into the gel, and charged samples of the same size are driven simultaneously in the direction of the anode inside the gel as discrete invisible band samples. The distance that the sample moves into a gel depends on the mass of its sample and the size of the mesh opening of the gel. The smaller macromolecule sample moves further than the larger ones. A charged molecule in an electric field depends on variables, the velocity of movement, $\nu = Eq / f$, where E is electric field strength, f is the frictional coefficient, and q is the net charge on the molecule [43].

Agarose is a polysaccharide gel matrix widely used for the separation of DNA molecules) shown in Scheme 1.3(a). It is composed of a repeating unit called agarobiose, which consists of galactose and 3,6-anhydrogalactose. The uniform charge distribution in nucleic acids makes agarose gel suitable for separating DNA molecules based on their mobility. It is often used to determine DNA molecular masses in electrophoresis. On the other hand, acrylamide is used to create a stronger gel for the separation of both proteins and nucleic acids. Acrylamide gel is formed by the polymerization of acrylamide, and a small amount of acrylamide is cross-linked with a methylene bridge (N,N′ methylene bis acrylamide) shown in Scheme 1.3(b). This cross-linking allows the formation of a gel with highly controlled porosity, making it mechanically strong and chemically inert. Acrylamide gels are preferred when higher resolution and tighter separation of molecules are required. Both types of gels are essential tools in gel electrophoresis techniques, playing a crucial role in separating biomolecules based on size, charge, or other characteristics for various applications in molecular biology and biochemistry.



Scheme 1.3 (a) Polysaccharide gel (b) Polymerization of acrylamide to form polyacrylamide gel[44].

Now for the discussion of cotton pretreatment. Cotton fabric must be pretreated, the mandatory process before dyeing, printing, and finishing, because cotton impurities make a hydrophobic layer to prevent water entrance. Scouring is the process of removing oils, fats, waxes, minerals, pectin, and proteins that act as a barrier during the wet process[45]. Sodium hydroxide is used for the chemical scouring process and enzymes are used for bio-scouring. Pectinase enzymes are commonly used for the bio-scouring process. Bio scouring is one of the alternative processes that has been investigated and studies have been undertaken into the application of cellulases, pectinases, proteases, and lipases in such a process. Of the enzymes investigated, pectinases are the most suitable as they are capable of removing impurities from raw cotton substrate without changing the properties of the substrate [46]. In the case of the pectinase enzyme, bio-scouring bath temperature is generally maintained at 60 °C to get optimum results in the presence of surfactant[47]. In conventional scouring, dilute sodium hydroxide (NaOH) solution is used at 95 °C which swells the cotton fibers and opens up the cell of the fibers [8].

Bleaching is the process of removing naturally produced colour in the fabric. Scouring and bleaching can be done in a single bath or separate bath. In a combined single bath scouring bleaching process, generally, the bath contains NaOH 4.0%, H_2O_2 3.0%-4.0%, surfactant 0.2-0.4%, sequestering agent 0.1-0.2%, stabilizer 0.5-1.0% for 60 minutes at temperature 100°C. In

a single bath chemical scouring, sodium hydroxide, sequestering agent, and surfactant are used. In the case of bio scouring enzymes and surfactants are used generally[48].

There are two types of bleaches: such as (a) reductive, and (b) oxidative [49]. Sulfur dioxide, sodium hydrosulfite, sulphoxylates, acidic sodium sulfite, sodium bisulfite [50] , thiourea dioxide ($CH_4N_2O_2S$)[51], and sodium dithionite ($Na_2S_2O_4$)[52] , sodium borohydride are reductive bleaching agent used in the reductive bleaching process [53]. Oxidative bleaching agents are divided into two categories; (1) chlorine-based bleaching agents, such as bleaching powder and sodium hypochlorite, and (2) peroxide-based bleaching agents: such as hydrogen peroxide [54]. Hydrogen peroxide (H_2O_2) is a commonly used bleaching agent. It is a milder reagent, capable of continuing the dissociation action simultaneously with the bleaching action, with less risk of yellowing after bleaching, no danger of equipment corrosion, and no unpleasant odors than hypochlorite bleaching [55]. Dissociation of hydrogen peroxide and formation of per hydroxyl ions (HO_2^-) to like as below:

$OH^- + H_2O_2 \rightarrow HO_2^- + H_2O$

[chromophore] + $HO_2^- \rightarrow$ Bleached Fabric

The mechanism of hydrogen peroxide bleaching is not well understood. Some of the chromophores oxidized during the bleaching process are undoubtedly conjugated double[56].

Bleaching is accomplished by solubilizing or removing chromophores or by destroying or altering unsaturated or conjugated chromophores. The dissociation of hydrogen peroxide occurs at a high pH (about 10-12). The formation of per hydroxyl ions has been widely proposed for the bleaching reaction. The dissociation of hydrogen peroxide increases with rising temperature, the concentration of the per hydroxyl ions depends to a much larger extent on the pH of the solution. At low temperatures and pH, hydrogen peroxide solutions generally react quite slowly. In practice, textile bleaching is performed at high temperatures under alkaline conditions using NaOH and Na_2CO_3 together with stabilizers and wetting agents. Stabilizers are added to minimize the decomposition of hydrogen peroxide and the per hydroxyl ion by forming complexes with heavy metal cations [57]. Even though there are lots of bleaching processes available in the current market, manufacturers are still looking for an economical way with enhanced bleaching performance. Very few studies compared the bleaches. Thiourea dioxide is an organic sulfur compound that is used by some textile dyeing industries. It acts as a robust reductive bleaching agent at pH 7.1. Thiourea dioxide produces sulfinate ions [53], which are

highly reactive as a bleaching agent. Thiourea is used for the production of thiourea dioxide according to the following reaction mechanism.

$H_2N\text{-}C(=NH)\text{-}SH + 2\ H_2O_2 \rightarrow H_2N\text{-}C(=NH)\text{-}SO_2H + 2H_2O$

$H_2N\text{-}C(=NH)\text{-}SO_2H + 2\ OH^- \rightarrow H_2N\text{-}C(=NH)\text{-}OH + SO_2^-$

$SO_2^- + [\text{chromophore}] \rightarrow SO_4^{-2} + [\text{reduced chromophore}]$

The oxidative bleaching of cotton is done by using the universal bleaching agent hydrogen peroxide. The peroxide bath is converted to a reductive bath by the addition of thiourea and bleaching continues without the need for a fresh batch for the reductive steps[58][59]. Some chromophores of cotton can be reduced by sulfinate ion and the resultant bleaching process increases the total whiteness than the oxidative bleaching process. The bleaching conditions developed with alkaline hydrogen peroxide bleaching at 60 °C for 60 min followed by the addition of thiourea, pH adjustment, and subsequent reductive bleaching at 60 °C for 25 min followed by washing and rinsing[60].The hydrogen peroxide activation by thiourea is suggested to proceed via a free radical mechanism that the first step is the formation of the intermediate hydrogen peroxide–thiourea complex which simultaneously decomposes to give hydroxyl free radicals (Eq. 1). These hydroxyl free radicals react with hydrogen peroxide to give per hydroxyl free radicals(Eq. 2), which in turn react with hydrogen peroxide to give hydroxyl free radicals and nascent oxygen(Eq. 3)[61].

$(NH_2)_2CS + H_2O_2 \rightarrow (NH_2)_2CS \cdot H_2O_2 \rightarrow (NH_2)_2CS + 2\ OH^{\bullet}$ ----- (1)

$OH^{\bullet} + H_2O_2 \rightarrow H_2O + HO_2^{\bullet}$ ---------- (2)

$HO_2^{\bullet} + H_2O_2 \rightarrow H_2O + HO^{\bullet} + 2[O]$ ---------- (3)

By adding the activating agent sodium borohydride into an aqueous solution containing thiourea dioxide, the reduction potential is raised, which value is higher than those exhibited by thiourea dioxide, and the activating agent each independently. This fact means that thiourea dioxide is activated[62].

Sodium borohydride was shown to be an extremely effective reducing agent in organic chemistry[63]. When the borohydride is used as a means of generating hydrogen, a rapid

reaction is desired[64]. The reduction of conjugated aldehydes and ketonos by sodium borohydride leads, in general, to substantial amounts of fully saturated alcohol products[65].

Reactive dyes are a type of colourant commonly used in the textile industry. They are called "reactive" because they chemically react with the fibres and they are applied to form a covalent bond between the dye and the fibre molecules. Substantivity refers to the ability of a dye to be attracted and retained by the fibres. The substantivity of reactive dyes varies depending on the specific dye and the conditions in which they are applied. Cotton fibres acquire a negative charge in the dyeing medium. The negative charge on cotton fibres can lead to repulsion between the fibre and the negatively charged dye anions during the dyeing process. To control the repulsion between the fibre and the dye, a large quantity of salt is used in the dye bath. The addition of salt helps to mitigate the repulsion forces, allowing the dye to be more effectively attracted to and taken up by the cotton fibres. Soda ash (sodium carbonate) is commonly used to adjust and control the pH of the dye bath. The use of reactive dyes involves carefully managing the repulsion between the negatively charged cotton fibres and the dye anions. This is achieved by incorporating a substantial amount of salt into the dye bath and controlling the pH with the addition of soda ash. These conditions ensure that the reactive dyes react effectively with the cotton fibres, resulting in durable and colourfast dyeing of the fabric[66].

The eco-friendly process emphasizes the need for sustainable processes in response to the growing global environmental condition. The goal is likely to reduce the environmental impact of traditional processes, possibly by minimizing the use of harsh alkaline treatments and promoting eco-friendly alternatives. The thesis approaches to investigate recombinant DNA technologies as a means to bypass the conventional scouring process in cotton treatment. This work suggests an interest in genetic engineering or gene modification of pectinase enzyme-producing bacteria to improve cotton bio-scouring, potentially making it more environmentally friendly and efficient in processing. The thesis proposes novel and efficient approaches to improve existing processes by applying low processing temperatures.

1.1 Aims and Objectives

This work aims to develop bio-engineered enzyme production and its application in the wet processing of cotton knit fabric to reduce the quantity of chemicals applied and process temperature**.**

The main objectives of this thesis are the production and application of the new recombinant and natural pectinase enzyme in cotton knit fabric scouring and asses by comparing the dyeability of natural and the new recombinant enzyme-treated fabric. Another objective is to improve the whiteness of enzyme-treated bio-scoured fabric to get dyeability of light colouration of cotton fabric.

1.2 Outline of the Thesis

- At first, designing the overlapping gene segments, PCR amplification, and full gene assembly through Gibson assembly will be followed. Regarding the modification, gene length will be checked to assess the ligation and to get the desired length of the gene.
- The restriction digestion of selected gene and plasmid will be done, then gene fragments will be ligated and transformed by heat shock culminating in the selection of positive clones through checking of antibiotic resistance, presence of transgene, and enzymatic activity. Eventually, PAGE analysis regarding protein molecular weight to confirm the correct pectinase protein is also one of the benchmarks of the thesis.
- The bio scouring has been modified by lowering processing temperature by growing bacterial production at 37 °C and recombining bacterial DNA with fungal DNA to get the pectinolytic activity in the bacterial enzyme.
- Cotton knit fabric needed to be bio-scoured with the recombinant and natural pectinase enzyme at 37 °C to check the pectinolytic activity of the produced enzyme. Moreover, pectin removal from cotton knit fabric will be checked by wicking test and FTIR analysis.
- Bleaching with bio-scoured fabric, which is not usual practice, will be applied to increase the whiteness of bio-scoured fabric with oxidative bleaching and reductive bleaching. Reactive dyeing performance will be also examined for enzyme-treated scoured and bleached samples and evaluated by the performance of achieved colour attributes and fastness properties. All types of investigation will be discussed.

Chapter 2
Materials and Methods

Chapter 2
Materials and Methods

2.1 Preparation and Production of Recombinant Pectinase Enzyme

In the recombinant enzyme preparation overview, the first step was to identify the gene of interest that wants to transfer into the bacteria. This gene could code for a protein with a particular function that wants to be introduced into the bacteria. Once identified the gene, needs to collect its fragments. These fragments were amplified through PCR (Polymerase Chain Reaction). PCR is commonly used to amplify specific DNA sequences. Overlapping primers are designed to target specific regions of the gene fragments, allowing them to be amplified separately. Add a signal peptide to the gene sequence to facilitate its expression and secretion in the bacteria. Restriction sites were also added to the gene fragments to aid in the ligation process. The amplified gene fragments were then ligated together using DNA ligase. This process joins the fragments in the correct order to reconstruct the full-length gene sequence. The gene construct was purified to remove any contaminants and ensure that have a clean sample for further experimentation. Both the inserted gene and the plasmid were cut using restriction enzymes, which act as molecular scissors, at specific recognition sites. This creates sticky ends on both the inserted gene and the plasmid. The cut insert gene was then ligated (joined) into the plasmid vector. DNA ligase enzyme was used to catalyze the formation of phosphodiester bonds between the insert gene and the plasmid, creating a recombinant DNA molecule. The recombinant plasmid was introduced into *Escherichia coli* bacteria through a process called transformation. The *E. coli* cells were made competent (capable of taking up foreign DNA) by subjecting them to a brief heat shock, followed by rapid cooling. During this brief period of increased permeability, the recombinant plasmid can enter the bacterial cells. The plasmid used for transformation usually carries a gene for antibiotic resistance (e.g., ampicillin). After transformation, the bacterial culture was plated onto agar plates containing the antibiotic. Only the bacteria that had successfully taken up the recombinant plasmid would be able to grow on these plates, as they possess the antibiotic resistance gene. The creation of genetically modified E. coli bacteria that express the desired gene were checked and exhibiting the desired phenotype, such as antibiotic resistance and enzymatic activity was also assessed.

2.1.1 Designing the recombinant plasmid

The gene of interest, choosing the vector, and insertion site designing are fundament factors before the transformation of the recombinant plasmid into the competent host bacterial cells which must bear the transgene under suitable promotor of a plasmid for achieving successful expression.

2.1.1.1 Designing the gene of interest

Designing the gene was done by the GEB department of the University of Dhaka, and involved selection of gene segments, codon optimization, designing the gene segments, primer designing, addition of signal peptide and restriction sites, structure prediction, and *in silico* affinity analysis. The PHYRE2 protein fold recognition server was used to predict the structure of the newly designed Ast protein (Figure A3 and Figure A4). The default normal settings were used for this purpose. PROCHECK software was used to validate the quality of the prediction. The Auto Dock Vina software was used to compute the affinity of monomeric alpha-D-galacturonic acid and dimeric digalacturonic acid in the predicted protein structure (Figure A5). The ligand structures were downloaded from the online database PubChem.

2.1.1.1.1 Selection and optimization of gene

The gene segments and primers were collected from Macrogen (lot no. HO00068084, Seoul, South Korea). A fungal pectinase gene of the *Aspergillus steynii* species was selected from the NCBI gene database[67] for Gibson assembly[68] and inserted into the *E coli* bacteria. The gene sequence optimization was done by using The JCat software[69] for *E. coli* strain K12 and denoted by "Ast''.Ten fragmented segments were coded, considering an overlap of ~20 bp, from 112 to 133 base pairs(bp). For amplifying each of the gene segments two primers were designed according to Table 2.1. The alpha-amylase signal peptide of *Escherichia coli* was selected for use upstream of the Ast gene. Its corresponding sequence, along with the NheI restriction site, was introduced inside the first forward primer. The reverse primer of the last segment contained the restriction site for XbaI.

Table 2.1 Sequence for the recombinant pectinase enzyme

No	Sequence ID	Sequence (5'→3')	bps
1	A.st_Seg_1_For	TCGTCGTCGGCTAGCATGAAACTGGCGGCGTGCT TTCTGACCCTGCTGCCGGGCTTTGCGGTGGCGAT GCGTTCTTTCCAGCTGCT	86
2	A.st_Seg_1_Rev	GGTAGCTTCAGCAGCAGAGG	20
3	A.st_Seg_2_For	CCTCTGCTGCTGAAGCTACC	20
4	A.st_Seg_2_Rev	CCTTCGAAGGTGATGGTGGT	20
5	A.st_Seg_3_For	ACCACCATCACCTTCGAAGG	20
6	A.st_Seg_3_Rev	GAGAACCGTCACCGTCGATC	20
7	A.st_Seg_4_For	GATCGACGGTGACGGTTCTC	20
8	A.st_Seg_4_Rev	CCGGGGTGTTTTTGATGTTCAG	22
9	A.st_Seg_5_For	CTGAACATCAAAAACACCCCGG	20
10	A.st_Seg_5_Rev	CCAACGTCGAACGCGTCAGT	20
11	A.st_Seg_6_For	ACTGACGCGTTCGACGTTGG	20
12	A.st_Seg_6_Rev	CACCAGAGCAGGTAGCACCA	20
13	A.st_Seg_7_For	TGGTGCTACCTGCTCTGGTG	20
14	A.st_Seg_7_Rev	GGTTTTGATACGGATACCGTTGTC	24
15	A.st_Seg_8_For	GACAACGGTATCCGTATCAAAACC	20
16	A.st_Seg_8_Rev	CCACCGTTTTTGTAGTCCTG	20
17	A.st_Seg_9_For	CAGGACTACAAAAACGGTGG	20
18	A.st_Seg_9_Rev	CAGAACCGCACAGGATGTAG	20
19	A.st_Seg_10_For	CTACATCCTGTGCGGTTCTG	20
20	A.st_Seg_10_Rev	GGAGGAGGAGGATCTAGATTAGCAGCTGACACCCGAT	37
21	A.st_TempSeg_1	ATGCGTTCTTTCCAGCTGCTGGGTCTGGCTGCTG TTGGTTCTCTGGTTTCTGCTGCTCCGGCTCCGTCT CTGACCAAAAAAGACTCTTCTTGCACCATCACCT CTGCTGCTGAAGCTACC	120
22	A.st_TempSeg_2	CCTCTGCTGCTGAAGCTACCGAATCTGTTTCTGG TTGCTCTAAAGTTGTTGTTAAAGACGTTAAAGTT CCGGCTGGTGAAACCCTGGACCTGTCTAAAGTT GACGACGGTACCACCATCACCTTCGAAGG	130

No	Sequence ID	Sequence (5'→3')	bps
23	A.st_TempSeg_3	ACCACCATCACCTTCGAAGGTACCACCACCTTCG AATACAAAGAATGGAAAGGTCCGCTGATCCGTA TCGAAGGTAAAGAAATCACCGTTAACATGGCTG AAGGTGGTCTGATCGACGGTGACGGTTCTC	130
24	A.st_TempSeg_4	GATCGACGGTGACGGTTCTCGTTGGTGGGACTCT AAAGGTACCAACGGTGGTAAAAAAAAACCGAA ATTCCTGTACGCTCACAAACTGGAAGACTCTACC ATCTCTGGTCTGAACATCAAAAACACCCCGG	131
25	A.st_TempSeg_5	CTGAACATCAAAAACACCCCGGTTCAGGCTATCT CTGTTCAGGCTACCAACACCCTGTTCGAAAACAT CCACATCGACAACTCTGACGGTGACTCTAACGG TGGTCACAATACTGACGCGTTCGACGTTGGT	132
26	A.st_TempSeg_6	ACTGACGCGTTCGACGTTGGTGAGTCTGACGGTG TTCAGATCCGTGGTGCTGTTGTTAAAAACCAGGA CGACTGCCTGGCTATCAACTCTGGTAAAAACATC GAATTCTCTGGTGCTACCTGCTCTGGTG	130
27	A.st_TempSeg_7	TGGTGCTACCTGCTCTGGTGGTCACGGTATCTCT ATCGGTTCTATCGGTGGTAGGGACGACAACACC GTAAGCAACGTTACCATCGCTGACTCTACCGTTA CCAACTCTGACAACGGTATCCGTATCAAAACC	133
28	A.st_TempSeg_8	GACAACGGTATCCGTATCAAAACCATCGTTGAC GAAACCGGTGACGTTTCTGACGTTACCTACTCTA ACATCGAACTGTCTAAAATCCACAAAAAAGCTA TCGTTATCCAGCAGGACTACAAAAACGGTGG	131
29	A.st_TempSeg_9	CAGGACTACAAAAACGGTGGTCCGACCGGTGAA CCGTCTAACGACATCCCGATCAAAGGTCTGACC GTTGACGGTATCACCGGTTCTGTTGACTCTGACG CTGTTCCGATCTACATCCTGTGCGGTTCTG	130
30	A.st_TempSeg_10	CTACATCCTGTGCGGTTCTGGTTCTTGCACCGAC TGGACCTGGTCTGGTGTTGACCTGTCTGGTGGTA AAGACTCTACCTGCAAAAACCAGCCATCGGGTG TCAGCTGCTAA	112

2.1.1.1.2 Selection of insertion site

The pGLO plasmid sequence was mapped to search for the insertion site, the insertion site is NheI and XbaI. NheI and XbaI sites had the nucleotide positions - 1345 (Nhe1) and – 2090 (XbaI) a difference of 745 base pairs. The GFP gene coding region is highlighted in gray in the following sequence. It spans from nucleotide number 1342 to 2061 – with a length of 720 bps. The NheI site is in blue, XbaI site is in purple.

ACTTTAAGAAGGAGATATACATATGGCTAGCAAAGGAGAAGAACTTTTCACTGGA
GTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGT
CAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTATT
TGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTA
TGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCA
AGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGA
CGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAAT
CGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACACA
AACTCGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAA
GAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACATTGAAGATGGATCCGTT
CAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTT
ACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAA
AAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGG
CATGGATGAGCTCTACAAATAATGAATTCGAGCTCGGTACCCGGGGATCCTCTAG
AGTCGACCTGCAGGC

2.1.2 Fungal gene insert and pGLO plasmid vector preparation

2.1.2.1 Stock solutions preparation of primers and fragments

For making a final concentration of 100 pmoles/µL of primers and fragments, the recommended ddH_2O (double distilled water) was added, written on each given tube, and mixed properly to make a stock solution.

2.1.2.2 PCR of the gene segments

Polymerase chain reaction is commonly known as PCR. It is used to produce multiple copies of a gene or DNA of interest. The steps of polymerase chain reaction are denaturation, primer annealing, and extension of primers [70]. The PCR condition according to Figure 2.1 were used to amplify ten fragmented genes. The PCR tubes were labeled and the master mix was formulated according to Table 2.2. The components were added sequentially from top to bottom. New England Biolabs company supplied buffer and the Q5 High-Fidelity DNA polymerase (lot

no. 10032989). A 2 µL volume template and 2 µL volume of corresponding segments primer mix (forward and reverse) were added to each tube. Axygen® MaxyGene II thermal cycler was used to perform PCR.

Table 2.2 PCR recipe for amplifying the Ast gene segments

SL No.	PCR elements	Reaction master mix amount (µL)
1	Q5® buffer (5×)	5.0
2	Diluted 10mM dNTP mix	1.0
3	PCR water	14.8
4	Q5 DNA polymerase	0.2
5	Total volume (each tube)	21.00

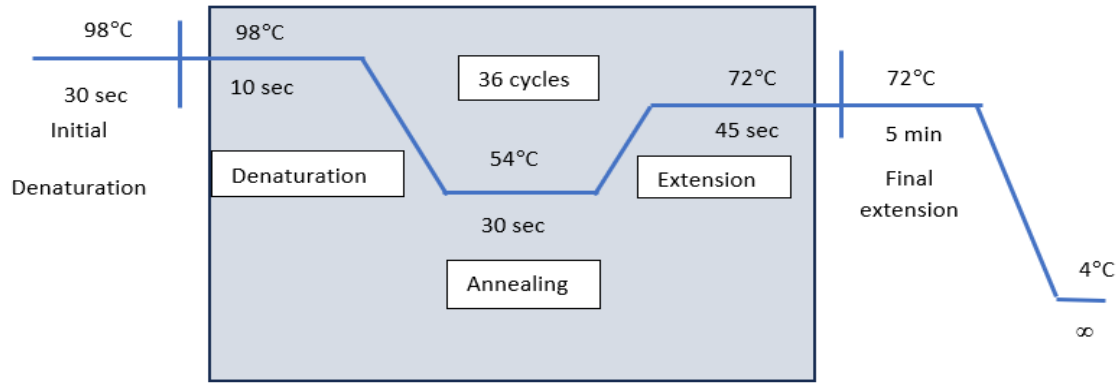


Figure: 2.1 PCR conditions for amplification of the individual Ast gene segments

2.1.2.3 Method of checking the size of DNA

Agarose gel electrophoresis is a globally recommended and acceptable method of checking the integrity and estimating the size of DNA [71]. Dissolved agarose powder in tris-acetate-EDTA buffer was taken to prepare the gels. 1% agarose (molecular grade, MP Biomedicals LLC™, catalog no: AGAH0100) gel was used for band visualization and extraction of DNA samples. The gel solution was made by measuring the agarose powder in an electronic balance (Kern & Sohn GmbH, Serial no- WB1210380), and 0.5× TAE (Tris-acetate EDTA) buffer (20 mM Tris (VWR life science, lot no.- 2617C433) 10 mM acetate and 0.5 mM EDTA). In a conical flask these were mixed and heated for 2 minutes in a micro-oven (Samsung, Korea) at 850 watts. Before the addition of ethidium bromide (Genaxxon bioscience, Germany, lot-No. 4L011634), the solution was kept for cooling down for 1 minute, and after cooling the solution was poured onto the gel trays. GeneOn 50-bp DNA ladder was used afterward as the DNA size marker in

agarose gel electrophoresis. The gel documentation system Fusion Pulse 6, (Vilber Lourmat, France) was used for visualization of the DNA band in agarose gel.

2.1.3.4 Purification of the gene segments after PCR

The FavorPrep™ Gel/PCR purification mini kit had catalog no: FAGCK001, lot no: BI307118307 was used to purify PCR products using their manual (Figure A1). 125 µL FADF buffer was added to 25 µL of PCR products for individual tubes and all tubes were vortexed properly. The FADF columns were kept in collection tubes. The first mixtures were poured into the FADF column, centrifuged at 11,000× g for 30 seconds, and finally the flow-through was discarded. Wash buffer was added to ethanol and 750 µL of ethanol-wash buffer mixture was poured into the FADF column, centrifugation at 11,000× g for 30 seconds, and then the flow-through was discarded. After drying the column matrix, a microcentrifuge tube was set under the FADF column. An elution buffer of 20 µL was added to the middle of the column membranes and kept for 10 minutes in standing condition. At last, centrifugation was done at 11,000× g for 50 seconds and the elution of DNA was obtained.

2.1.2.5 Assembly of the ten segments of genes of interest

The ten fragmented gene segments were ligated by the Gibson assembly method. NE Builder® HiFi DNA assembly master mix from the company New England Biolabs was used for the Gibson assembly (catalog no N2611A, lot no 10056330). An amount of 1 µL each Ast gene segment was taken in a tube by micro pipetting to make 10 µL and another 10 µL assembly master mix was added to assemble the fragmented DNA. The tube containing 20 µL mixture was incubated for 1 hour at 50 °C in a PCR. After incubation, the DNA solution was transferred to the silica matrix in a spin column. After PCR each gene segment was purified and the ligated products were also purified using the FavorPrep Gel/PCR purification mini kit following the procedure described in subsection 2.1.3.4. Here at the first stage, 20 µL of the ligated gene mixture was poured into the microcentrifuge tube and added with 100 µL of FADF buffer that was vortexed thoroughly. A PCR was run according to Figure 2.2 using the reagent mixture of five times concentrated 5 µL Q5® buffer, 1.5 µL of diluted 10mM dNTP mix (10 mM), and 1.5 µL of primer (mixture of segment1 forward primer and segment 10 reverse primer).

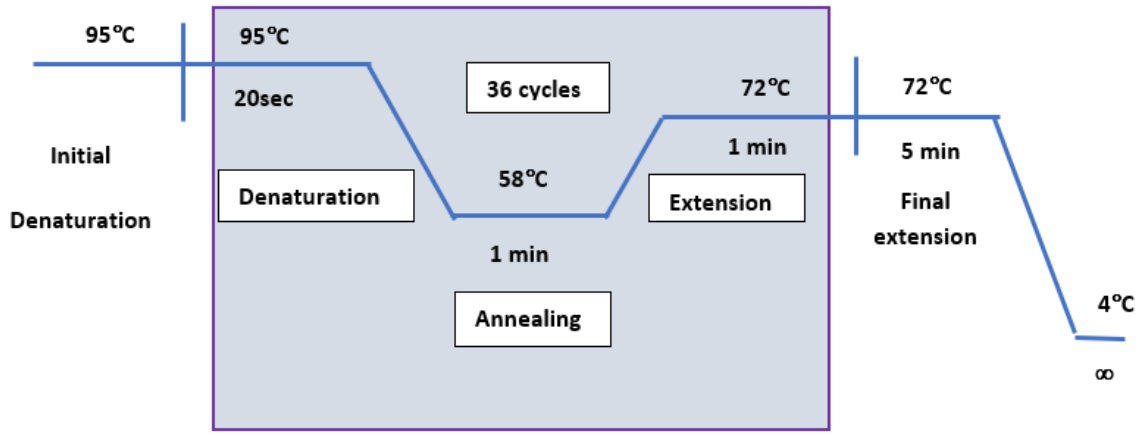


Figure: 2.2 PCR conditions to amplify the ligated Ast gene

The PCR product (6 µL) was run in an agarose gel with GeneON-BioScience One-for-all ladder as a DNA size marker.

2.1.2.6 Purification of gel for the assembled gene

A gel electrophoresis apparatus (Cleaver Scientific Ltd., UK) was used to gel run with 170 µL purified PCR product added with loading dye ethidium bromide (10 × i.e. 1 volume of dye with 9 volume of sample mix was run in the gel electrophoresis system), and then purification was done with Favorgen® Gel/PCR purification mini kit shown in Figure 2.3. The DNA band gel slices were cut out according to the area of the coloured portion from agarose gel and kept in two micro centrifuges tubes by measuring their weight. Each gel-containing tube was vortexed with 500 µL of FADF buffer and incubated at 55 °C for 10 minutes in a water bath (Model LSB-015S, Daihan Labtech, Korea). After cooling, these FADF columns were set into collection tubes. 750 µL of the mixture was poured into the FADF columns, centrifuged at 11,000× g for 30 seconds, and the flow-through was discarded. 750 µL of ethanol-added wash buffer was mixed to the FADF columns. Again, centrifugation was done at 11,000× g for 30 seconds, and then flow through was discarded. A wash-step was performed to remove impurities. The FADF columns were kept in a new microcentrifuge tube, 70 µL of elution buffer was added to each tube and the columns were allowed to stand for 10 minutes. Again, centrifugation was done at 11,000× g for 50 seconds to complete the elution of DNA. 6 µL of this purified product was run in an agarose gel with GeneON-BioScience One-for-all ladder as a DNA size marker.

Figure: 2.3 Purification of the ligated gene by mini kits(FavorPrep)

2.1.2.7 Plasmid purification to obtain pGLO plasmids

pGLO plasmids were purified from transformed DH5α *E. coli* cells using the Favor Prep plasmid extraction mini kit manual (Figure A2). Well-grown bacterial cultures (2µL) were taken into centrifuge tubes, centrifuged at 11,000× g for 50 sec to pellet the cells, and the supernatant was discarded. 200µl of FAPD1 buffer (RNase A added) was added to the pellet, and the cells were resuspended in the buffer for pellet dissolution. FAPD2 buffer (200µL) was added to the tubes and gently inverted several times to act as a harsh cell lysis step. For lysate neutralization FAPD3 (300µL) buffer was added to each tube. To clarify the lysate, centrifugation was performed at 18,000× g for 5 minutes. The supernatant was transferred carefully to the FAPD column (inserted inside a collection tube) and centrifuged at 11,000× g for 50 seconds. The flow-through was discarded and the columns were placed back on the collection tubes. After that WP buffer (400µL) was added to the FAPD column and centrifuged at 11,000× g for 50 sec. Discarding the flow, the columns were put on the collection tubes. Wash buffer (700µL)

was added to the FAPD columns and centrifuged at 11,000× g for 50 sec. The flow-through was discarded and the columns were placed in the collection tubes. After drying the FAPD columns, it was set on a centrifuge tube (1.5µL). 50µl of elution buffer was added to the center of each of the FAPD columns. The column was allowed to stand as like for 10 minutes. Centrifugation was done for 1 minute at 11,000× g to elute the plasmid DNA. These solutions were poured into PCR tubes and stored at -20 °C for further requirements

2.1.3 Production and selection of recombinant *E.coli*

2.1.3.1 Restriction digestion of Ast gene and pGLO plasmid

The plasmids and the purified gene were used for restriction digestion by NheI (which was sourced from *Neisseria mucosa*) and XbaI (which was sourced from *Xanthomonas badrii)*. The plasmids were used for alkaline phosphatase treatment to prevent self-ligation. Tango buffer was used for double digestion (digesting a DNA substrate with two restriction endonucleases simultaneously) of DNA with restriction enzymes. The reaction materials and conditions were followed according to Table 2.3 and Figure 2.4 respectively.

Table 2.3 Amounts of reaction materials for restriction digestion

Components	Plasmid(µL)	Ast gene of interest(µL)
Tango buffer (10×)	5	5
Extract or PCR product	20	20
Alkaline phosphatase	1.0	0.0
Water	22	23
XbaI	1.0	1.0
NheI	1.0	1.0
Total	50.0	50.0

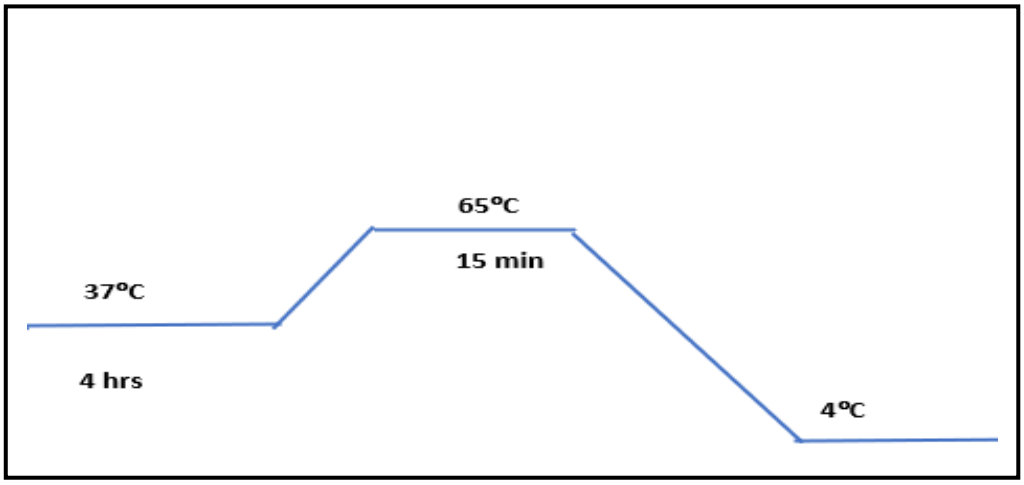


Figure: 2.4 Restriction digestion protocol

2.1.3.2 Purification of the digested product and quantification

The digested products were loaded into 1% agarose gel in separate lanes and the desired bands were purified using the gel extraction kit (Monarch® DNA, New England Biolabs). The desired bands of the agarose gel section were taken using a 1 mL pipette and appropriate tips under intense blue light and an orange filter. The gel slices were transferred to two microcentrifuge tubes. 4 volumes of Monarch gel dissolving buffer were added to the gel slice, here a mass of gel in micrograms was taken as an equivalent volume in microliters. The samples were incubated in a heat block (Daihan Labtech, Namyangju, Korea) at 55 °C for 10 minutes with intermittent vortexing. Two columns were placed into collection tubes and samples were loaded onto the respective columns. Centrifugation was performed at 11,000× g for 50 seconds, and the flow-through was discarded. 200 µL DNA wash buffer (ethanol-added) was loaded onto the column and again centrifuged at 11,000× g for 50 seconds. Flow-through was discarded. A blank spin was performed for another 50 seconds at 11,000× g. The columns were transferred to new clean 1.5 microcentrifuge tubes. 15 µL of elution buffer was added at the center of the membrane and allowed to stand for 10 minutes. A centrifugation at 11,000× g eluted the purified restriction digested products. Afterward, spectrophotometric readings were taken in a OneDrop microvolume spectrophotometer (Serial no.- HSZ6010-1812-002J, Biometrics Technologies Co. Ltd., New Taipei City, Taiwan). The molar concentrations were created using the 'DNA µg & pmol converter' web tool (GeneInfinity).

2.1.3.3 Ligation of the digested products

To maintain the molar ratio of the vector: insert the optimum for ligation[72] , in between 1:3 to 1:5, the ligation reaction by T4 DNA Ligase (Thermo Scientific) was done with the following components and conditions: in Table 2.4 and Figure 2.5 respectively. The ligation mixture was kept at -20 °C for the next use. T4 DNA Ligase is a ligation enzyme that is used to join DNA fragments by catalyzing the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3' hydroxyl termini in double-stranded DNA. T4 DNA Ligase buffer designed for use with T4 DNA Ligase to catalyze the joining of two cohesive- or blunt-ended strands of DNA between the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides. The formulation of the T4 Ligase buffer (10×) is 300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP.

Table 2.4 Components for the ligation mixtures

SL No.	Components	Amount (µL)
1	10× buffer (T4 Ligase)	5
2	Purified pGLO digest	30
3	Purified Ast gene digest	10
4	Water	4.5
5	T4 Ligase	0.5
	Total	50

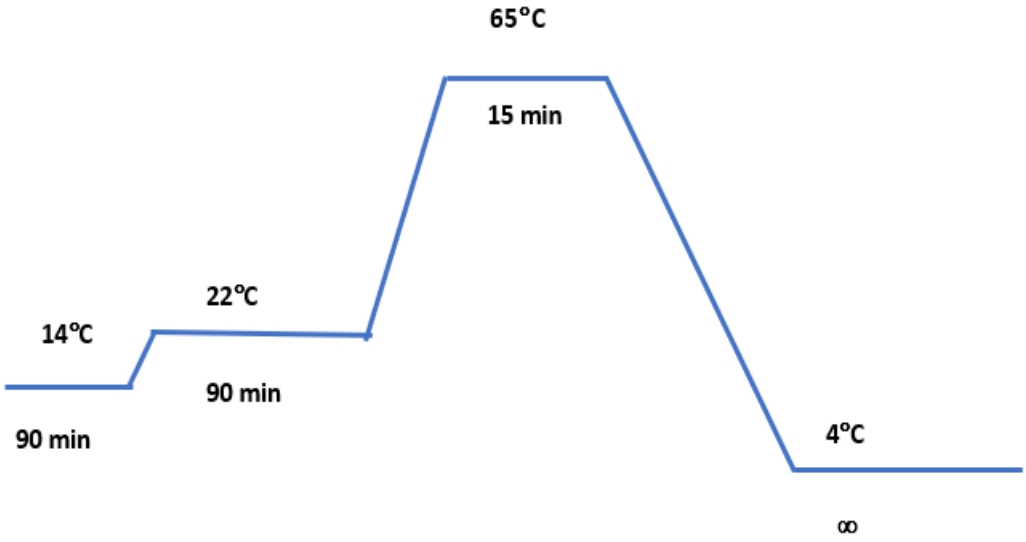


Figure 2.5 Protocol for the ligation reaction

2.1.3.4 Preparation of stock cultures of competent bacterial cells

The following three steps were done to make stock cultures of competent cells. To begin with, the preparation of calcium chloride buffer solutions, Secondly, the culture of bacteria, and finally, calcium chloride solutions were used for the competency and viability of bacteria during storage in a deep freezer.

2.1.3.4.1 Calcium chloride buffer preparation

100 mL 1 M $CaCl_2$ stock solution was prepared (by mixing 11.1 g of anhydrous $CaCl_2$ with distilled water). The stock solution was filtered and sterilized. 0.1 M $CaCl_2$ solution was prepared by mixing 10 mL of stock $CaCl_2$ solution with 90 mL distilled water and finally, 0.1 M $CaCl_2$ with 15% glycerol solution was prepared.

2.1.3.4.2 Overnight culture of bacteria

The *Escherichia coli* strains DH5α and BL21 were cultured in individual 15 mL tubes having a media composition of 2% Luria-Bertani (LB) medium (Liofilchem, Italy, lot no-121119503). After inoculation, the culture was incubated at 37 °C at 120 revolutions per minute (rpm) for 16 hours. Optical density values at 600 nm were taken using the blank media to auto-zero. In this thesis, the competent untransformed *E coli* DH5α and BL21 strains will be referred to as UTD and UTB, respectively.

2.1.3.4.3 Competent cell generation by $CaCl_2$ wash

The liquid culture tubes were taken and kept on ice for 20 minutes, the tubes were centrifuged at 4 °C and 4000 rpm for 10 minutes, the supernatant was decanted and the remaining media was aspirated using a pipette. The pellets in the tubes were resuspended in 10 mL 0.1 M $CaCl_2$ and incubated on ice for 30 minutes, centrifugation was continued at 4 °C at 4000 rpm for 10 minutes, and the supernatant was discarded. 50 mL of 0.1M $CaCl_2$ with 15% glycerol working solution was kept on ice and used to resuspend the cells. 50 µL of this mixture was transferred to each microcentrifuge tube, sealed, labelled, and stored in a -80 °C deep freezer.

2.1.3.5 Competency checking of stock competent cells

2.1.3.5.1 Preparation of recovery media

It is necessary to observe if the stock of bacteria stays viable and competent, and whether bacteria are naturally resistant to used ampicillin. For this reason, super optimal broth with catabolite repression (SOC) media was taken as recovery media. The SOC media was prepared according to the below recipe given in Table 2.5, which was taken from the "SOC Medium" recipe of Cold Spring Harbor Protocols [73].

Table 2.5 Super optimal broth with catabolite repression (SOC) media composition

SL No.	Components	Amounts
1	Peptone	2% (w/v)
2	Yeast extract	0.5% (w/v)
3	Glucose	20 mM
4	$MgCl_2$	10 mM
5	KCl	2.5 mM
6	NaCl	10 mM

All components were dissolved in distilled water (except $MgCl_2$ and glucose) and autoclaved at 121 °C for 15 minutes. Glucose and $MgCl_2$ stock solutions of 1M strength were prepared immediately just before their addition through filtration using filters (0.22 μm pore).

2.1.3.5.2 Heat-shock transformation

A protocol (modified version) developed by Choi et al[74] was followed to get transformation. A hot water bath was set to carry the temperature of 42 °C. The stock solutions of bacteria species DH5α and BL21 of the *E. coli* were kept on ice for 15 minutes. Competent cells (50 μL) were taken in individual tubes and 5 μl of pGLO plasmid (48.608 pmol/mL) was added and held on ice for about 30 minutes. The tubes were set in that pre-heated water bath for 30 seconds and immediately put on ice for 2 minutes. SOC medium (1 mL) was added to each tube at room temperature and incubated at 37 °C at 120 rpm for 1 hour

2.1.3.5.3 Selective media preparation and inoculation

LB-agar medium was prepared with the composition of 1.2% (w/v) agar (Liofilchem, Italy, 031916504), 2% (w/v) Luria-Bertani (LB) (Liofilchem, Italy, lot no-121119503), 0.75% (w/v) arabinose (RESEARCH-LAB, Mumbai, India, cat no.- 00184, batch no.- 184020221), and 10 mg/mL ampicillin sodium stock solution (Duchefa Biochemie, Haarlem, Netherlands, cat no.- A0104.0005, lot no.- 015449.04). The all above components (except the ampicillin solution) were mixed in distilled water and autoclaved at 121 °C for 15 minutes. Petri dish, micropipettes, marker, autoclaved pipette tips, alcohol solution, spreader, and spirit lamp were put in a vertical type clean bench (BC-01E, Lab companion, Korea) cabinet, and treated with ultra violate radiation for 20 minutes. The following steps were performed inside the biosafety cabinet. After cooling the media, ampicillin was added from its aqueous stock solution using a micropipette to reach the concentration of 100 μg/mL. 25 mL of shaken media were poured into each plate and allowed for some moments for solidification. After the solidification process, 100 μL of the cultures in SOC-media were taken onto their respective plates by pipetting and spread on the whole plate by using a glass spreader which was sterilized using alcohol and a spirit lamp flame. Untransformed DH5α and BL21 competent cells were similarly spread onto plates as control. All plates were incubated at 37 °C for 24 hours and after incubation, visualized under ultra violate light. Green colonies under UV light will be denoted as untransformed colonies.

2.1.3.6 Preparation of the fungal recombinant pectinase-producing bacteria and preliminary selection

A new optimized protocol was developed and described below for successful transformation because of the low concentration of recombinant plasmid.

2.1.3.6.1 Heat-shock transformation

The success of bacterial transformation depends on various factors, and the specific conditions may vary based on the exact protocol, the type of competent cells used, and the DNA being transformed. Thaw stock solutions of bacterial species DH5α and BL21 on ice for 15 minutes. 50 µl of competent cells were added to separate tubes. 25 µL of ligation solution was added to each tube. Place the tubes in a water bath set to 42°C for 60 seconds. Immediately transfer the tubes to ice and leave them for 2 minutes. Add 1 ml of SOC medium (formulated according to subsection 2.1.4.5.1) at room temperature to each tube. Incubate the tubes at 37°C with shaking at 120 rpm for outgrowth for 1 hour.

2.1.3.6.2 Selection plate formulation and inoculation

Eight plates (4 for DH5α and 4 for BL21) were prepared as for selection media with the composition of 2% LB, 0.75% arabinose, and 5% agar were mixed in distilled water and autoclaved at 121 °C for 15 minutes, 2 plates with composition without arabinose, 2% LB and 1.5% agar were prepared as control media. These components also were mixed in distilled water and autoclaved at 121°C for 15 minutes. Plastic petri dishes or plates, alcohol solution, micropipettes, autoclaved pipette tips, spreader, marker, and spirit lamp were kept in a vertical-type clean bench biosafety cabinet, and treated with UV-radiation for 20 minutes. The steps were performed using aseptic techniques inside the biosafety cabinet. The plates were appropriately labelled. After cooling the media to bearable temperatures, ampicillin was added to the selective media using a micropipette from its aqueous stock solution to reach the desired final concentration of 100 µg/mL. The media was shaken and 25 were poured into each plate and allowed to solidify. After the plate's solidification, 250 µl of cultures from SOC-media were pipetted onto their respective plates and spread on the entire plate using a glass spreader sterilized using alcohol and a spirit lamp. As a control, untransformed DH5α and BL21 competent cells were similarly spread onto the control plates. The plates were sealed with two strips of parafilm and incubated for 24 hours at 37 °C for the growth of colonies.

2.1.3.6.3 Pure cultures plates and assay plates preparation

2.1.3.6.3.1 Pure culture plate preparation

Pure culture plates were made with the composition of 2% LB, 1.5% agar, and 100 μg/ml ampicillin. First of all, individual colonies were numbered using markers. Individual colonies were streaked onto newly produced individual culture plates in four consecutive streaking zones, each time burning the inoculation loop wire to a red-hot state. The plates were closely sealed with two strips of parafilm and incubation was done for 24 hours at 37 °C. After bacterial growth, the pure cultures were used for inoculating assay plates, after which the pure culture plates were carefully re-sealed and stored at 4 °C in a refrigerator.

2.1.3.6.3.2 Transformed bacterial cultured plates for DH5α and BL21

LB media was prepared for broth culture with the composition of 2% LB, and 1.5% agar and sterilized for 15 minutes at 120 °C. After cooling the media 100 μg/ml ampicillin was added to the LB media and transferred to different petri dishes. After solidification bacteria were streaked into the solid media on petri dishes from the main transformed plates of DH5α and BL21 and cultured for 24 hours in an incubator at 37 °C. Seventy colonies were found from the main plates. An identical colony was selected by numbering A, B, and, C, and by inoculating the C colony in LB, it was found several individual colonies in the petri dish after incubation. After that colony 13 was again selected from that dish and again inoculated in LB media for sub-subculturing and got different new colonies. From that dish D2, D3, D8, and D25 colonies were selected for DH5α. A similar subculturing process was continued for transformed BL21 bacterial main plates and B2, B6, B22, B23, B24, and B25 colonies were selected from the sub-subculture shown in Figure 2.6. After that, the pure culture plates were prepared carefully re-sealed, and stored at 4 °C in a refrigerator. The pure cultures were used for inoculating assay plates.

Figure: 2.6 Transformed bacterial different cultured plates for DH5α and BL21

2.1.3.6.3.3 Assay plate preparation

The assay plate was carried with 2% LB, 1.5% agar, 0.75% arabinose, 0.2% pectin, and 100 μg/mL ampicillin sodium. Assay plates were divided into four quadrants, each of which was used to inoculate a small streak from each of the corresponding clones from pure culture plates. The culture plates were sealed with parafilm and incubated at 37 °C. After 24 hours of incubation, 0.1% Congo red solution was poured onto the plates and stained for 30 minutes at 40 rpm in an orbital shaker (serial no.- R160000268, Bibby Scientific, PRC). The plates were destained using 1 M NaCl (Wako Pure Chemical, lot no. – WDJ2158) solution for 1 hour. The NaCl solution was decanted, and again destaining was performed with 1-hour duration.

2.1.3.6.4 Isolation of plasmid and gene amplification

Ten falcon tubes were labelled serially B2, B6, B22, B23, B24, B25, and D2, D3, D8, D25 for their respective denoted bacterial plates respectively. LB media was made for liquid culture (100 μg/ml ampicillin and 2% LB) and sterilized. After cooling, ampicillin was added to the LB media, and 20 mL media was poured into each (50 mL) falcon tube. Bacteria were streaked into the marked falcon tubes solution accordingly from previous B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 well-grown bacterial petri dish, and these falcon tubes were kept overnight in a shaking incubator at 120 °C. Bacterial growth was observed for ten falcon tubes of two (DH5α and BL21) bacterial strains and used for plasmid extraction.

2.1.3.6.4.1 Plasmid extraction from bacterial culture

Plasmid extraction was held by a plasmid extraction kit (FavorPrep, Favorgen biotech corp.) that contains FAPD 1 buffer, FAPD 2 buffer, FAPD 3 buffer, W1 buffer, wash buffer, elution buffer, FAPD column, collection tube, and RNase A shown in Figure 2.7. RNase A was stored at 20 °C. 0.5 M FAPD 1 buffer was added to an RNase A tube and mixed by vortexing the tube. FAPD 1 buffer was stored at 4 °C. Wash buffer was prepared by adding 100 % ethanol. B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 well-grown 1.5 mL bacterial cultures were transferred to ten labeled centrifuge tubes according to their respective B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 labels (Figure 2.8). The tubes were set on a centrifuge machine to separate bacterial pellets by maintaining momentum and ran for 5 minutes at 11,000 × g. After centrifugation supernatant was discarded completely from the all tubes. 200 μL FAPD 1 buffer (added RNase A) was mixed into the cell pellets and complete resuspension of the bacterial cells was confirmed by pipetting. After resuspension, no bacterial cell pellets were visible in the solution. Again 200 μL FAPD 2 buffer was added to each tube and inverted the tubes gently 10 times to make the lysate clear. The sample mixtures were incubated for 5 minutes at room temperature to lyse the cell and time was carefully maintained. 300 μL FAPD 3 buffer was added to each tube and inverted the tubes gently 10 times immediately to neutralize the lysate. Centrifuged at 18000 × g for 5 minutes to clarify the lysate. The FAPD column was placed during centrifugation. The supernatant was transferred carefully to the FAPD column and centrifuge at 11,000 × g for 30 seconds.

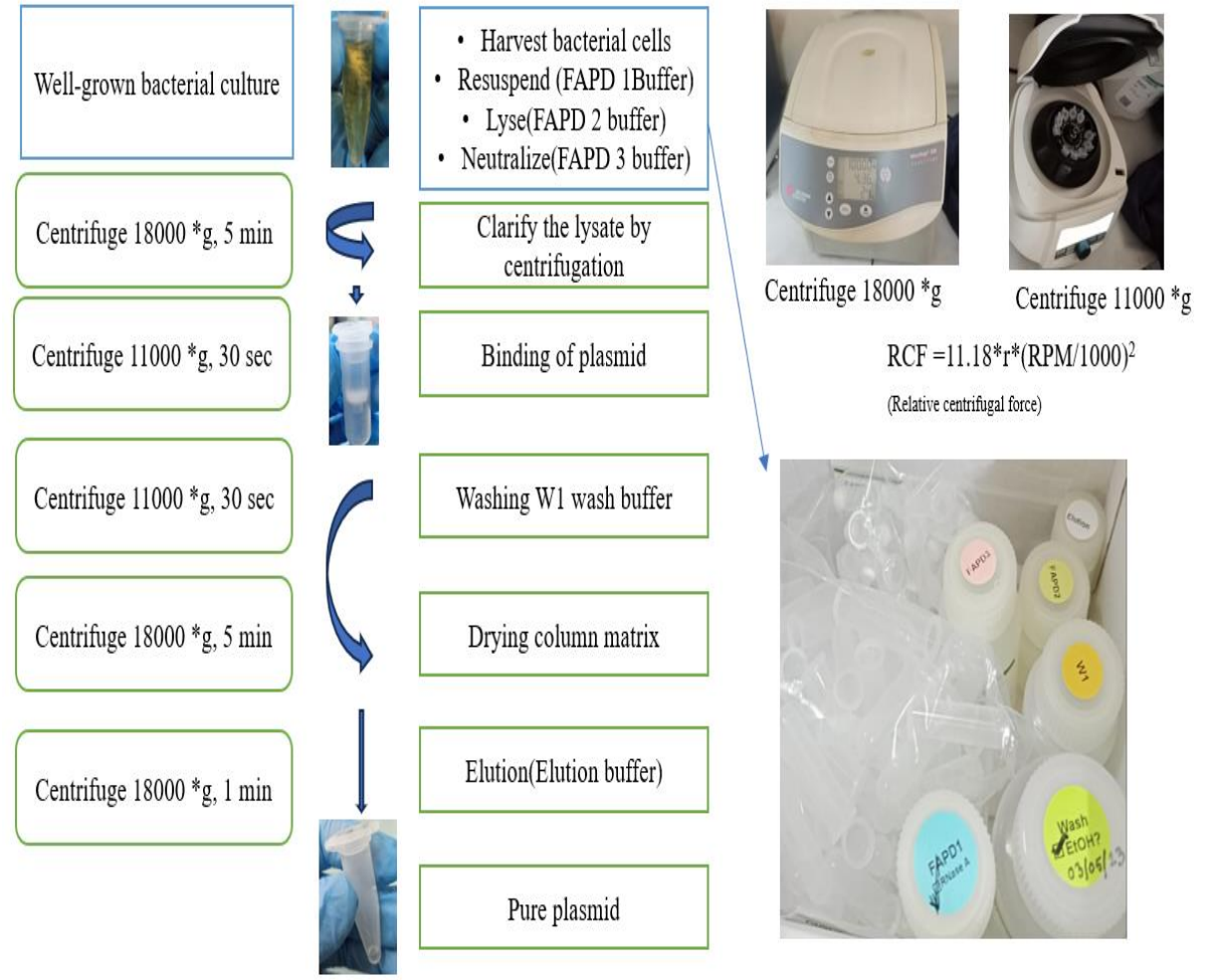


Figure: 2.7 Process flow chart of plasmid extraction from well-grown bacterial culture

The flow-through was discarded and the column was placed back in the collection tube. 400 µL W1 buffer was added to the FAPD column and centrifuged at 11000 × g for 30 seconds. The flow-through was discarded and the column was placed back in the collection tube. Again 700 µL wash buffer (added ethanol) was added to the FAPD column and centrifuged at 11000 × g for 30 seconds. The flow-through was discarded and the column was placed back in the collection tube. The FAPD column was dried by additional centrifugation at 18000 × g for 3 minutes to remove the residual liquid thoroughly. The FAPD column was placed in a new 1.5 mL microcentrifuge tube and 50 µL elution buffer was added to the FAPD column. The FAPD column stood for 1 minute and was centrifuged at 18000 × g for 1 minute to elute plasmid DNA.

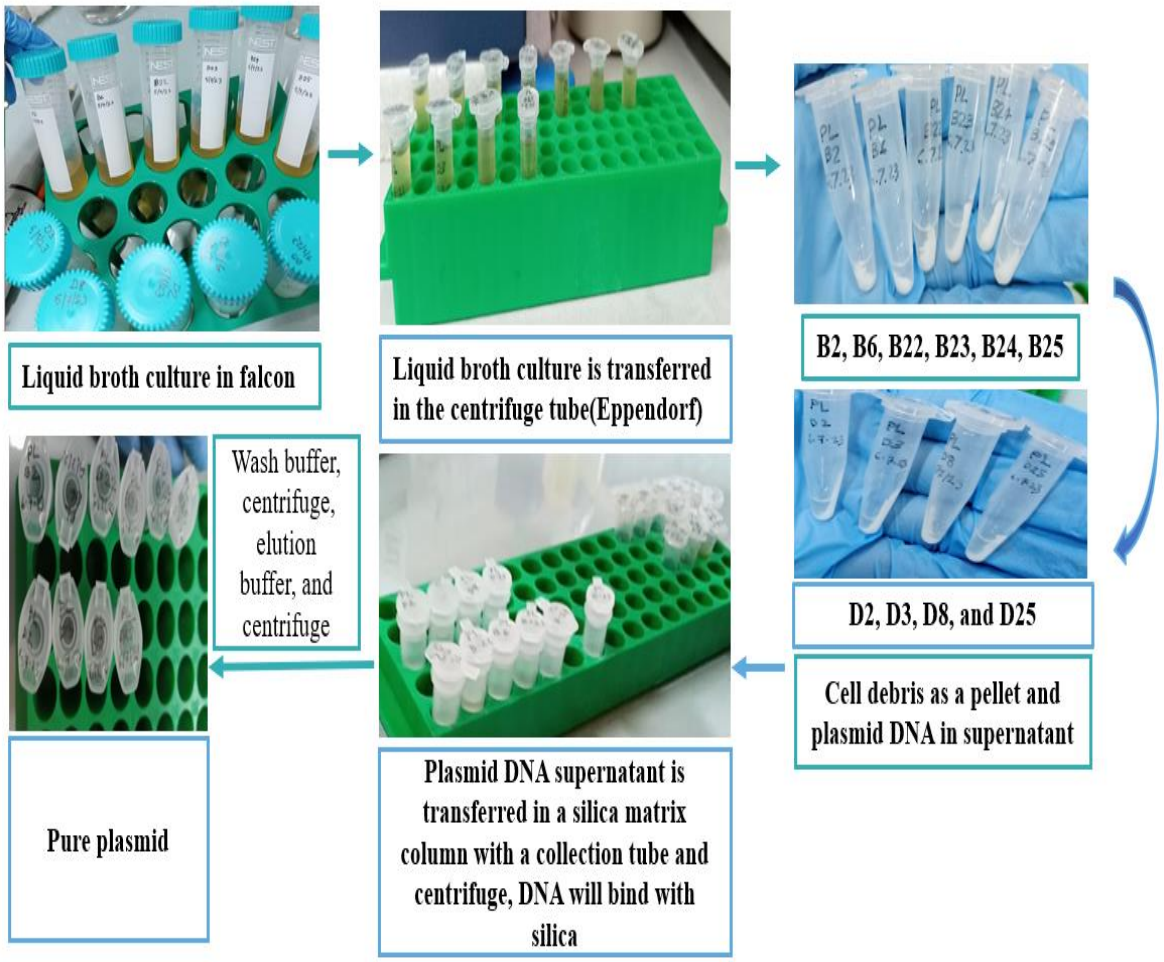


Figure: 2.8 Plasmid extraction from B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 well-grown bacterial culture

Table 2.6 PCR mix for amplification of Ast gene from extracted plasmid

SL No.	Components	Master mix (µL)
1	Phusion High Fidelity Buffer (5×)	5
2	Diluted 10mM dNTP mix	1.25
3	PCR water	15.85
4	Primer mix (Forward segment 1 + Reverse segment 10)	1.25
5	DNA Taq polymerase	0.15
6	Extracted plasmid	1.5
7	Total (volume/tube)	25

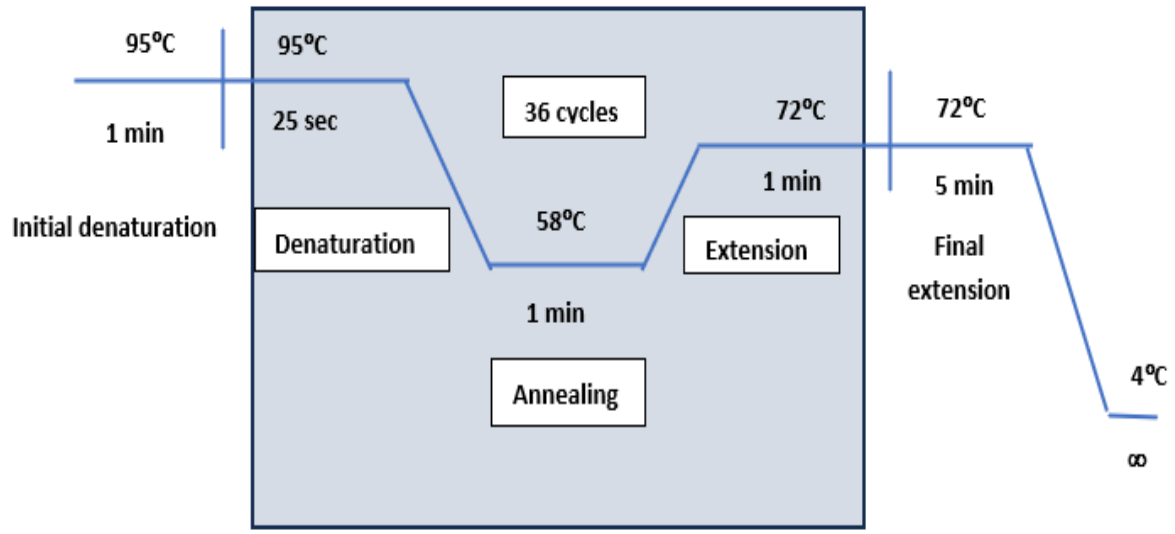


Figure: 2.9 Ast gene amplification (using phusion polymerase)

2.1.3.6.4.2 Plasmid isolation

The purifying process of the plasmids was done by using the FavorPrep plasmid extraction kit, a similar process of subsection 2.1.3.7 was followed.

2.1.3.6.4.3 Ast gene PCR for gene confirmation

PCR is required to confirm the presence of the Ast gene inside the transformed clones. PCR mix for amplification of Ast gene from extracted plasmid was used to amplify according to Table 2.6 and Figure 2.9 respectively.

2.1.3.7 Protein identification by PAGE run

The following lysis buffer was made to extract proteins from cells by breaking the cell wall and Laemelli buffer was used to load the extracted samples to the PAG wells to visualize the samples in the gel.

2.1.3.7.1 Reagent preparation

2.1.3.7.1.1 RIPA lysis buffer preparation

The RIPA lysis buffer was prepared with the following reagent concentration in Table 2.7 with double distilled water. 25 mL ddH_2O was taken in a 50 mL falcon tube and all the components were added, vortexing was performed to solubilize the components, and ddH_2O was filled up to get the 50 mL mark.

Table 2.7 RIPA lysis buffer composition

SL No.	Reagent	The final concentration for 5× RIPA lysis buffer
1	NaCl	750 mM
2	Tris-HCl (adjusted to pH 8.0)	250 mM
3	Sodium deoxycholate (Wako, lot no.- WDM6329)	2.5%
4	Triton ×-100 (non-ionic surfactant)	5%
5	Sodium dodecyl sulfate (VWR life science, lot no.- 2716C363)	0.5%

2.1.3.7.1.2 4× Laemelli buffer preparation

Laemelli buffer was used as a loading buffer, it contained the following components for a 5 mL buffer given in Table 2.8.

Table 2.8 Loading buffer composition

SL No.	**Components**	**Amount**
1	0.25 M Tris HCl (adjusted to pH 6.8)	0.25 mL
2	10% Sodium dodecyl sulfate (SDS)	0.4 mL
3	10% Beta- mercaptoethanol	0.5 mL
4	30% Glycerol	1.5 mL
5	0.02% Bromophenol blue	0.001 g
6	ddH_2O	Up to 5 mL

2.1.3.7.1.3 10× Tris-glycine running buffer preparation

2 litters of the 10-times concentrated polyacrylamide running buffer were made and the procedure is stated below.

Table 2.9 Running buffer composition

SL No.	**Components**	**Amount**
1	SDS (VWR life science, lot no.- 2716C363)	20 g
2	Tris base (VWR life science, lot no.- 2617C433)	60.4 g
3	Glycine (MP Biomedicals, France, lot no.- N60236)	288 g
4	dH_2O	Up to 2 L

400 mL of dH_2O were poured into a flask and set on a magnetic stirrer bar, 1/4th of the amount of each component from Table 2.9 was slowly added to the solution under the influence of magnetic stirring, the stirrer was removed after complete dissolution and dH_2O was added up to 500 mL. This process was repeated 3 more times to get the desired 2 liters of 10× Tris-glycine running buffer solution. The 10× buffer was diluted to 1× Tris-glycine running buffer with the addition of 9 volumes of dH_2O in the PAGE tray.

2.3.7.2 Polyacrylamide gel preparation

A 10% acrylamide with bis-acrylamide gel was prepared using the components given in Table 2.10.

Table 2.10 10% polyacrylamide gel composition

SL No.	Components	Amount
1	dH_2O	3.95 mL
2	30% Acrylamide + 1% bis-acrylamide mixture (Carl Roth GmbH Co.)	7 mL
3	N, N, N′, N′ -Tetramethyl ethylenediamine (TEMED) (VWR life science, lot no.- 2147C484)	5 µL
4	10% Ammonium persulfate (APS) (VWR life science, lot no.- 0175C191)	112.5 µL
5	Tris HCl 1.5 M	4.05 mL
6	20% SDS (VWR life science, lot no.- 2716C363)	0.1 mL

A 50 mL beaker was cleaned with dH_2O before mixing and the different solutions were added to the beaker given in Table 2.10, which was set on top of a magnetic stirrer machine with a magnet. Except for TEMED and APS, serological pipettes were used for loading the components and these two were loaded onto the beaker using micropipettes. The mixture was poured between two glass plates appressed against each other (Cleaver Scientific, UK) of a gel-running apparatus and a comb was inserted on the top part between the appressed glass plates, the gel was allowed to solidify for 30 minutes, the gel-running apparatus was inserted into the tray with 1× tris-glycine buffer. The comb was removed carefully and the buffer layer was maintained by addition so that the wells were completely immersed in the buffer.

2.1.3.7.3 Bacterial broth culture preparation

For this purpose, the bacterial clones B2, B6, B22, B23, B24, B25, and D2, D3, D8, D25 and as control, untransformed DH5α strain (UTD), untransformed BL21 strain (UTB) and pGLO-transformed DH5α (GFP) strains were cultured in a sterile liquid media containing 2% LB, 0.75% arabinose and 100 µg/mL ampicillin sodium. A blank media without inoculation and control UTD were also cultured using the same media composition except for ampicillin. 20 mL of the media was poured into 50 mL centrifuge tubes. After inoculation of a single colony from pure culture plates, the tubes were kept half-screw loose and incubated at 37 °C for 24 hours. The pure culture plates were re-sealed with parafilm before storing at 4 °C.

2.1.3.7.4 Protein extraction from bacterial culture for SDG PAGE run

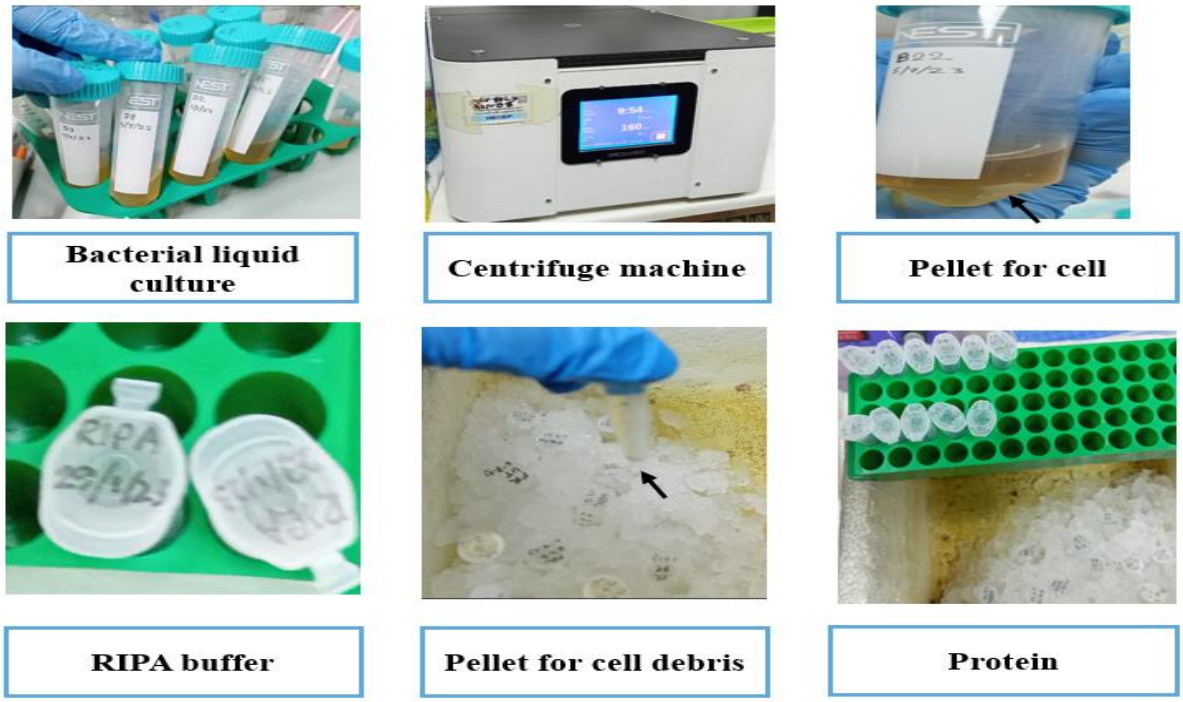


Figure: 2.10 Protein extraction from B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 well-grown bacterial culture

B2, B6, B22, B23, B24, B25, and D2, D3, D8, and D25 well-grown 20 mL bacterial cultures were centrifuged (Figure 2.10) to spin down the bacterial cells for 15 minutes at 4800 × g at 4 °C. 1 mL of PBS was used to dissolve the pellet and transferred to 1.5 µL microcentrifuge tubes, which were centrifuged at 16,000× g for 10 minutes at 4 °C. In the cell pellets 50 µL 5× RIPA buffer and 200 µL PBS (phosphate buffered saline) were added and mixed well and vortexed to dissolve the pellets. After that the cells were lysed using a syringe cycle for 5 minutes and all tubes were then centrifuged for 10 minutes at 4 °C and 16,000× g.

The supernatant was stored in separate tubes. Each 15 µL of the collected protein extract was mixed with 5 µL 4× Laemelli buffer to visualize and loaded in wells of the prepared polyacrylamide gel. The loaded protein samples were run for 6 hours in the gel. The proteins were run in the gel at 90 Volts, 400 milliamperes for 6 hours. A protein ladder was used, Thermo Scientific™ PageRuler™ Plus Prestained, and 3 µL was loaded as a size marker.

2.1.3.7.5 Process for protein visualization

For this procedure, molecular grade Emsure® methanol (Merck, Germany, Lot no.-I621109203) and Emprove® glacial acetic acid (Merck, Germany, Lot no.- K48810456432) were used as reagents. The polyacrylamide gel was separated after gel run completion from the two glass plates carefully using a spatula and kept in a tray containing fixative solution (10% acetic acid, 50% methanol, 40% dH_2O). After 10 minutes, the fixative solution was decanted and staining solution was added (50% methanol, 10% acetic acid, 39.75% dH_2O, 0.25% Coomassie brilliant blue). The gel was incubated overnight with gentle shaking for proper staining. After 16 hours, the staining solution was decanted. The destaining solution (67.5% dH_2O, 7.5% acetic acid, 25% methanol) was poured into the tray and was shaken for 1 hour. The destaining solution was discarded and a new destaining solution was added to cover the gel. 4 times repetition was done and an overnight destaining incubation was continued. For imaging of the gel, it was used the gel documentation system Fusion Pulse 6.

2.1.4 Lugol's iodine test

Lugol's iodine was applied to test for the activity of pectinase[74] . This solution was made using 4% potassium iodide (ref-1.05040.1000, Merck, Germany) and 2% iodine (Vetec, Sigma-Aldrich, India, cat no.-V800218-100G, lot no.- BGBB9480V) in distilled water. Assay plates were prepared with 1.25% LB, 0.75% pectin, 1% arabinose, 1.5% agar, and 100 µg/mL ampicillin. The plates were inoculated with target bacteria streaked into a small circle. The plates were then incubated for 2 days. These were stained by flooding with exactly 6 mL of Lugol's iodine solution for 5 minutes and kept on an orbital shaker at 40 rpm. Lastly, the plates were destained using 10 mL distilled water for 10 minutes at 40 rpm, and after decanting the spend dH_2O, this step was performed for another 10 minutes.

2.1.5 DNA sequencing to confirm the identity of bacteria and inserted gene

DNA was isolated from bacteria using Chelex beads (a chelating material used to purify other compounds via ion exchange). Bacterial was broth cultured in 15 mL centrifuge tubes. 2 mL of culture was taken in microcentrifuge tubes and centrifuged at 16,000× g for 10 minutes. The supernatant was discarded and the pellet was dissolved in 1 mL PBS. Another centrifugation was performed at 16,000× g for 10 minutes. The supernatant was discarded. 5% Chelex solution was added and heated at 100 °C for 10 minutes with intermittent vortexing. The tubes were then centrifuged at 14,500× g for 5 minutes and the supernatant was collected. This contained the bacterial DNA. Two sets of PCRs were performed. One uses a set of 16S primers which can generate an amplicon of about 1:100 bp, and the other reaction uses the forward primer of the first segment and the reverse primer of the last segment for the Ast gene. Then 16S primers sequence is as follows:

Forward - CTCCTACGGGAGGCAG and reverse – GGTTACCTTGTTACGACTT

2.1.5.1 Analysis of 16S sequence

The sequence from the forward primer was trimmed before searching for similarities using the Basic Local Alignment Search Tool (BLAST) to find regions of local similarity between sequences.[75]. The first 90 nucleotides were disregarded and the next 460 nucleotides were used as a query in the nucleotide BLAST tool. In the case of the sequence using the reverse primer, a similar procedure was followed. However, the trimming was done to select the 467 nucleotides after the first 83 bp.

2.1.5.2 Analysis of Ast sequence

The forward primer of the first segment was used for sequencing and the best-quality sequence of 314 nucleotides from the middle of the sequence was used for pairwise BLAST with the codon-optimized original sequence.

2.1.6 DNS test for crude protein extract

The dinitro salicylic acid reagent (DNS) method[76] was used for pectinase enzyme assay based on the determination of produced reducing sugars as a result of enzymatic hydrolysis of pectin. A mild cell lysis procedure was done to break the cell wall for analysis of their pectinolytic activity.

2.1.6.1 Reagents preparation

1. Wash buffer: 1 tablet (Life Technologies, Maryland, USA, ref- 003002, lot no.- 751506615) was dissolved in 100 mL distilled water to make a PBS (phosphate buffered saline) solution was used as a wash buffer, that had concentrations of NaCl – 140 mM, KCl – 2.7 mM, Na_2HPO_4 – 10 mM, KH_2PO_4 – 1.8 mM and pH – 7.3.
2. Lysis buffer: Lyophilized lysozyme powder was added in an amount of 10 mg/mL to the wash buffer.
3. DNS solution: 20 mL of the solution was made using the following formulation of Table 2.11

Table 2.11 Composition of the DNS solution

1	Reagent	Amount
2	NaOH (Wako Pure Chemicals, China, lot – LKE1030)	0.2 g
3	3, 5-Dinitrosalicyclic acid (DNS) powder (SIGMA-ALDRICH, USA, lot - 102576417)	0.2 g
4	Potassium sodium tartrate (SIGMA-ALDRICH, USA, product – 217255-500G)	6 g
5	Sodium thiosulfate	0.01 g
6	Phenol crystal (TCI, Japan, lot- 52×5C)	0.04 g

At first, 20 mL dH_2O was taken in a 50 mL screw-cap bottle to measure and label the level of the solution of this volume. The water was then discarded and 14 mL dH_2O was taken in the bottle. The components were added, and dH_2O was added to make the 20 mL volume. The components were dissolved using a magnetic stirrer.

2.1.6.2 Glucose standard curve and correlation curve generation

Dextrose (D-glucose) anhydrous (lot no.- 3586C015, Life Science AMRESCO, VWR) solutions were prepared of concentrations 0 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, and 10 mM using PBS as the solvent. A final volume of 500 μL solution was used for the DNS test. The solutions were mixed with 1 mL of the DNS solution and kept in boiling water for 10 minutes. Optical density

values were taken at 600 nm using the UV-Vis spectrophotometer. Here, PBS solution was used as blank to perform auto-zero of absorption. From these values, a correlation curve was derived using the Microsoft Excel software, which shows how much the change in absorption (OD value) will correspond to the change in concentration of reducing ends of sugar, in the working range of the DNS test.

2.1.6.3 Cell lysis and reducing end concentration determination

The individual clones UTD, UTB, GFP, D8, and B24 were grown in liquid broth in 50 mL falcon tubes for 24 hours. The cultures were centrifuged at 4,800× g for 10 minutes and supernatant was discarded. Then the cell pellet was dissolved in 1 mL wash buffer and transferred to a 1.5 mL microcentrifuge tube, centrifugation was performed at 16,000× g for 10 minutes, and the supernatant was discarded. 500 µL lysis buffer was added to lyse the cell wall and was incubated at 37 °C for 15 minutes. 5 mL syringes were used to help lyse the cells using the force of repeated needle syringe aspiration and release. The tubes were then centrifuged at 16,000× g for 10 minutes at 4 °C. 250 µL of clean supernatant was collected in a separate tube. 250 µL pectin (Tokyo Chemical Industries, lot no.- F7F4I-JN) solution (dissolved in PBS) was added such that the final concentration of pectin in this mixture becomes 0.1%. The crude enzyme-pectin mixture was incubated in a water bath of 37 °C for 2 hours. 1 mL of DNS solution was mixed and run for 10 minutes at boiling temperature. Optical density reading at 540 nm was taken PBS solution as blank to perform auto-zero.

2.2. Natural Pectinase Producing Bacteria

2.2.1 Isolation of pure cultures of bacteria

The source of bacteria was obtained from a mixture of soil and chia seeds, that mixture was serially diluted. Several culturing plates were prepared from the different diluted solutions. From the subculturing plates, individual colonies A and B were selected which is shown in Figure 2.11. Pure cultures were obtained from 2% LB and 1.5 % agar medium. The crude natural enzyme collection process was the same as the recombinant enzyme described before.

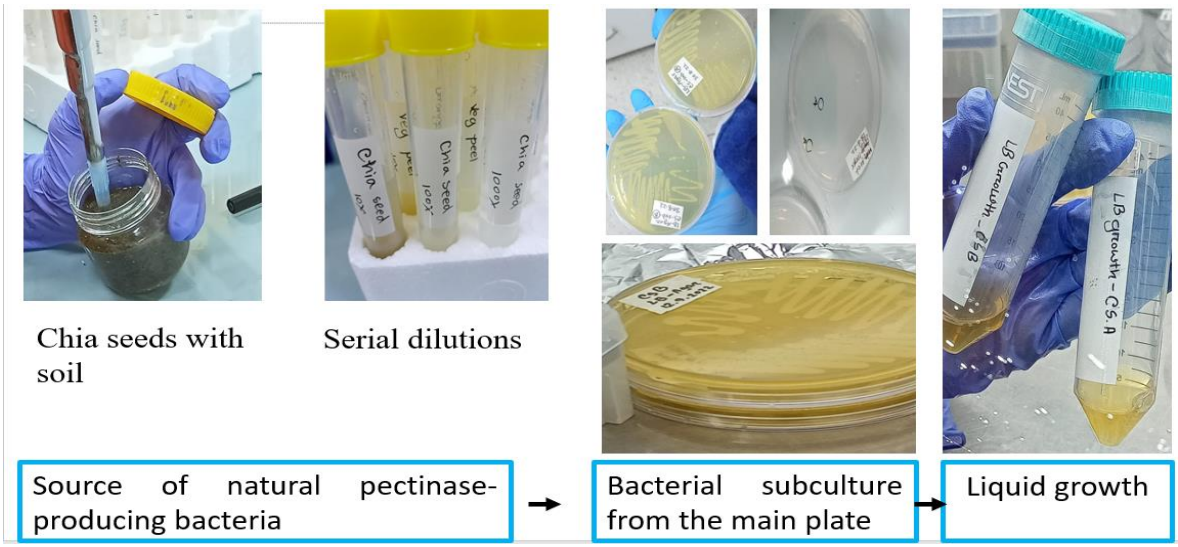


Figure: 2.11 Chia seeds with soil as a source of natural pectinase-producing bacteria

2.2.2 Screening of pectinase-producing bacteria and qualitative assessment

Bacteria from pure culture plates were streaked on a minimal agar medium containing pectin (0.5%) as the sole carbon source. In each of these pectin-minimal agar media plates, the non-pectinolytic Escherichia coli was inoculated as a control. Following incubation of these streaked plates at 37 °C for 48 h, pectin degradation was determined by flooding the plates with freshly prepared 0.1% (w/v) Congo Red solution in water, followed by de-staining with 1 M NaCl solution. A clear zone around the growth indicated the ability to break down pectin. To further assess pectinase activity, this strain was streaked on a pectin-LB-agar medium (2% (w/v) LB, 1.2% (w/v) agar, and 0.5% (w/v) pectin) [77]. Here, as well, two non-pectinolytic E. coli bacterial strains were used as negative controls. One of these was a natural strain of E. coli, and the other was a transformed E. coli harboring a pGLO plasmid.

2.2.3 Molecular identification and characterization of the screened isolate

The pectinase-producing bacteria were cultured in LB (2% (w/v)) broth at 37 °C for 16 h in a shaking incubator at 140 rpm. The culture was centrifuged at 16,000 × g for 10 min in a 1.5-ml tube, the supernatant was removed, and the pellet was washed with 500 µl of PBS, followed by centrifugation at 16,000 × g for 10 min and removal of the supernatant. The pellet was then mixed with 150 µl of 10% (w/v) Chelex®-100 (C7901, Sigma) in ultrapure water, vortexed vigorously, and incubated at 95 °C for 10 min with intermittent vortexing. The solution was centrifuged at 16,000 × g for 10 min, and the supernatant with the genomic DNA was collected for amplifying the 16S ribosomal RNA gene (rDNA) [78]. 16S rDNA gene sequence was

amplified in a reaction total reaction volume of 25 µl containing 1.5 µl of the DNA sample, 2.5 µl of 10 × PCR buffer (EP0702, Thermo Fisher Scientific), 1.0 µl of dNTP mix (10 mM), 0.5 µl of each forward (16S_357F: CTCCTA CGGGAGGCAGCA G) and reverse (16S_1100R: AGGGT TGC GCT CGTTG) primers (10 µM), 0.2 µl of Taq DNA polymerase (EP0702, T hermo Fisher Scientific), and nuclease-free water using a thermal cycler following an initial denaturation step at 94 ℃ for 3 min, then 30 cycles—each with denaturation at 94 ℃ for 30 s, annealing at 61 ℃ for 1 min, and elongation at 72 ℃ for 1 min., followed by the final extension at 72℃ for 5 min. The amplified products were resolved in 1.2% (w/v) agarose gels using 1 × tris acetate EDTA (TAE) buffer along with a DNA size marker (300003, GeneON). Amplified DNA sequences were visualized in a gel documentation system following electrophoresis in 1.2% (w/v) agarose gels [79]. PCR products were purified using the FavorPrep™ GEL/PCR Purification Kit (FAGCK 001, Favorgen Biotech Corp.) following the manufacturer's protocol, and purified PCR products were sequenced following the Sanger sequencing with a commercial service. To identify the nearest neighbors of the pectinase-producing isolate, the resulting DNA sequence was subjected to the NCBI Basic Local Alignment Search Tool (BLAST) [75]. The BLAST search was restricted to 16S rRNA (Bacteria and Archaea). Additionally, models (XM/XP) and uncultured/environmental samples were also filtered out. In the MEGA11 software [80], this sequence was aligned with the top 50 high-similarity sequences obtained from the BLAST search using the ClustalW program [81], and a phylogenetic tree was constructed by the neighbor-joining (NJ) method with 1000 bootstrap.

2.3 Raw Materials, Chemicals and Dyestuffs for Cotton Knit Fabric Treatment

Cotton knit fabric samples (single jersey), 155 GSM (Gram per square meter), collected from Microfibre Group, T.K Bhaban (10th & 6th Floor), 13, Kawran Bazar, Dhaka-1215, Bangladesh.

2.3.1 Pretreatment chemicals, dyestuffs and auxiliaries

For bio-scouring, the detergent Imerol jet was used and sourced from Archroma. Reactive dye (Yellow 4GL and Navy Blue RGB) and dyeing chemicals such as Glauber salt, soda ash, levelling agents sequestering agents, and anti-creasing agents were collected from Tubingen Chemicals (BD) Ltd., Bangladesh. BS 200 enzyme was set as standard and used for comparison which is the commercially available pectinase enzyme was sourced from Orient Chem. Tex Ltd, Dhaka, Bangladesh.

In the case of the chemical scouring and bleaching process, sequestering agent Jintex 2UD for demineralization, detergent KS-10 from Tubingen Chemicals (BD) Ltd, Bangladesh, sequestering agent SQ114FE for pre-treatment chemicals, anti-creasing agent JinsofEco CBA from Jintex Corporation, Taiwan was collected. Hydrogen peroxide (30%), thiourea (99%), acetic acid (99.5%), caustic soda (97.97%), sodium borohydride (98%) were purchased from Merck, Mumbai, and dyestuff reactobond Yellow 4GL and Navy blue RGB (dye) and dyeing auxiliaries such as leveling agent, Glauber salt, soda ash were purchased from Orient Chem. Tex Ltd, Dhaka, Bangladesh.

2.3.2 Testing Chemicals

Non-ionic detergent was collected from Archroma Bangladesh Ltd, AHN Tower, (4th Floor), 13 Sonargaon Road, Dhaka - 1000. The multifibre fabric for colour fastness to washing and rubbing cloth for rubbing tests were also collected from James H. Heal.

2.3.3 Pretreatment process and reactive dyeing of cotton knit fabric

2.3.3.1 Bio-scouring and reactive dyeing of cotton knit fabric

Commercial enzyme BS 200 is used in the textile industry for fabric bio-scouring. The enzyme is applied at a concentration of 1 g/L in a pretreatment bath, along with 0.2 g/L detergent. The treatment is carried out for 60 minutes at 60°C. After the enzymatic pre-treatment, a hot wash is performed with 0.5 g/L detergent in a wash bath. The hot wash is conducted for 20 minutes at 90°C. The fabric is treated in a bath containing 1 g/L crude extract with 0.2 g/L detergent for 60 minutes at 37°C. Fabric weight for enzymatic treatment is 20 gm with a material-to-liquor ratio of 1:10. Fabric weight for dyeing is 5 gm, and the material-to-liquor ratio is maintained at 1:10. Dyeing is performed with a 2% reactive dye solution at 60°C for 90 minutes. The dye solution contains 46 g/L Glauber salt, 18 g/L soda ash, 10 g/L levelling agents, 10 g/L sequestering agents, and 20 g/L anti-creasing agents. Chemicals, auxiliaries, dyes, and fabric are placed in the dye pot with water adjustment for 50 mL. The dyeing machine is set at 60°C. After 30 minutes, soda ash is added to the dye bath, and the process continues for an additional 60 minutes. Dyed fabrics undergo a washing sequence: cold wash for 7 minutes at room temperature, acid wash (50% acetic acid, 1 g/L), first hot water wash for 7 minutes at 95°C, soap wash, second hot water wash, and third hot water wash. An ECO dyer (ECO-18, Xiamen Rapid Co., Ltd. China) is used for both the enzymatic pre-treatment and dyeing processes. Sample identification for different processed samples is given in Table 1.12.

Table 2.12 Sample identification for different samples

Process parameter	**Sample ID**
Chia seeds enzyme	CS
Chia seeds enzyme form clone A treatment at 37 °C	CS A 37
BL21 enzyme	BL
BL21 enzyme from 24 no. clone	BL24
BL21 enzyme treatment from clone 24 treatment at 37 °C	BL 37
DH5α enzyme from 8 no. clone	D8
DH5α enzyme from 8 no. clone treatment at 37 °C	D8 37
Untransformed bacteria	UT
Untransformed bacteria treatment at 37 °C	UT 37
Without enzyme treatment	WE
Without enzyme treatment at 37 °C	WE 37
Without enzyme treatment at 60 °C	WE 60
Chemical scouring process	CSP
Chemical scouring and bleaching process	CSBP
Chemical scouring and bleaching process with 10 g/L H_2O_2	CSBP 10
BS 200 enzyme	BS
BS 200 enzyme treatment at 60 °C	BS 60
BS 200 enzyme treatment at 37 °C	BS 37
Bleaching with 30% H_2O_2 with CS enzyme-treated fabric	CS HP30
Bleaching with 30% H_2O_2 with BL21 enzyme-treated fabric	BL HP30
Bleaching with 30% H_2O_2 with D8 enzyme-treated fabric	D8 HP30
Bleaching with 30% H_2O_2 and sodium borohydride with CS enzyme-treated fabric	CS HP30+SB
Bleaching with 30% H_2O_2 and sodium borohydride with BL21 enzyme-treated fabric	BL HP30+SB
Bleaching with 30% H_2O_2 and sodium borohydride with D8 enzyme-treated fabric	D8 HP30+SB
Bleaching with 50% H_2O_2 with CS enzyme-treated fabric	CS HP50
Bleaching with 50% H_2O_2 with BL21 enzyme-treated fabric	BL HP50
Bleaching with 50% H_2O_2 with D8 enzyme-treated fabric	D8 HP50
Bleaching with 50% H_2O_2 and sodium borohydride with CS enzyme-treated fabric	CS HP50+SB
Bleaching with 50% H_2O_2 and sodium borohydride with BL21 enzyme-treated fabric	BL HP50+SB
Bleaching with 50% H_2O_2 and sodium borohydride with D8 enzyme-treated fabric	D8 HP50+SB

2.3.3.2 Chemical scouring bleaching and reactive dyeing of cotton knit fabric

The demineralization process (P1) of removing mineral matter and salts from grey fabric was done by using a proposed recipe given in Table 2.13. Combined scouring-bleaching processes (P2) were performed with demineralization (P1) and without demineralization of grey fabric following the recipe mentioned in Table 2.14. After that 6g/L thiourea was added to processes P2 bleached baths and again run for 25 minutes at 60 °C temperature which was denoted process P3. The conventional combined scouring-bleaching process (P2a) is a regularly used recipe considered for the control sample. The recipe of the proposed sequential bleaching process (P4) is tabulated in Table 2.15 and Table 2.17. Afterward, all pre-treated samples were dyed with reactive dye Yellow 4GL according to the common recipe in Table 2.18 to evaluate the dyeability of the pre-treated samples and to compare them with the conventionally pre-treated samples. All the processes were done in ECO dyer and reduction potential was measured by ORP tester (AD14, Adwa, Hungary).

After conventional bleaching with 4 g/L hydrogen peroxide, the bath showed a pH near about 11.5, fabrics were treated according to Table 2.16 for reductive bleaching with acid addition, and whiteness was checked after reductive bleaching. Whiteness was found high for pH 6.5. and the declining order was seen for lowering pH value. Fabrics were treated according to Table 2.15 for reductive bleaching without acid addition.

Table 2.13 Recipe for demineralization process (P1)

Process code	KS-10 (Detergent)	2UD (Sequestering Agent)	Temperature	Time (min)	M:L
P1	0.2 g/L	1 g/L	80°C	20 Min	1:10

Table 2.14 Recipe for combined scouring and bleaching (P2)

Process Code	KS-10 (g/L)	CBA (g/L)	SQ-114FE (g/L)	Stabilizer (g/L)	Caustic Soda (g/L)	H_2O_2 (g/L)	Temperature (°C)	Time (min)	M:L
P2a	0.7	1	1	0.5	2	4	98	45	1:10
P2b	0.7	1	1	0.5	2	6	98	45	1:10
P2c	0.7	1	1	0.5	2	8	98	45	1:10
P2d	0.7	1	1	0.5	2	10	98	45	1:10

Table 2.15 Recipe for bleaching with thiourea and sodium borohydride without acid addition (P4)

Process code	Thiourea (g/L)	$NaBH_4$ (g/L)	Temperature (°C)	M:L	Time (min)
P4a	6	1	60	1:10	25
P4b	6	3	60	1:10	25
P4c	6	5	60	1:10	25
P4d	6	7	60	1:10	25

Table 2.16 Recipe for bleaching with thiourea after conventional bleaching in different pH

Process code	Thiourea (g/L)	pH (Acetic Acid)	Temperature (°C)	M: L	Time (min)
P3.7	6	3.7	60	1:10	25
P4.0	6	4	60	1:10	25
P4.5	6	4.5	60	1:10	25
P5.0	6	5	60	1:10	25
P5.5	6	5.5	60	1:10	25
P6.0	6	6	60	1:10	25
P6.5	6	6.5	60	1:10	25

Table 2.17 Recipe for bleaching with thiourea and sodium borohydride with acid addition

Process code	Thiourea (g/L)	$NaBH_4$ (g/L)	pH (Acetic Acid)	Temperature (°C)	M: L	Time (min)
P4e	6	1	6.5	60	1:10	25
P4f	6	3	6.5	60	1:10	25
P4g	6	5	6.5	60	1:10	25
P4h	6	7	6.5	60	1:10	25

Table 2.18 Recipe for dyeing

Shade	Glauber Salt	Soda Ash	Levelling Agent	pH	Temperature	Time	M: L
0.5%	16g/L	9g/L	2g/L	11	60 °C	1.5 hr	1:10

2.3.4 Bleaching for enzyme-treated fabric

Bio-scoured fabric bleached at 98 °C for 45 minutes with 10 g/L of hydrogen peroxide with detergent 0.7g/L, stabilizer 0.5 g/L, anti-creasing agent 1g/L, and sequestering agent 1g/L except 2 g/L caustic soda for bio scouring bleaching process (BSBP). In the chemical scouring process (CSP), 2 g/L caustic soda, 1 g/L sequestering agent and 0.7 g/L detergent was used for 45 minutes at 98 °C. After BSBP, further added 3g/L sodium borohydride in the bleached baths and again was run for 25 minutes at 60 °C.

2.3.5 Testing Methods

2.3.5.1 Wicking test

The wicking rate of natural and recombinant enzyme-treated cotton fabric was assessed by vertical wicking test, as per the AATCC (American Association of Textile Chemists and Colourists) standard method AATCC 197, 2022 Edition. The vertical wicking test typically involves immersing a cut edge of the fabric into a liquid (in this case, a 1% dye solution) and observing the liquid's ascent into the fabric due to capillary action. A cut edge of the fabric was likely used to expose the fabric structure and facilitate capillary action. Enzyme-treated fabric is marked above 1 cm and 4 cm from the bottom. The test specimen was immersed in 1 cm of dye solution and the travelling rate of the dye solution was noted by counting the time required to travel 4 cm height and wicking distance after 5 minutes (wet condition) and 10 minutes (dry condition).

2.3.5.2 Measurement of DE and K/S value

CIE provides standardized systems for colour representation and measurement. The Data colour 850 (spectrophotometer) measured the reflectance percentage and calculated DE between standard and chemical or enzyme-treated samples, likely employing CIE principles[82]. The shade will be acceptable if their difference remains equal to or less than 1 [83].The colour difference is the separation between two coloured samples. The 1976 CIE Lab colour space is the most widely used three-dimensional space. The L axis is a scale with values from 0 (black) to 100 (white). The L value correlates with the level of lightness/darkness of a colour. The a is the red/ green axis; positive and negative a describe redness and greenness, respectively. The b is the yellow/ blue axis; positive and negative b describe yellowness and blueness, respectively shown in Figure 2.12. The CIE Lab units included the asterisk (*) to differentiate the CIELAB system from the units of other colour systems[84]. Chroma (C*) and hue (h*) can be extracted from the a* and b* values as below.

$$C^* = ((a^*)^2 + (b^*)^2)^{\frac{1}{2}}$$

$$h^\circ = arctan\frac{b*}{a*}$$

$$\Delta E^* = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{\frac{1}{2}}$$

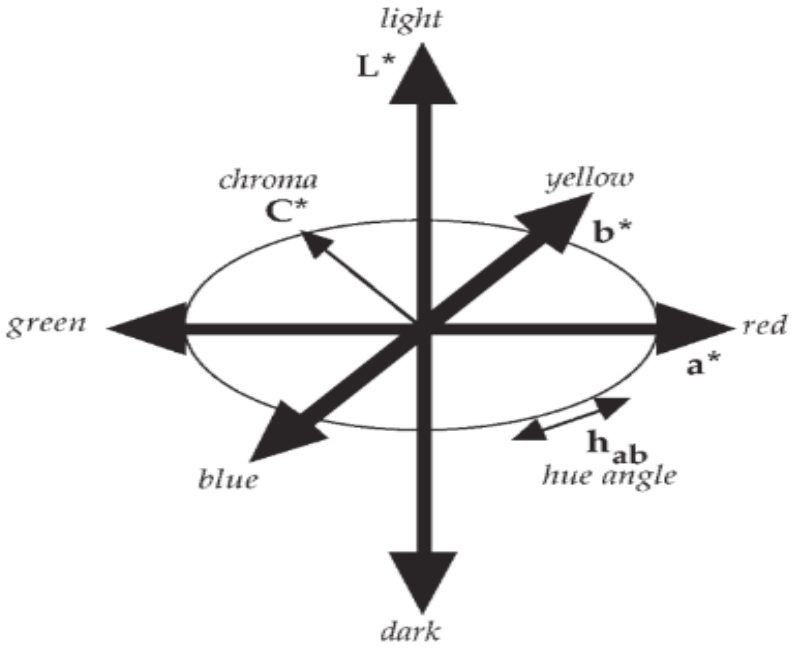


Figure: 2.12 Cylindrical representation of CIELab colour space [84]

The colour depth of the dyed sample in terms of K/S value, was determined by the AATCC TM182- 2011 standard method.

The K/S value is derived from the Kubelka-Munk equation- $K/S = (1-R)^2/2R$

Where K, S and R are the absorption coefficients, scattering coefficient, and reflectance of dyed fabric that can be generated by Data colour 850.

2.3.5.3. Fourier transform infrared spectroscopy (FT-IR) analysis

Fourier transform infrared spectrometer is a powerful tool for analyzing the chemical composition of materials based on their interaction with infrared light. The use of a FT-IR spectrometer, specifically the ABB MB3000 model, for the analysis of fabric samples is a common and powerful technique in materials science. The FT-IR spectrometer recorded the infrared spectra for each sample. The recorded data likely includes information about the percentage of infrared light transmitted through the fabric samples. The data was plotted, likely as a transmittance percentage against wave number (cm^{-1}) graph, using Origin software. This type of plot is common in FT-IR spectroscopy to visualize peaks and patterns associated with different molecular vibrations.

2.3.5.4 SEM

SEM utilizes a focused beam of electrons to create high-resolution images of a sample's surface. The technique excels at revealing surface features at the micro and nanoscale. The magnification was systematically varied, ranging from 5000× to 30,000×. Images taken at different magnifications facilitate a comprehensive analysis of how treatments impact surface topography. SEM is instrumental in visualizing changes on the surfaces of fabric samples resulting from treatments, coatings, or alterations. SEM facilitated the visualization of surface features, such as fibres, textures, and any alterations made to the fabric[85].

2.3.5.5 Colour fastness

The colour fastness of dyed materials is a critical aspect in assessing the durability and stability of colour in textiles. Colour fastness to wash and rubbing was done. Colour fastness is the ability of dye molecules to adhere to a substance, ensuring the resistance of colour to fading or disappearing over time. Colour fastness was evaluated using Gyro wash from James H. Heal and Co., UK, following the guidelines outlined in ISO 105–C06–C2S:2010.

2.3.5.6 Bursting strength

The bursting strength test is essential in the textile industry, as it provides valuable information about the durability and strength of fabrics, helping manufacturers ensure that their products meet quality standards. Bursting strength was done by the ISO 13938-2:1999 method. The test specimen was placed over the diaphragm and clamped in the circular holder. Fastened the safety cover in the position and pressure was applied to the fabric until the fabric bursts. After bursting the fabric, the main air valve was closed and bursting pressure was noted. The recorded bursting pressure provides a quantitative measure of the fabric's bursting strength. Higher bursting pressures generally indicate stronger and more durable fabrics.

Chapter 3

Results & Discussion

Chapter 3

Results & Discussion

3.1 Recombinant Pectinase Gene and Enzyme Production

3.1.1 Insert fungal gene design assessment

3.1.1.1 Recombinant gene design

Gene fragments were codon optimized by the GEB department of Dhaka University for efficient translation in *E. coli*. Ten overlapping segments of the gene "Ast" were designed for Gibson assembly, widely adopted as a preferred cloning method[86]. The forward primer of the first segment contains the NheI restriction site GCTAGC and the coding region for the alpha-amylase signal peptide MKLAACFLTLLPGFAVA. The reverse primer of the last segment contains the XbaI restriction site TCTAGA.

3.1.1.2 Individual gene segments and the full length of the gene assessment

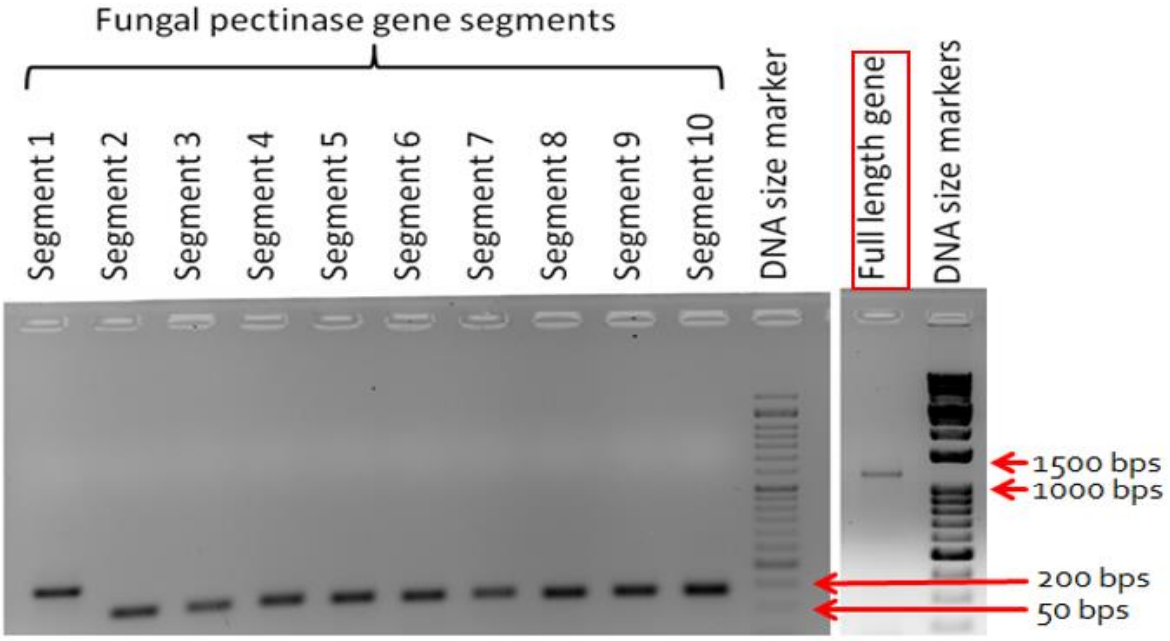


Figure: 3.1 Gene segments visualization under UV light

Ten synthesized gene fragments were amplified in a PCR thermocycler and checked by electrophoresis test with 1% agarose gel with a ladder to confirm their expected size [87]. Ten

segments were also amplified separately to check their size. Extension of overlapping gene segments by PCR is a simple, versatile technique for site-directed mutagenesis and gene splicing [88]. A PCR product is analyzed by agarose gel electrophoresis, it appears as a double-stranded DNA fragment[89]. A gel documentation system was used to visualize the segments under UV light. The ligated gene length was between 1000 to 1500 base pairs which is in the expected region (1143 bps) shown in Figure 3.1(right side). This indicates that the ligation processes were successful in generating the desired gene fragment. On the left side, darker bands are at the desired size of the individual fragments. This indicates that individual segments of the gene are present in their desired fragment sizes (50 -200 bps) from their separate PCR products.

3.1.1.3 pGLO plasmid

The pGLO plasmid is 5371 base pairs (bp) long[90], and a DNA band between 5000 and 6000 bp was visible in a gel electrophoresis image, as shown in Figure 3.2. This suggests that the pGLO plasmid was successfully isolated and analyzed using gel electrophoresis, and the observed DNA band corresponds to the expected size range for the plasmid.

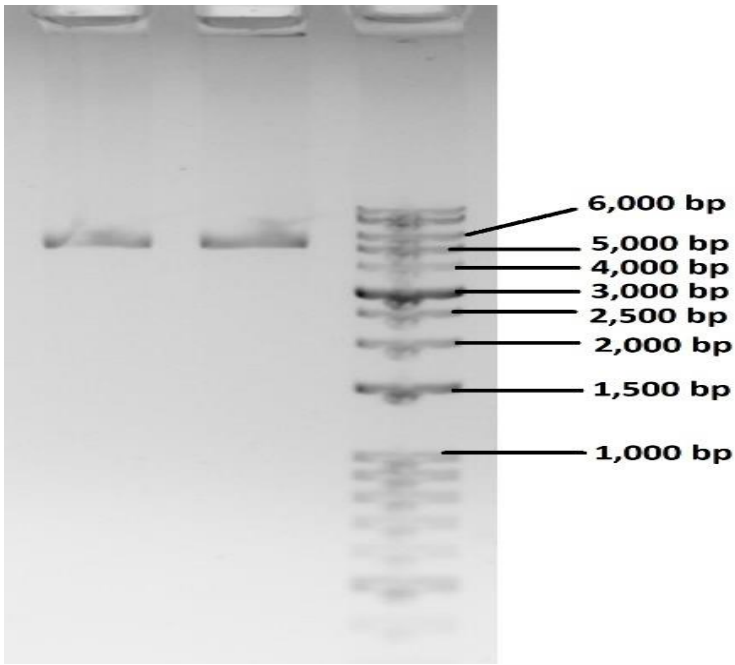


Figure: 3.2 Purified pGLO plasmids in the left two lanes, and on the right the GeneOn One-for-all ladder

3.1.2 Bacteria production and selection

3.1.2.1 Restriction digestion and ligation

Table 3.1 Absorbance and concentration of restriction digestion products

Sample	A_{260}	Concentration (μg/mL)	Concentration (pmol/mL)
pGLO digest	0.038	1.906	0.626
Ast digest	0.122	6.091	8.032

The concentrations of the restriction digest products obtained from the gel using a microvolume spectrophotometer is presented in Table 3.1. Interestingly, the digested gene appeared to be more concentrated compared to the digested plasmid. This observation suggests that the gene fragment was present in higher abundance in the original sample compared to the plasmid. So, the final vector: insert molar ratio was 1:4.277 [91] by taking 30 μL of pGLO digest and 10 μL of Ast digest during the ligation process.

3.1.2.2 Preparation of competent bacterial cells

Optical density was measured at 600 nm for the liquid culture of bacterial two types of strains after overnight inoculation in 2% LB media (Table 3.2). The DH5α strain solution showed higher turbidity than the BL21 strain solution[92] to check the ability of strains to the expression.

Table 3.2 Optical density measurement due to turbidity of bacterial growth for two types of bacterial strain

Bacterial Strain	OD at 600 nm
Blank Media	0.000
Untransformed DH5α	0.786
Untransformed BL21	0.681

3.1.2.3 Checking competency of transformed and untransformed bacterial growth plates under UV light

Transformation of the ligated gene was primarily confirmed by the glowing effect (green light) under UV light. It green means there is no pectinase gene in the plasmid and transformed bacteria will not glow[93]. The transformed bacterial-grown area was white and the untransformed was green (Figure 3.3 and Figure 3.4). The transformed bacteria should be able

to grow in ampicillin as the pGLO plasmid contains an ampicillin resistance gene (coding for a β-lactamase)[94]. Therefore, colonies were observed that can grow in LB-agar media with a concentration of 100 µg/mL of ampicillin. The untransformed inoculums were not grown in the ampicillin-added media proving 100 µg/mL of ampicillin can be used to select for the transformed colonies.

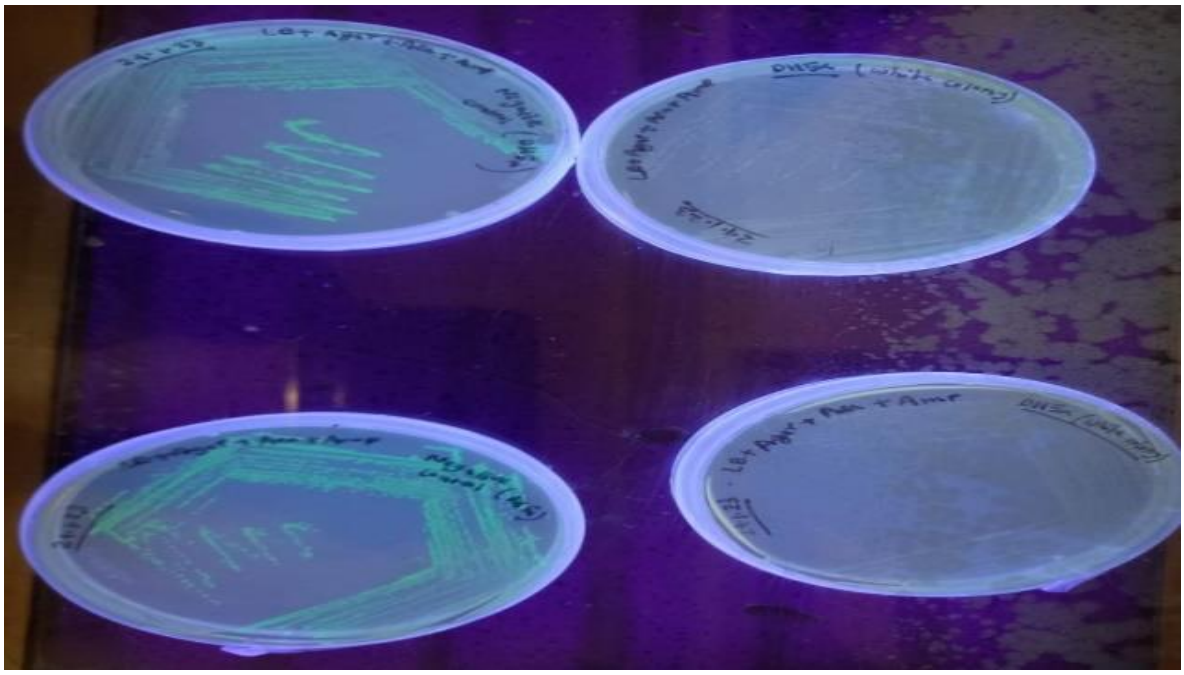


Figure: 3.3 Transformed DH5α strains inoculated into the plates on the right-side column. The control *E. coli* (Untransformed) cells were inoculated on the plates to the left side

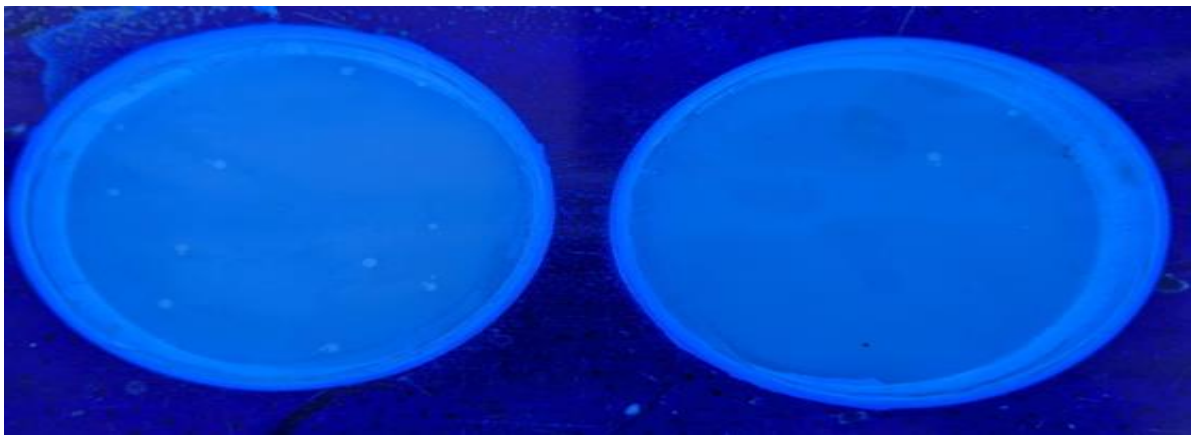


Figure: 3.4 Transformed BL21 strains inoculated into the plates on the left side column. The control untransformed BL21 strain's cells were inoculated on the plates to the right and no colonies were present in the ampicillin medium

3.1.2.4 Recombinant pectinase producing bacterial preparation

The DH5α selection plates and BL21 selection plates were found to contain individual colonies after 24 hours of incubation from stored sub-cultured plates and different clones were selected from the plates for the next growth plates. After fluorescence and colony morphology checking pure culturing process was done and plating for the assay was successful with a surrounded clear zone (Figure 3.5).

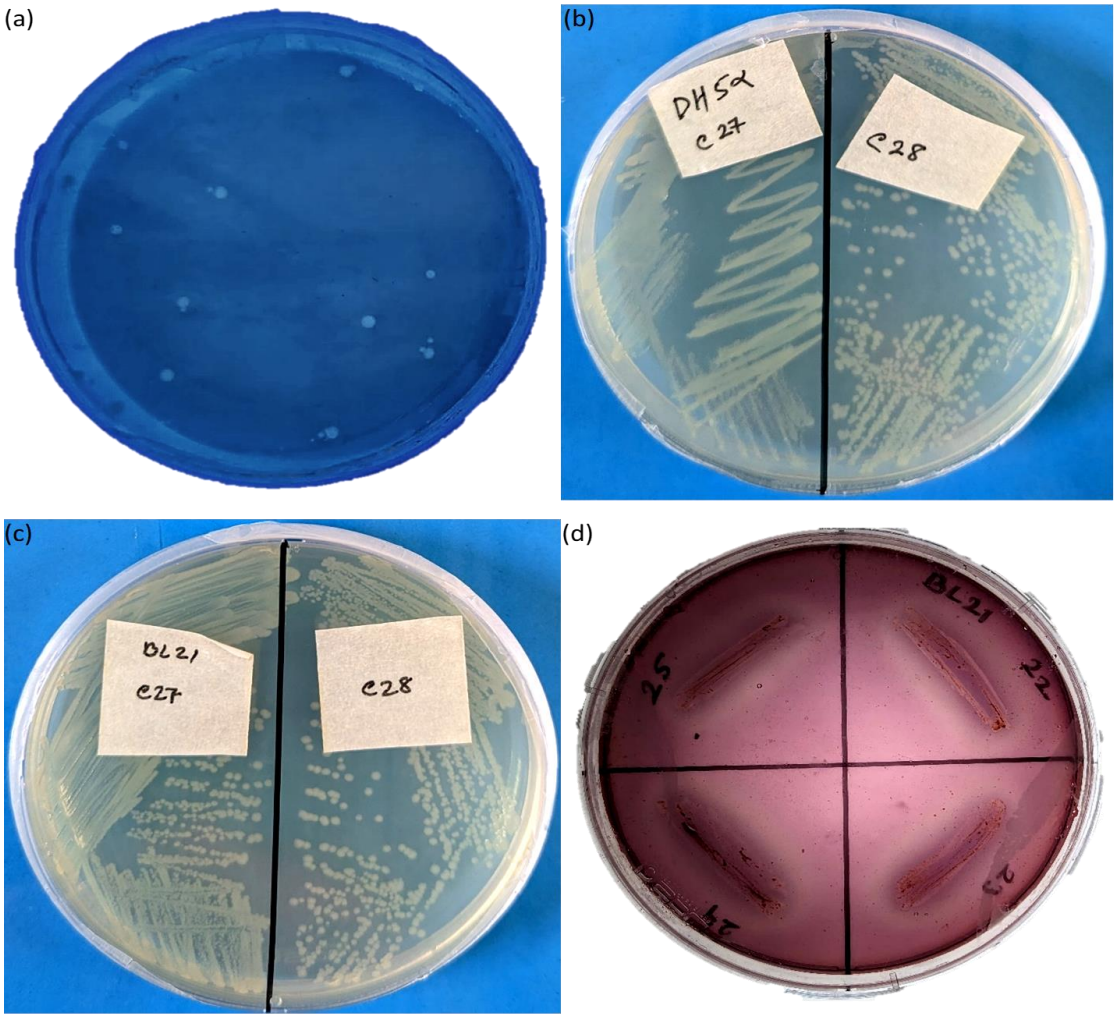


Figure: 3.5 Isolation of the transformed colonies-

(a) Transformed colonies' plate under UV light, (33% colour saturation),

(b) Pure cultures of 27th and 28th transformed colonies of DH5α *E coli* strain,

(c) Pure cultures of 27th and 28th transformed colonies of BL21 *E coli* strain,

(d) Assay plate for selection of the 22nd, 23rd, 24th and 25th transformed colonies of the BL21 strain, stained with congo red and destained twice.

3.1.2.5 PCR amplification of the Ast from clones

The clones B2, B6, B22, B24, D2, D3, D8, and D25, through PCR amplification, contain the Ast gene when they were loaded into separate lane of agarose gel as shown in Figure 3.6, where gene length was between 1000 bps to 1500 bps indicated by the size marker with gel documentation system. It proves that presence of trans pectinase gene in the mentioned clones.

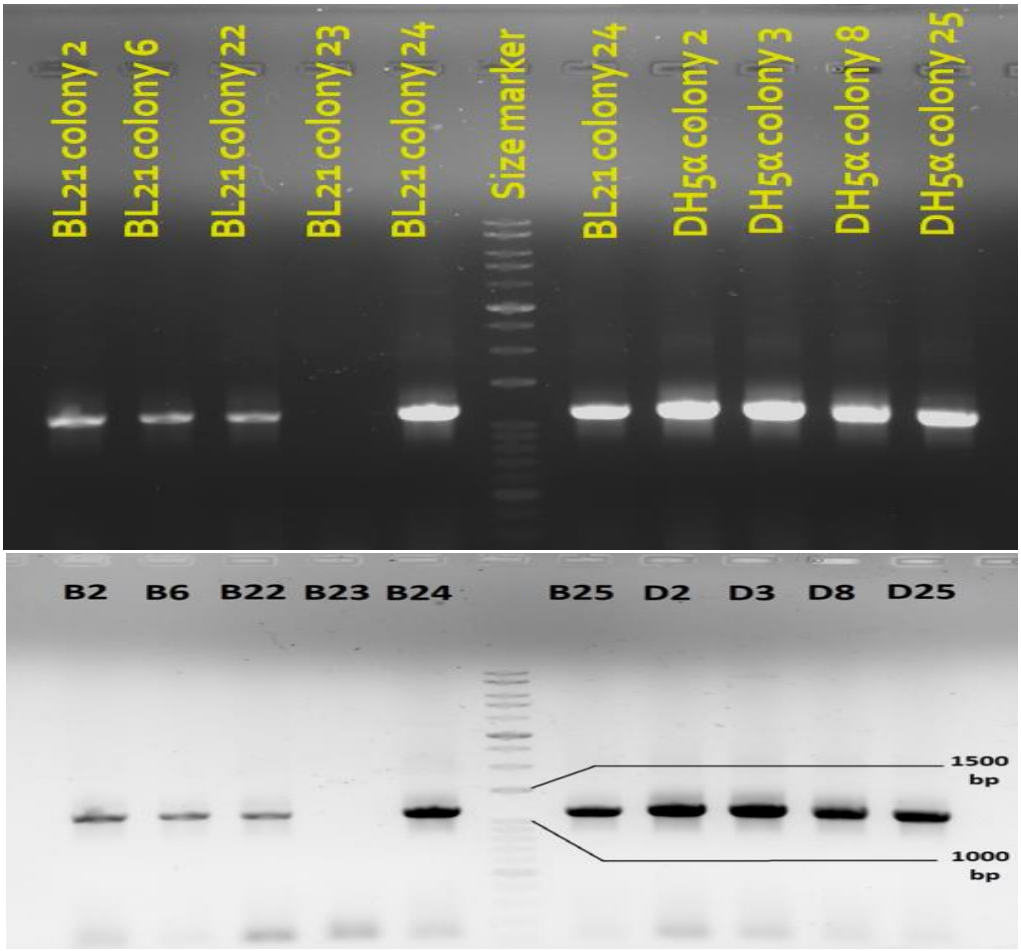


Figure: 3.6 Screening by agarose gel run for presence of the Ast in different colonies with the gel documentation system

3.1.2.6 Protein identification by PAGE report

SDS PAGE report for protein band was above 35 KDa (whole protein) i.e. the molecular weight of the Ast pectinase was 37.81985 kDa. The molecular weight of Green Florescent Protein encoded in the pGLO plasmid is 26.86722 kDa which was calculated using the ExPASy server[95]. In Figure 3.7, the polyacrylamide gel electrophoresis of extracted proteins was displayed and the recombinant protein should be slightly above the 35 kDa of marker line. The B6, B22, B24, B34, D3, D8 colonies displayed the desired band. Although the band was mild,

it was not present in the untransformed lane. These bands are shown in the light-yellow bands in the following image. The GFP shows a heavy and diffused band in the ‘GFP’ lane around the 25 kDa size marker.

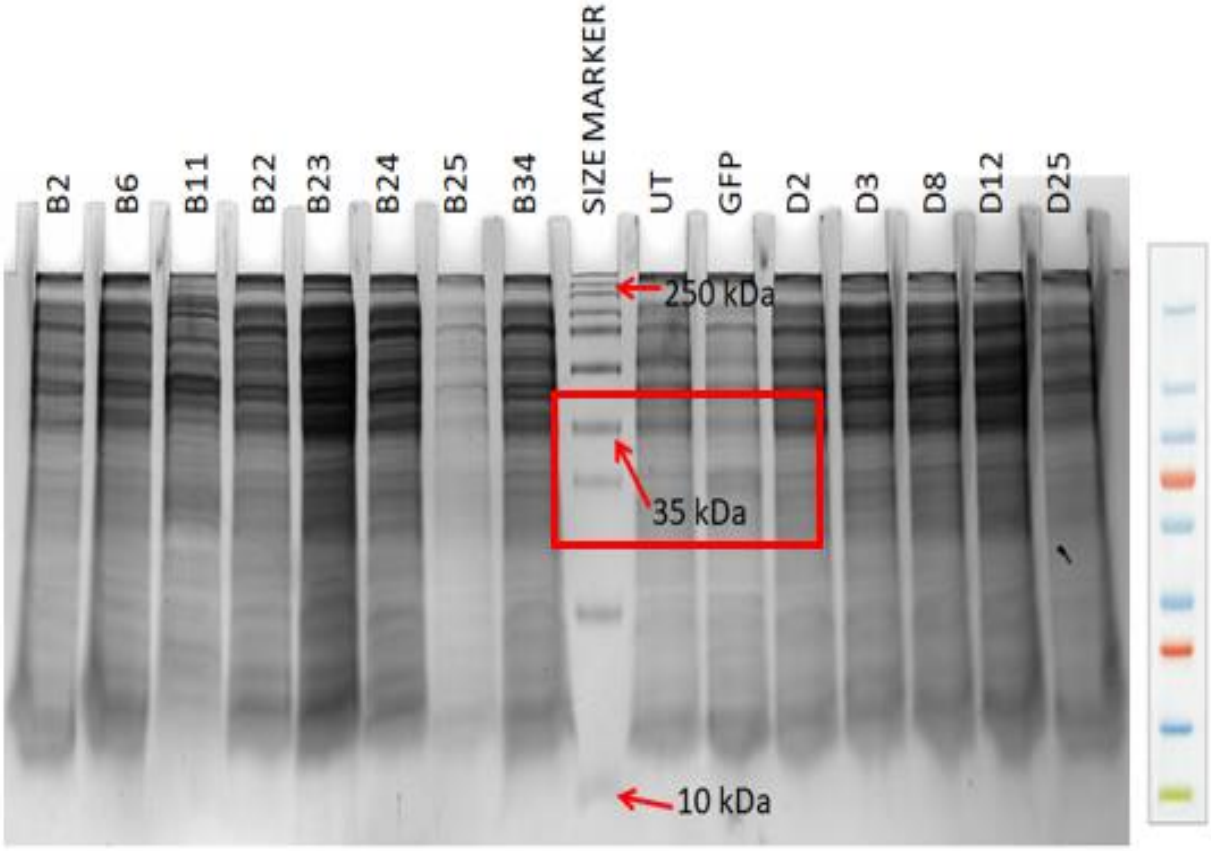


Figure: 3.7 Polyacrylamide gel electrophoresis of the extracted proteins.

In Figure 3.7, the untransformed DH5α sample is marked as ‘UT’, and the sample of DH5α strain transformed with the pGLO plasmid is referred to as ‘GFP’. The clones deriving from the BL21 strain bacteria are denoted with the prefix ‘B’ followed by the clone number. Similarly, in the case of clones of the DH5α strain, the prefix is ‘D’ followed by the clone number.

3.1.3 Analysis of sequencing data

The concentration of the DNA templates sent for sequencing is given below in Table 3.3.

Table 3.3 Template concentration obtained for 16S and Ast samples

Sample ID	Concentration (ng/µL)
16S rDNA	20.1
Ast	30.0

3.1.3.1 Bacteria identification

16S rDNA sequence similarity was shown the highest similarity (100% identity for the sequence from the forward primer and 99.79% for the sequence from the reverse primer), by the BLAST tool, the bacterial sequence with *Escherichia coli*, which can be seen in Figure 3.8 and Figure 3.9.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Escherichia coli strain LZSFT34 chromosome, complete genome	Escherichia coli	850	5954	100%	0.0	100.00%	4945961
Escherichia coli strain LZSFZT33 chromosome, complete genome	Escherichia coli	850	5954	100%	0.0	100.00%	5056393
Escherichia coli strain XL1Blue chromosome, complete genome	Escherichia coli	850	5948	100%	0.0	100.00%	4633417
Shigella sp. strain TRM82590 16S ribosomal RNA gene, partial sequence	Shigella sp.	850	850	100%	0.0	100.00%	1495
Shigella sp. strain TRM82563 16S ribosomal RNA gene, partial sequence	Shigella sp.	850	850	100%	0.0	100.00%	1394
Shigella sp. strain TRM82198 16S ribosomal RNA gene, partial sequence	Shigella sp.	850	850	100%	0.0	100.00%	1436
Shigella sp. strain TRM82197 16S ribosomal RNA gene, partial sequence	Shigella sp.	850	850	100%	0.0	100.00%	1463
Escherichia coli strain AB1 16S ribosomal RNA gene, partial sequence	Escherichia coli	850	850	100%	0.0	100.00%	1361
Escherichia coli strain JM83 chromosome, complete genome	Escherichia coli	850	5954	100%	0.0	100.00%	4558296
Escherichia coli strain PNUSAE145745 chromosome, complete genome	Escherichia coli	850	5954	100%	0.0	100.00%	5458846
Escherichia coli strain PNUSAE145743 chromosome, complete genome	Escherichia coli	850	5954	100%	0.0	100.00%	5458821

Figure: 3.8 BLAST search result using the trimmed 460 nucleotide sequence from the forward primer.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Shigella flexneri strain Uyi_45 16S ribosomal RNA gene, partial sequence	Shigella flexneri	857	857	100%	0.0	99.79%	1412
Escherichia fergusonii strain NA-29 16S ribosomal RNA gene, partial sequence	Escherichia fergusonii	857	857	100%	0.0	99.79%	1318
Escherichia coli strain UFV 251 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1353
Escherichia coli strain DPRA3 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1451
Escherichia coli strain DPRA2 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1451
Escherichia marmotae strain LMEM304 16S ribosomal RNA gene, partial sequence	Escherichia marmotae	857	857	100%	0.0	99.79%	669
Escherichia coli strain CAU1710 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1380
Salmonella enterica subsp. arizonae strain NS10 16S ribosomal RNA gene, partial sequence	Salmonella enterica subsp. a...	857	857	100%	0.0	99.79%	984
Escherichia coli strain B66 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1112
Escherichia coli partial 16S rRNA gene, isolate C33	Escherichia coli	857	857	100%	0.0	99.79%	824
Escherichia coli strain FBC1029 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1273
Escherichia coli strain FBC1018 16S ribosomal RNA gene, partial sequence	Escherichia coli	857	857	100%	0.0	99.79%	1367

Figure: 3.9 BLAST search result using the trimmed 460 nucleotide sequence from the reverse primer

3.1.3.2 Ast gene sequence analysis and integrity check

The inserted pectinase gene was sequenced using the forward primer of the first segment, and a snapshot of the sequence. ab file (Figure 3.10), opened with chromas, and pairwise BLAST alignment between the codon-optimized sequence and the middle 314 bps of the sequence results are given in the image Figure 3.11 below. A 3-base pair deletion and 5 mismatches were found in this region.

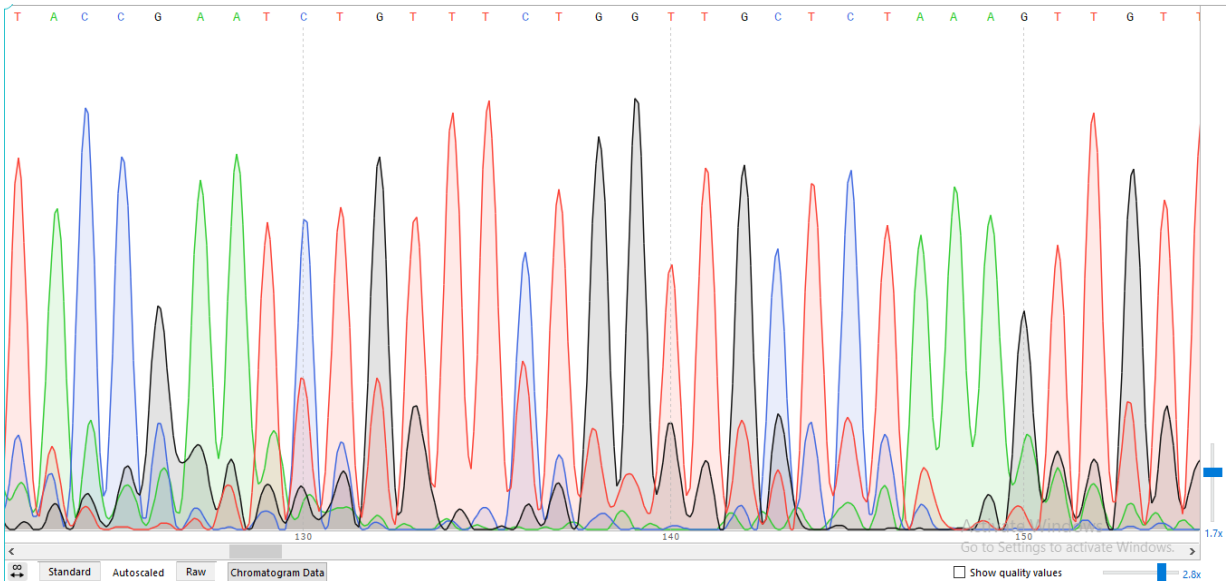


Figure: 3.10 A snapshot of the sequence. ab file

Score	Expect	Identities	Gaps	Strand
538 bits(291)	3e-157	309/317(97%)	3/317(0%)	Plus/Plus

```
Query  84   TGTTGGTTCTCTGGTTTCTGCTGCTCCGGCTCCGTCTCTGACCAAAAAAGACTCTTCTTG  143
           ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1    TGTTGGTTCTCTGGTTTCTGCTGCTCCGGCTCCGTCTCTGACCAAAAAAAACTCTTCTTG  60

Query  144  CACCATCACCTCTGCTGCTGAAGCTACCGAATCTGTTTCTGGTTGCTCTAAAGTTGTTGT  203
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CACCATCACCTCTGCTGCTGAAGCTACCGAATCTGTTTCTGGTTGCTCTAAAGTTGTTGT  120

Query  204  TAAAGACGTTAAAGTTCCGGCTGGTGAAACCCTGGACCTGTCTAAAGTTGACGACGGTAC  263
           |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  121  TAAAGACGTTAAAGTTCCGGCTGGGGAAACCCTGGACCTGTCTAAAGTTGACGACGGTAC  180

Query  264  CACCATCACCTTCGAAGGTACCACCACCTTCGAATACAAAGAATGGAAAGGTCCGCTGAT  323
           |||||||||||||||||||||||||    |||||||||| ||||||||||||||||||||
Sbjct  181  CACCATCACCTTCGAAGGTACCACAC---CGAATACAAAAAATGGAAAGGTCCGCTGAT  237

Query  324  CCGTATCGAAGGTAAAGAAATCACCGTTAACATGGCTGAAGGTGGTCTGATCGACGGTGA  383
           |||||||||||||||| ||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  238  CCGTATCGAAGGTAAAAAAATCACCCTTAACATGGCTGAAGGTGGTCTGATCGACGGTGA  297

Query  384  CGGTTCTCGTTGGTGGG  400
           |||||||||||||||||
Sbjct  298  CGGTTCTCGTTGGTGGG  314
```

Figure: 3.11 The pairwise BLAST alignment between the optimized sequence as Query and Ast sequence as Subject (Sbjct)

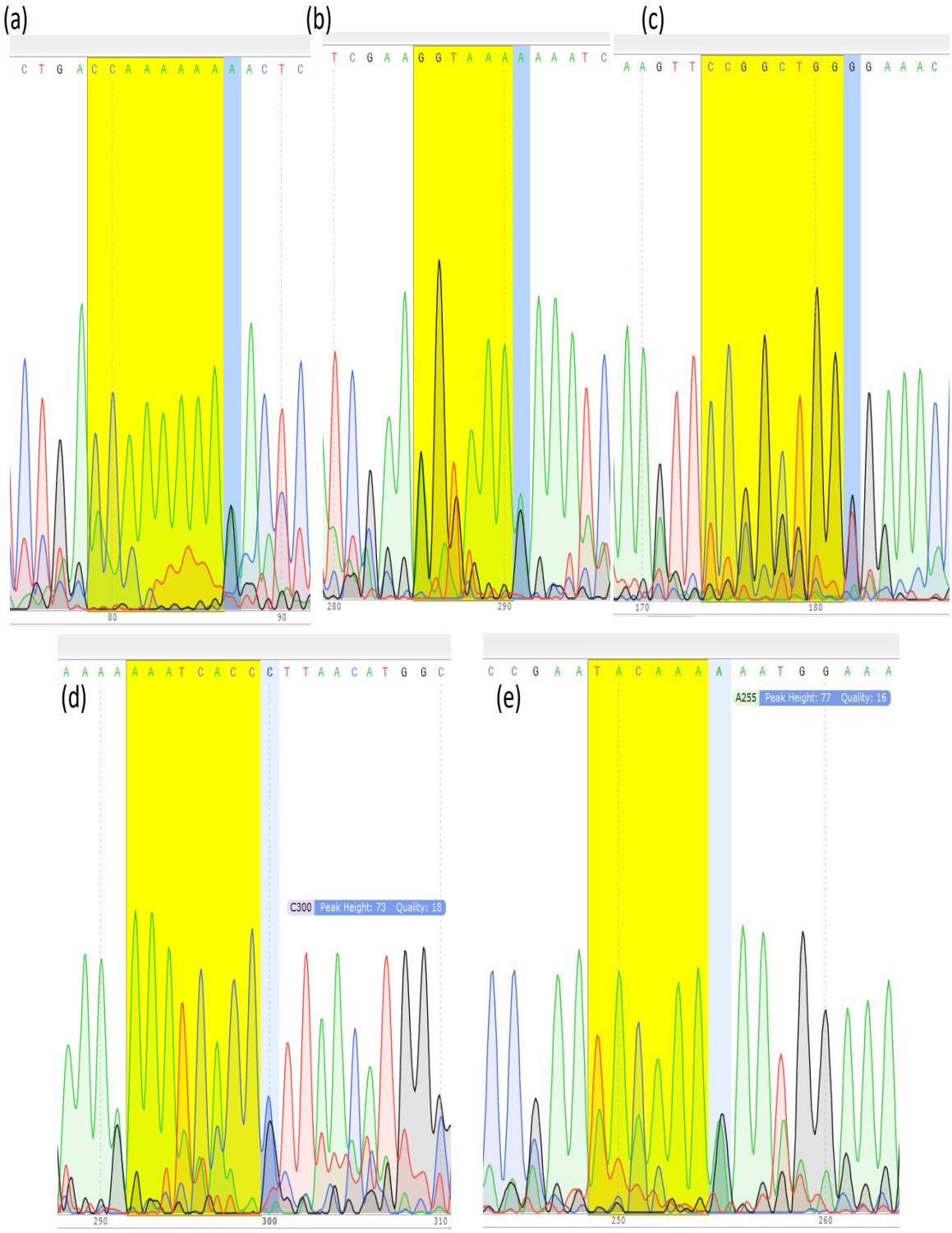


Figure: 3.12 Analyzing the sequences at the 5 mismatches in the pairwise alignment

The 5 bases of the mismatches were manually edited to the correct base and again subject to pairwise BLAST alignment. The pairwise BLAST result after the base editing operations is in Figure 3.12.

The 5 mismatches were analyzed using the SnapGene software and a problem was suspected with their base-calling. The genomic analyzer software could not differentiate a correct different base after a series of consecutive same bases. Snapshots of these mismatches are highlighted in Figure 3.12 a-e. Here, the blue-highlighted base is thought to be assigned wrong. The base calls of the yellow-highlighted region may have influenced the wrong base calling in the blue-highlighted base. The 3-nucleotide deletion, however, was true. But since it is a multiple of 3, only one amino acid would be deleted from the final protein and frameshift would not occur.

Score	Expect	Identities	Gaps	Strand
566 bits(306)	1e-165	314/317(99%)	3/317(0%)	Plus/Plus

```
Query  84   TGTTGGTTCTCTGGTTTCTGCTGCTCCGGCTCCGTCTCTGACCAAAAAAGACTCTTCTTG  143
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGTTGGTTCTCTGGTTTCTGCTGCTCCGGCTCCGTCTCTGACCAAAAAAGACTCTTCTTG  60

Query  144  CACCATCACCTCTGCTGCTGAAGCTACCGAATCTGTTTCTGGTTGCTCTAAAGTTGTTGT  203
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CACCATCACCTCTGCTGCTGAAGCTACCGAATCTGTTTCTGGTTGCTCTAAAGTTGTTGT  120

Query  204  TAAAGACGTTAAAGTTCCGGCTGGTGAAACCCTGGACCTGTCTAAAGTTGACGACGGTAC  263
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TAAAGACGTTAAAGTTCCGGCTGGTGAAACCCTGGACCTGTCTAAAGTTGACGACGGTAC  180

Query  264  CACCATCACCTTCGAAGGTACCACCACCTTCGAATACAAAGAATGGAAAGGTCCGCTGAT  323
            |||||||||||||||||||||||||   |||||||||||||||||||||||||||||||
Sbjct  181  CACCATCACCTTCGAAGGTACCACCAC---CGAATACAAAGAATGGAAAGGTCCGCTGAT  237

Query  324  CCGTATCGAAGGTAAAGAAATCACCGTTAACATGGCTGAAGGTGGTCTGATCGACGGTGA  383
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  CCGTATCGAAGGTAAAGAAATCACCGTTAACATGGCTGAAGGTGGTCTGATCGACGGTGA  297

Query  384  CGGTTCTCGTTGGTGGG  400
            |||||||||||||||||
Sbjct  298  CGGTTCTCGTTGGTGGG  314
```

Figure: 3.13 Pairwise BLAST alignment after manual base editing operations

The 5 bases of the mismatches were manually edited to the correct base and again subject to Pairwise BLAST alignment. The pairwise BLAST result after the base editing operations is in Figure 3.13.

3.2 Natural Pectinase Enzyme Production

3.2.1 Bacterial isolate with pectin degrading abilities

The bacterial isolate with pectinase enzyme was identified via the congo red assay on minimal agar plates with pectin as the sole carbon source (Figure 3.14 a). The clear zone formed by the

enzyme-mediated breakdown of the pectin demonstrated the isolate's pectin degradation capabilities[96]. In the region where the control *E. coli* strains grew, no such zone was seen. This activity was corroborated further by detecting a comparable clear zone in the congo red-washed pectin-LB-agar plate with the screened bacteria (Figure 3.14 b). In this instance, no clear zone for *E. coli* or *E. coli* harboring pGLO plasmid was observed.

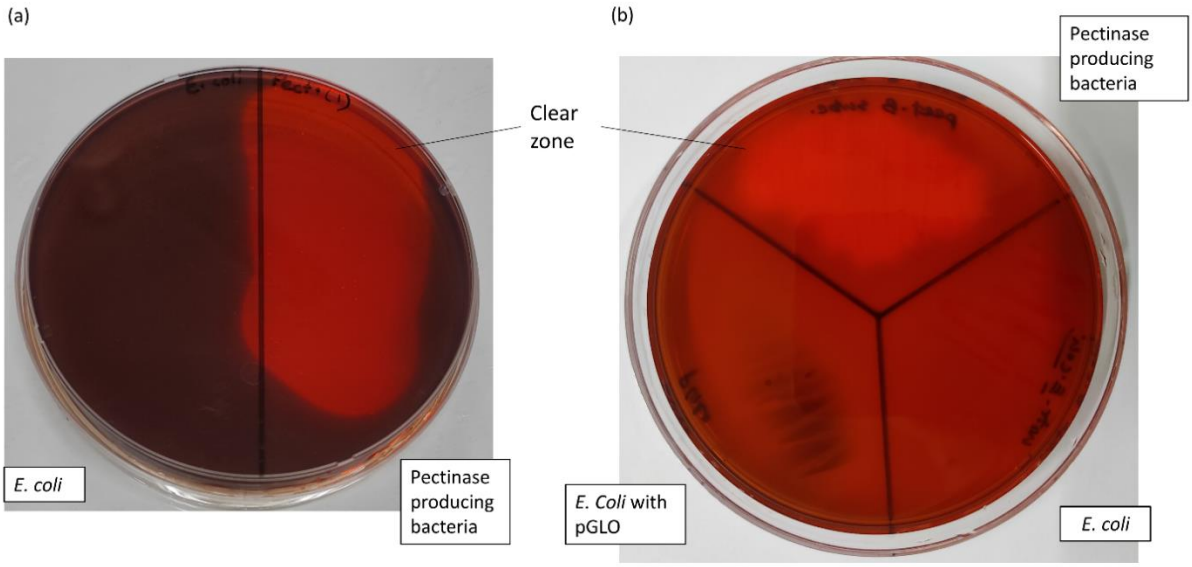


Figure 3.14 Screening of pectinolytic bacteria using the congo red assay. A clear zone was observed around the colonies of pectinase-producing bacteria in (a) minimal agar plates with pectin as the sole carbon source and (b) pectin-Luria-Bertani (LB) agar media. No clear zone was observed for *E. coli* as well as *E. coli* harboring pGLO palsmid.

3.2.2 Molecular identity of pectinase-producing bacteria

The nucleotide BLAST program query of our bacteria's 16S rRNA sequence revealed great similarity with the genus *Burkholderia* (Table A1), with the highest similarity (99.87% identity) to the species *Burkholderia cepacia.*

Multiple sequence alignments with 50 of the best hits from this search produced a neighbor-joining tree shown in Figure 3.15. It has shown the highest closeness with the species of *B. ubonensis, B. dolosa, B. latens, B. multivorans, B. cenocepacia, B. vietnamiensis, B. metalica, B. cepacia, B. terrtorri.*

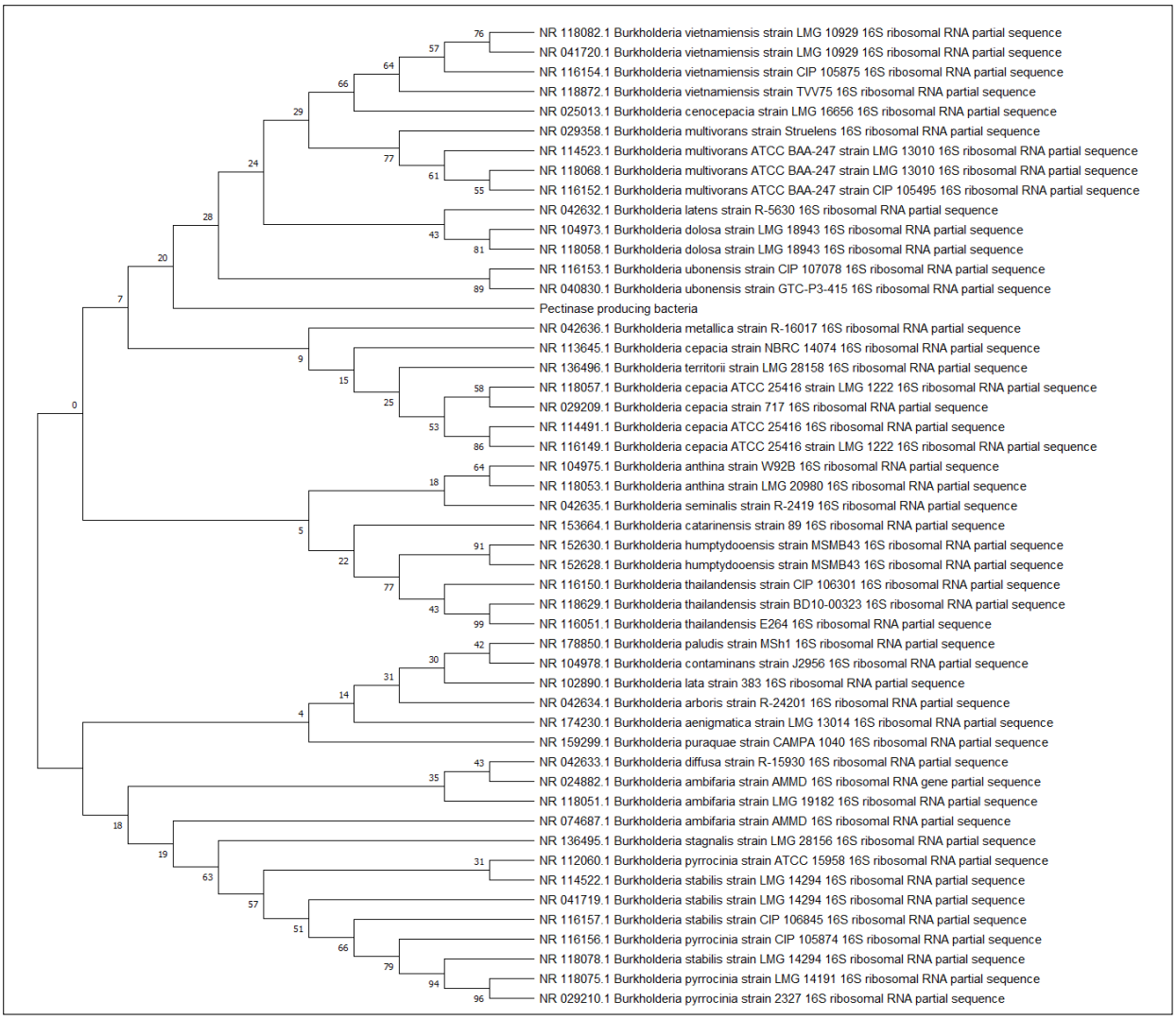


Figure 3.15 Neighbor-joining (NJ) tree based on the pectinase-producing isolate's 16S rRNA gene sequence. The numbers on the branches of the NJ tree represent bootstrap support values. All sequences' NCBI GenBank accession numbers are written in front of each species' name

3.3 Crude Pectinase Enzyme Activity Test

3.3.1 Lugol's iodine test

The strain was transformed carefully by streaking on a pectin-LB-agar plate using a non-pectinolytic untransformed strain (*E. coli*) and a transformed *E. coli* harboring a plasmid (GFP)as negative controls under UV light. Plates are flooded with iodine and KI solution and de-stained with water after 5 minutes. D8, B24, and CS colonies have the most pectinolytic activity (Figure 3.16 a and Figure 3.16 b). The iodine and polyiodide ions can preferentially bind to polymeric carbohydrates and therefore, the places with degraded polygalacturonic acid will not be able to retain the stain, resulting in a lighter zone around the colony is the indication of pectinase activity[97].

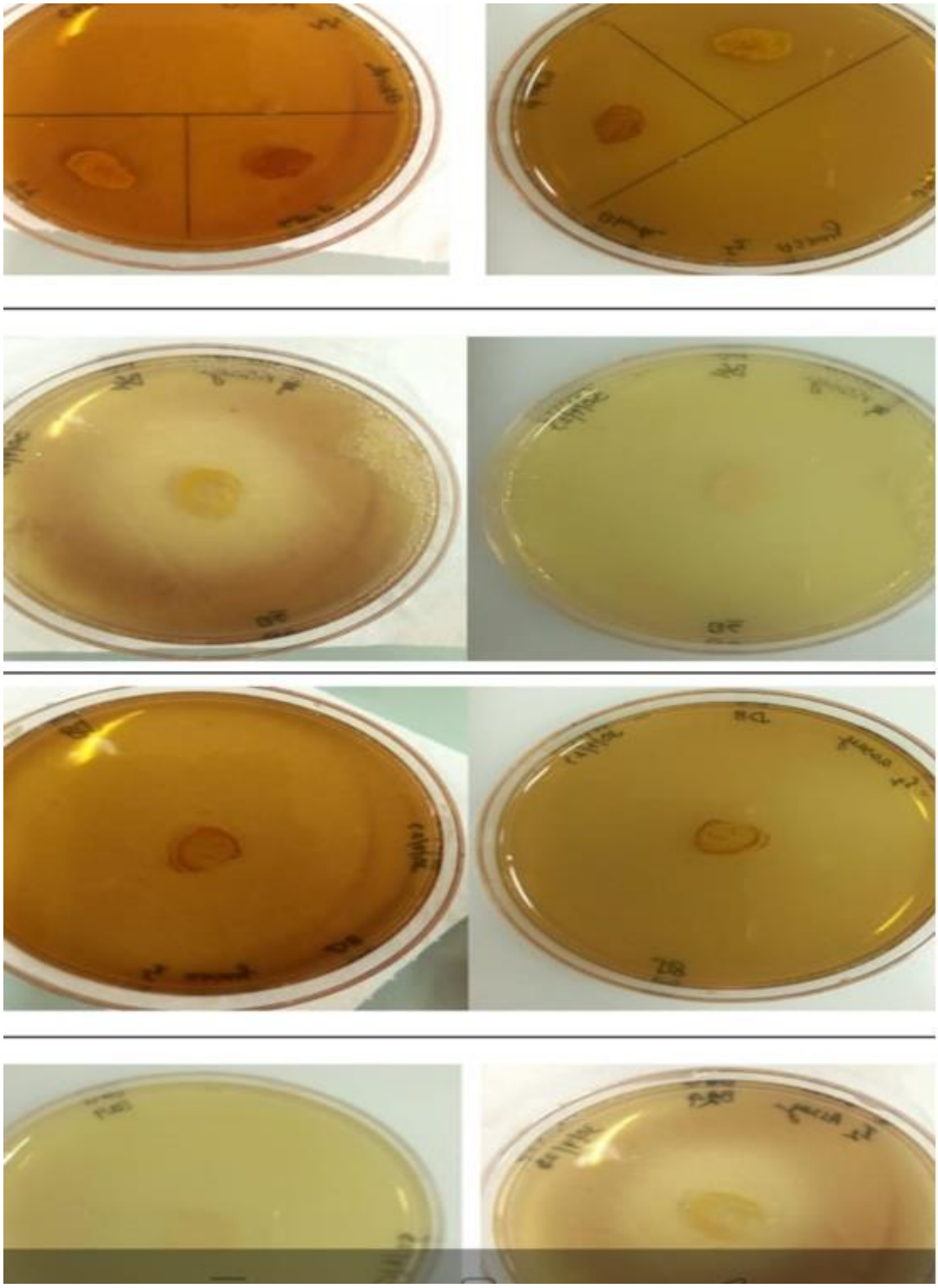


Figure: 3.16 a Lugol's iodine test on a circular streak of UT (Untransformed DH5α) and GFP (DH5α transformed with pGLO) (Top 1st row), B6 (2nd row), D8 (3rd row), and B24 (Bottom row)

Figure: 3.16 b Lugol's Iodine test for pectinolytic activity of the CS bacterial plate

3.3.2 Quantitative DNS test

3.3.2.1 Glucose standard curve generation

The glucose concentrations and optical density (OD) at 540 nm are given in Table 3.4.

Table 3.4 Absorption values at 540 nm for the DNS test of glucose standards

Glucose concentration (mM)	OD_{540}
0	0.220
0.05	0.175
0.1	0.241
0.25	0.263
0.5	0.478
1	1.016
1.5	1.438
2	1.949
2.5	2.533
3	2.7
4	2.857
5	2.87
6	2.871
7	2.872
8	2.876
9	2.884
10	2.885

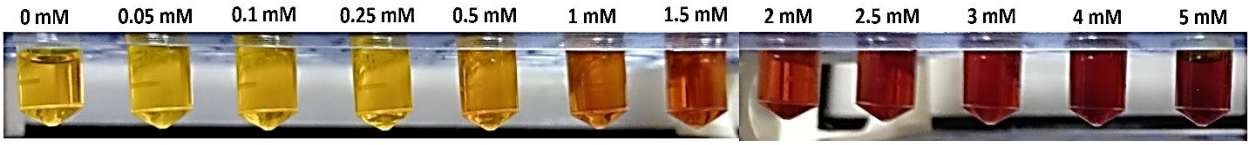


Figure: 3.17a Image of colours produced by the glucose standards

The standard curve can produce a consistent straight line in concentrations ranging from 0.25 mM to 2.5 mM. Considering this range, the equation of the linear line is y = 0.9974x – 0.0088 fits the points, which shows a linear regression value R^2 = 0.998. This correlation table and curve are as below.

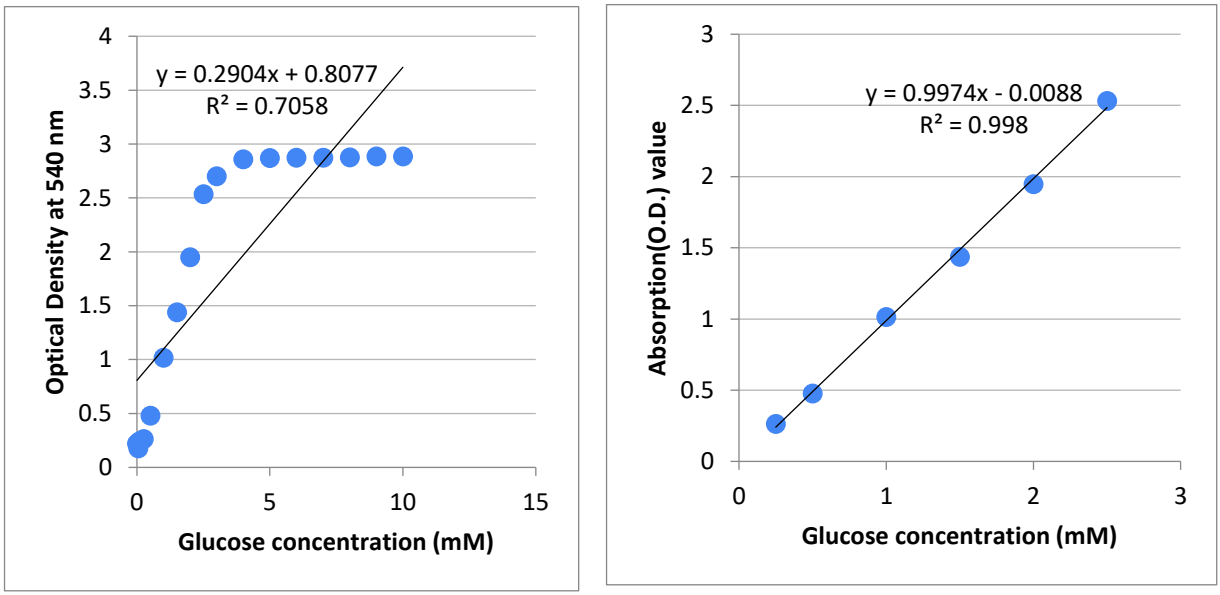


Figure: 3.17 b Plots of glucose concentration vs absorbance value at 540 nm

In Figure 3.17 b, on the left, all concentrations from 0.05 to 10 mM are considered to build the graph, on the right, 0.25 to 2.5 mM concentrations are used.

3.3.2.2 Reducing sugar correlation curve generation

The absorption values of glucose concentration in the range of 0.5 mM to 2.5 mM were used to correlate the difference in O.D. values with nascent carbohydrate-reducing end concentration is given in Table 3.5.

Table 3.5 Calculating the correlation of difference in O.D. values and sugar reducing end concentration.

Concentration of released reducing ends of sugars (mM)	Concentration values considered from the standard curve	Difference in O.D. values	Average Δ O.D.
0	-	0	0
0.5	0.5 and 1.0	0.538	0.51375
	1.0 and 1.5	0.422	
	1.5 and 2.0	0.511	
	2.0 and 2.5	0.584	
1.0	0.5 and 1.5	0.960	0.996
	1.0 and 2.0	0.933	
	1.5 and 2.5	1.095	
1.5	0.5 and 2.0	1.471	1.494
	1.0 and 2.5	1.517	

According to the Microsoft Excel calculation, the correlation plot of Figure 3.18a was generated and the equation derived was y = 1.0071x – 0.0062, where x is the difference in O.D. value and y is the reducing end concentration. This equation showed a linear regression R^2 of 0.9999.

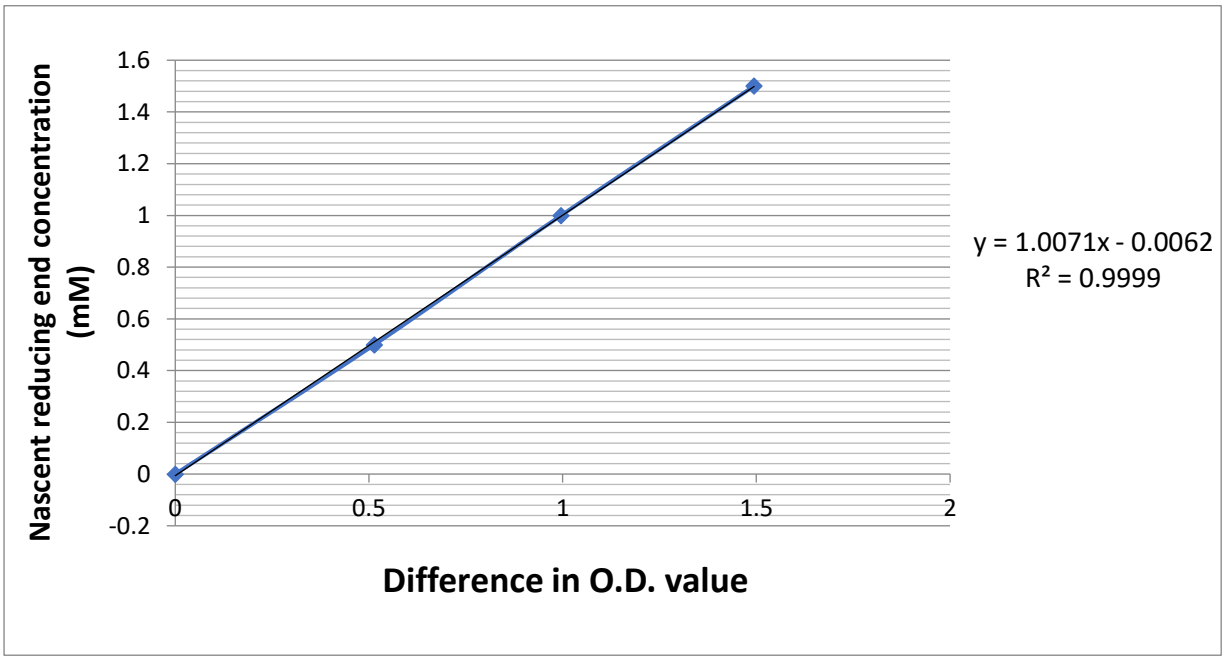


Figure: 3.18a Correlation curve of O.D. value and reducing end of sugars

3.3.3.3 Amount of nascent reducing end calculation

The 'GFP' sample was used as the reference to calculate the difference in O.D. values, ultimately the amount of released reducing ends (and concentration of endogalacturonase breaks) was found using the correlation equation.

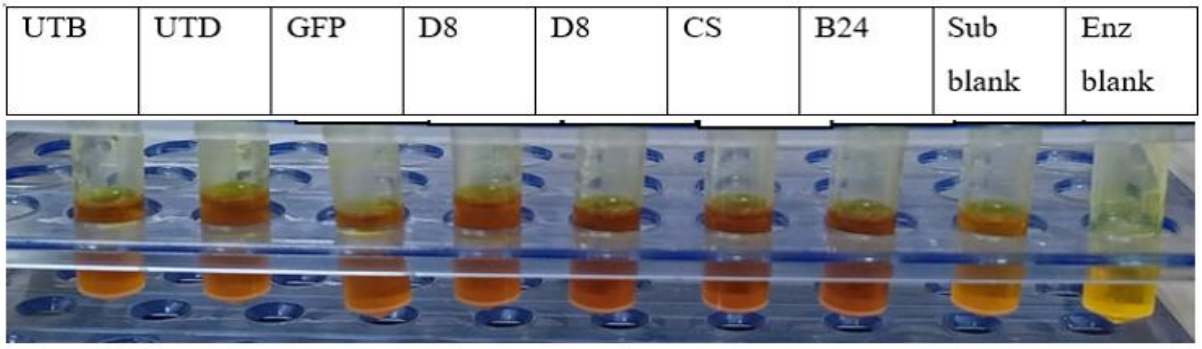


Figure: 3.18b Colour produced by the DNS test of transformed clones and control samples

Table 3.6 Calculation of amounts of enzymatic breaks in polygalacturonic acid chains using DNS test

Sample	O.D. @ 540 nm	Change in O.D. relative to GFP	Reducing ends of polygalacturonic acid (mM)
Sub blk	0.624	-0.351	-
Enz blk	0.215	-0.760	-
GFP	0.975	0.000	0.0
UTD	0.893	-0.082	-0.076
UTB	1.02	0.045	0.039
B24	1.102	0.127	0.122
CS	1.08	-	0.112
D8	1.424	0.449	0.446

Reducing sugars were observed in the pectin solution with DNS solution in Figure 3.18b by changing the yellow DNS solution to reddish-orange which indicates the ability to produce reducing ends of polygalacturonic acid as mentioned in Table 3.6 [98].

3.4 Crude Enzyme Activity on Cotton Knit Fabric

3.4 .1 Activity test of the crude enzyme by wicking of cotton knit fabric

The wettability was checked to observe the wicking rate in the untreated and produced natural and recombinant enzyme-treated fabric. The average wicking rate was encouraging because of increased wicking height in the case of transformed enzyme-treated fabric than untransformed enzyme-treated fabric at the same treatment parameters and washing conditions. This confirmed the cleavage of the pectic elements by enzymatic treatment[99]. Of course, the pectinase enzyme assisted in removing pectin along with the action of detergent on the fibre. Surfactant is a necessary component in the enzymatic composition for scouring and seems to have a high impact on the removal of waxes and fats at high temperatures, while the enzyme facilitates the removal of pectic substances. In the wicking test, transformed bacteria D8's extract-treated fabric at 37 °C had travelled more than untransformed ones, and it had the highest wicking distance among all the samples after 5 minutes (wet condition) (Figure 3.20) and 10 minutes (dry condition) (Figure 3.21). Wicking distance of recombinant DH5α, BL21, CS and BS enzymes was found 85 mm, 65 mm, 55 mm and 75 mm after ten minutes in dry condition and it was 65 mm, 50 mm, 45 mm and, 63 mm in wet condition. In the case of WE 37 and UT 37 had travelled 40 mm and 41 mm in wet condition and 41mm and 41 mm in dry condition respectively which is due to the detergency effects on waxy substance removal from the surface of the cotton. It is the also evidence of activity of enzymes in CS 37, BL 37, D8 37. On the contrary, it was taken 3.4sec, 4sec, 4.5sec, more than 5 minutes, 5 minutes and 3.5 minutes for treated fabric with D8 enzyme, CS enzyme, BL enzyme, without enzyme, untransformed bacterial solution and BS enzyme at 37 °C respectively to reach 4 cm height (Figure 3.19). Commercial enzyme takes more time to travel 4 cm height and the outer area was not uniform (Figure A 48) like other enzyme which indicates the commercial enzyme is not uniformly working at low temperature. The grey fabric was completely untreated and could not cross more than 1 cm because of the outer waxy layer that makes a barrier to penetration. This wicking test makes the strong evidence of pectinolytic activity of enzymes with their quantitative performance for the decomposition of pectin compounds in cotton knit fabric during bio scouring.

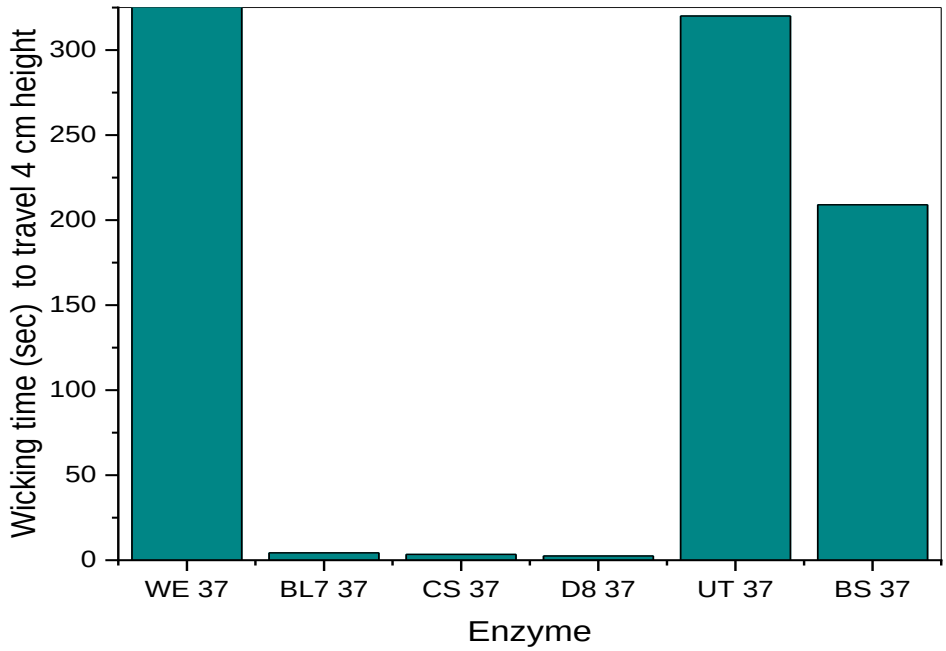


Figure: 3.19 Wicking time required to travel 4 cm height with the sample treated at 37 °C

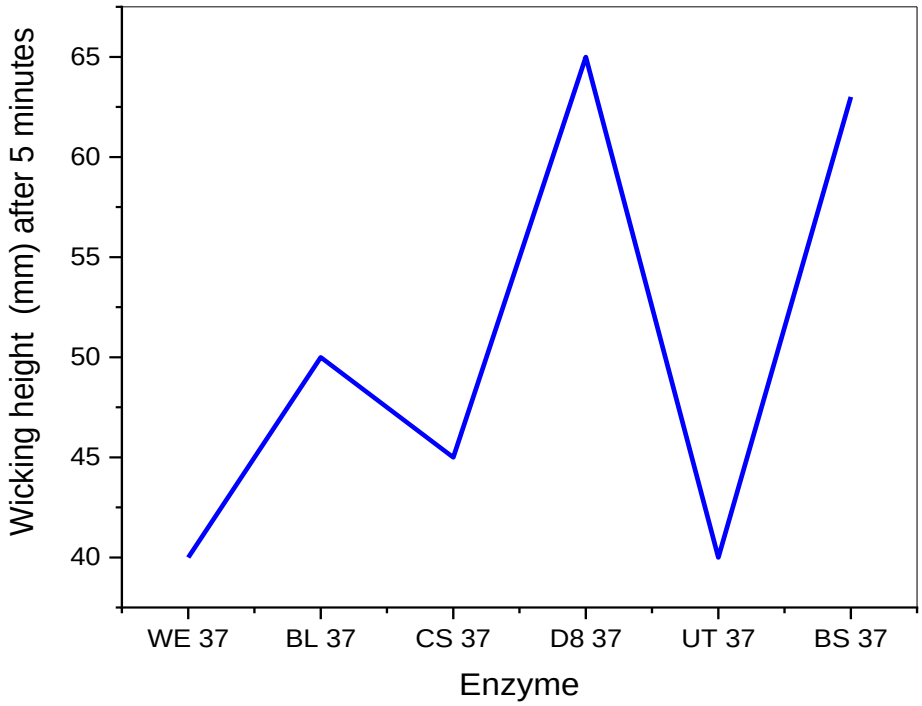


Figure: 3.20 Wicking height in wet condition with the fabric treated at 37 °C

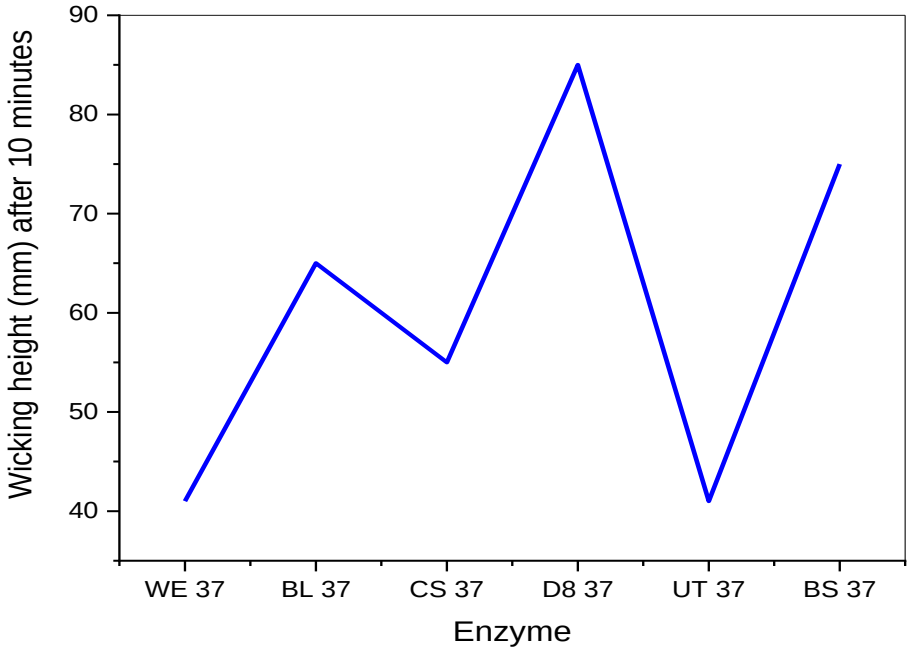


Figure: 3.21 Wicking height in dry condition with the fabric treated at 37 °C

3.4.2 FTIR Analysis of the untreated and new recombinant enzyme-treated cotton samples

The study focuses on the structural analysis of cellulose, cuticle, and pectin in the context of light transmittance measurements and the impact of pectic compound degradation. The graph from Figure 3.22 shows the weaker intensity of the characteristic bands of aliphatic CH stretch, hydroxyl, and ester carbonyl wax, and the cuticle layer of cotton. The mono and tri-esoteric components like wax, fat, and oil were removed with the detergent action. The weakened and moved-out bands in the region of 1700 ~ 1400 cm^{-1} refer to the partial removal of pectin[2]. In Figure 3.22, the intensity of the peak is noticeably changed in the mentioned region from grey, bands of CS 37, BL 37, and D8 37 are nearer to BS60, D8 37 is almost identical to the commercial enzymatic processed sample[2]. These bands are designated within the territory of C = O stretch (1745 cm^{-1}) for pectic acid; C = O stretching (1745 cm^{-1}), O-CH_3 stretching (1445 cm^{-1}), and -CH_3 bend (1234 cm^{-1}) for pectic ester; COO asymmetric stretch (1614 cm^{-1}) and symmetric stretch vibration (1425 cm^{-1}) for calcium pectate[100][101].

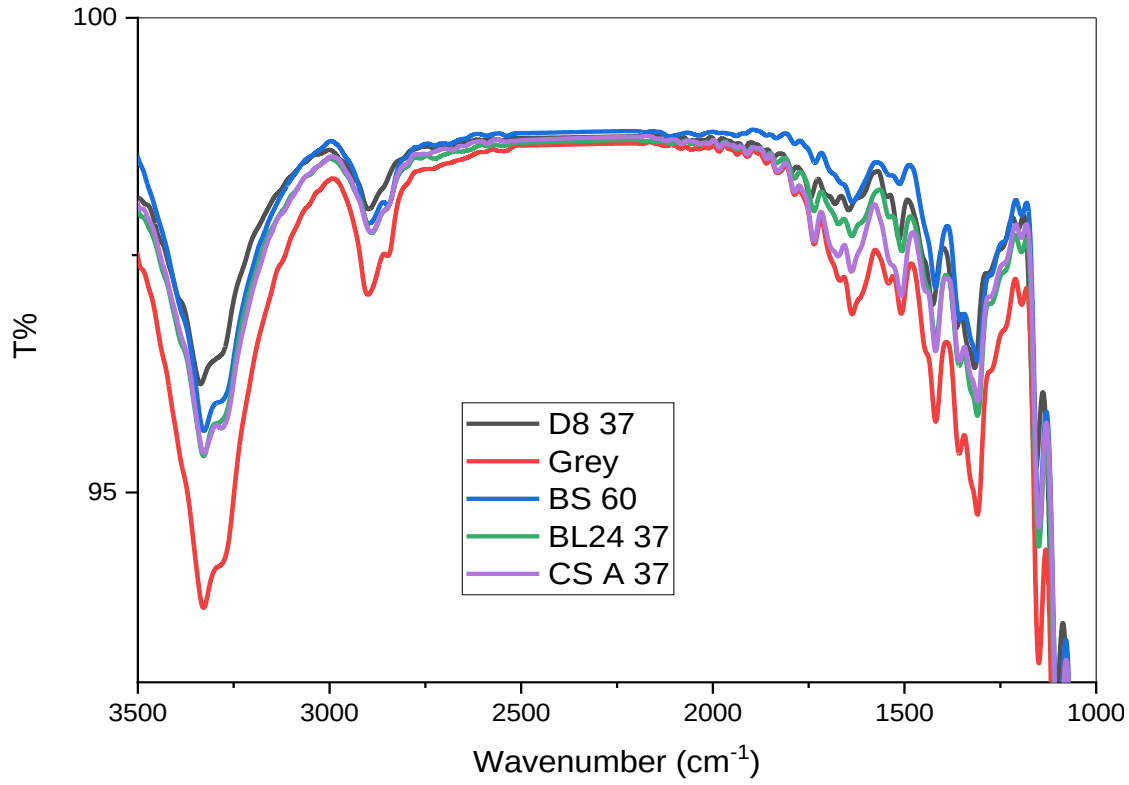


Figure: 3.22 FTIR analysis of the untreated, commercial, natural, and new recombinant enzyme-treated cotton samples

3.4.3 Bursting strength of the bio-scoured fabric

Non-cellulosic substances in the fibres act as a protective barrier, preserving the hydrophobic (water-repellent) nature of the fibres and preventing surface from degradation. This protection is beneficial in maintaining the integrity and structure of the cellulose fibres. Pectinase activity with detergent can be changed on the surface of the cotton fibre[102]. D8 pectinase enzymes from recombinant technology causes the decomposition of pectin substances enormously was visible in FTIR peak with the assistance of detergent to remove the outer waxy layer, others pectin component elimination and cellulose degradation decreases the strength of D8 pectinase enzyme-treated fabric than chemical scoured and commercial scoured enzyme-treated fabric, whereas BL21 enzyme causes slightly less effect and natural enzyme has almost similar effect like commercial enzyme on bursting strength shown in the Figure 3.23.Here, it was known CSBP 10 had greater loss of strength than CSP , and was used for verifying the acceptability of others strength .

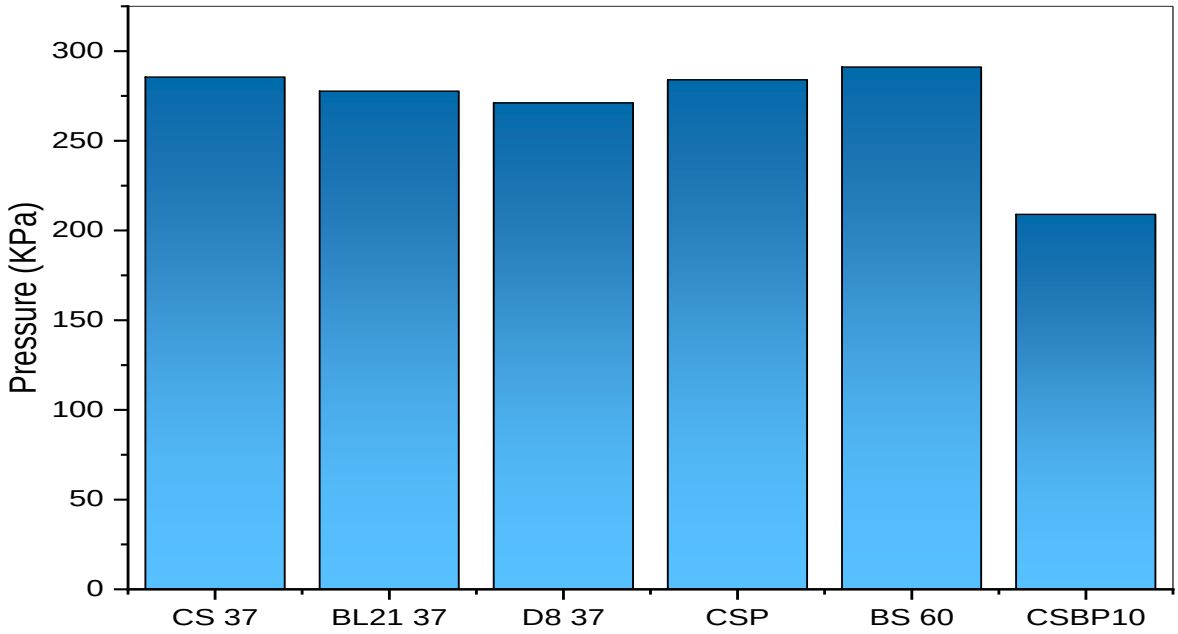


Figure: 3.23 Bursting strength of the bio-scoured, chemical-scoured, and chemical-scoured bleached fabric

3.4.4 WI YI Tint of the bio-scoured fabric

The relationship between whiteness (expressed by Whiteness Index, WI) and yellow-ness (expressed by Yellowness Index, YI) and tint is correlated in fabrics. Using CIELAB colour space for simulations and data estimation helps systematise colour quality control. The negative correlation between WI and YI implies that yellowness tends to decrease as whiteness increases, and vice versa. However, the correlation coefficients vary depending on the colour or fabric hue. The statement also notes that colours with the same distance from the white point in CIELAB exhibit different rates of change in WI and YI based on hue. Specifically, when WI decreases, the YI of reddish and yellowish-tinted colours decreases more than that of bluish-tinted colours. In grey fabric, whiteness is considered 0, tint is negative and it contains some yellowness [103]. WE 37, CS37, BL37, D837, UT 37. WE 60, BS 60, CSP and CSBP10 samples have different effects on whiteness, yellowness, and tint which are shown in Figure A6, Figure A7 and Figure 3.24. Processing type, treatment condition, time, and temperature had a great influence on WI and YI. With the increase in processing time, temperature and the number of processes, cotton impurities like fat, wax, and pectin compounds which contain some colour, are significantly removed in CSP than bio scouring and further bleaching process chromophore group are oxidized or reduced and the fabric gets more whiteness[61].Therefore CSBP 10 had the highest value in whiteness and other two values were lower. All enzyme-treated fabrics had a lower whiteness effect than CSP and among the three enzymes, D8 showed more whiteness.

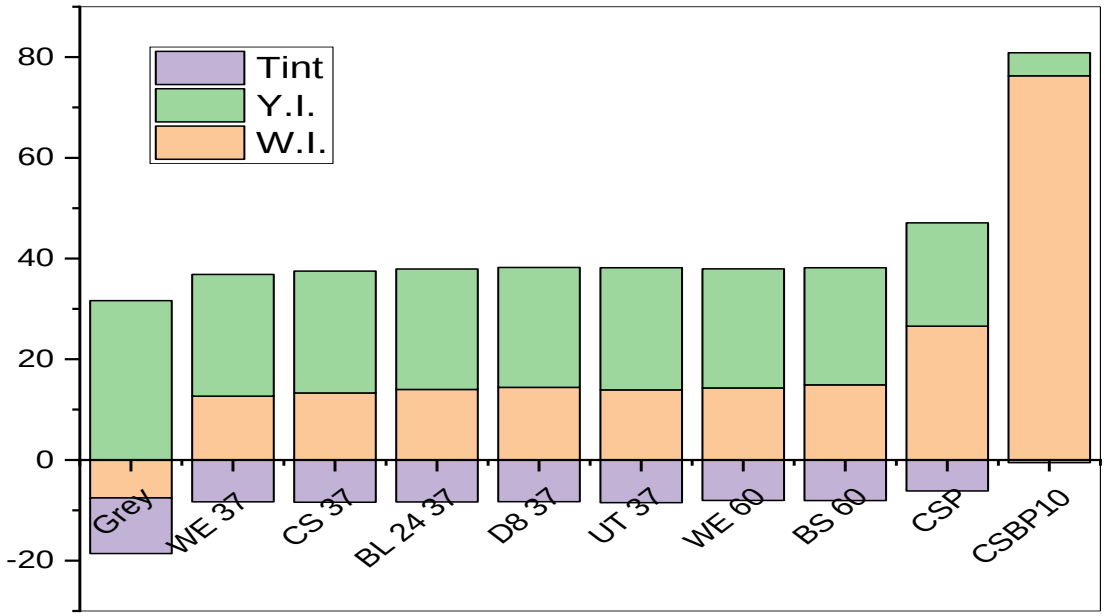


Figure: 3.24 Whiteness index, Yellowness index and Tint of the different types of treated fabric

3.4.5 Colour strength of the bio-scoured fabric

Colour strength (K/S) is a term to express the ability of a dye to impart colour to a material. When a fabric contains some colour before colouration, the pure white fabric gives pure colour strength, it increases the resultant colour strength after colouration which is shown in Figure A14, A15, A16, A17, A18, A19, Figure 3.25 and Table 3.7 for dyed fabric with, WE 37, CS37, BL37, D837, UT 37, WE 60, BS60, CSP and CSBP10 samples. Only detergent action without pectinase, WE 37, carries more colour-bearing components due to pectin [48], bio-scoured fabric contains more tint than chemical-scoured and bleached fabric[104], and therefore resultant colour strength for the bio-scoured fabric is higher than only detergent-washed fabric. Chemical scouring and combined scouring bleaching processes eliminate much more tint due to the alkali and alkali with hydrogen peroxide at higher temperatures respectively. For that reason, chemical scouring processed (CSP) and combined scouring bleaching processed (CSBP) samples showed less K/S values as compared to bio-scoured samples. Bio-scoured fabric D8 37 contains less tint and more W.I. than BL 37 and CS 37, for that reason, D8 37 had less colour strength than BL 37 and CS 37. Consequently, the L value of combined scouring bleaching processed (CSBP) samples showed 27.89, which means the highest lightness value of the dyed samples. CSP is shown less than CSBP. Among the three-enzyme treated dyed fabrics D8 had a 26.99 L value which was more than the other two enzymes but less than CSP and CSBP. The L value (lightness or darkness) of the dyed samples (Figure A8, A9, A10, A11, A12, A13)

indicates the amount of colourant present in the samples which justifies the presence of tint or W.I. in the sample which is why bio-scouring is suitable for dark coloration.

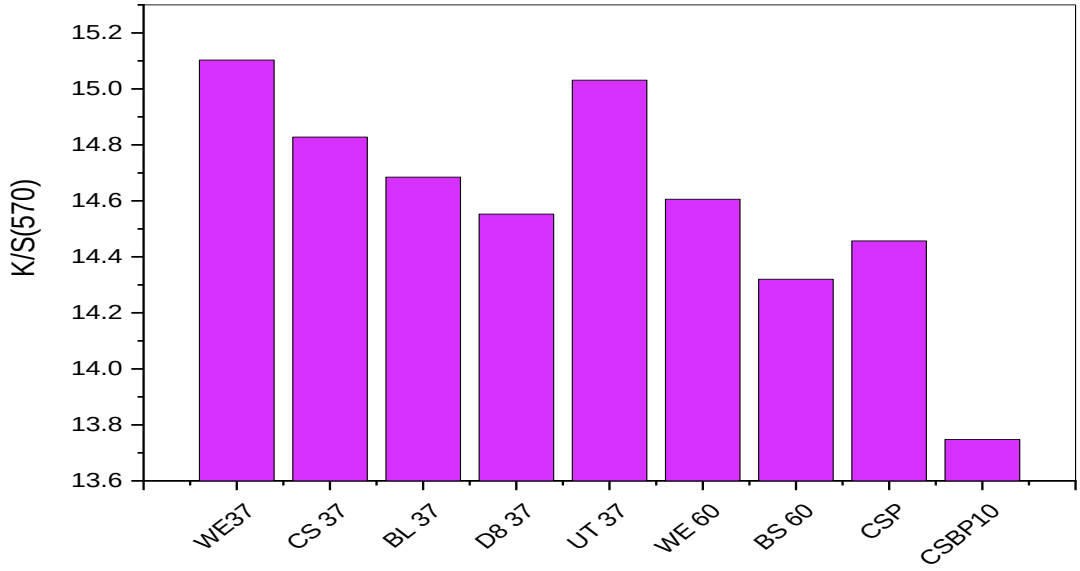


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Figure: 3.25 Colour strength of the different types of treated fabric

Table 3.7 The colour attributes of different dyed samples with Navy blue RGB (2%)

Sample	CIE L Values	CIE a Values	CIE b Values	CIE C Values	CIE h Values	CIE DE Values	CMC DE Values
BS 60	26.99	-8.36	-15.85	17.92	242.19	-	-
CS 37	26.58	-8.15	-15.75	17.73	243.63	0.68	0.47
UT 37	26.27	-8.20	-15.72	17.73	243.46	0.74	0.50
BL 37	26.73	-8.17	-15.82	17.80	242.68	0.32	0.24
D8 37	26.69	-8.28	-15.81	17.85	242.37	0.31	0.21
WE 37	26.99	-8.25	-15.77	17.80	242.38	0.30	0.20
WE 60	26.69	-8.21	-15.77	17.78	242.50	0.43	0.29
CSP	27.17	-8.44	-16.08	18.16	242.32	0.31	0.20
CSBP 10	27.89	-8.52	-16.29	18.39	242.38	1.02	0.67

a.Grey

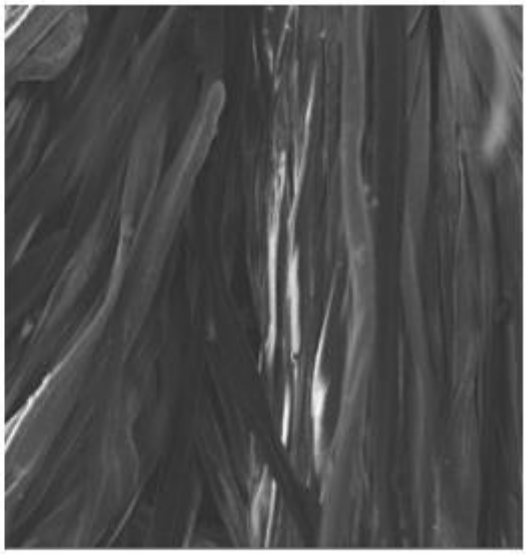
b.D8

c.BL21

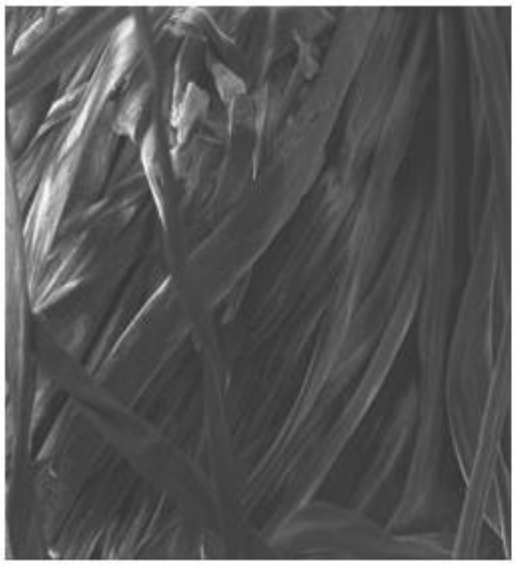
d.CS

Figure: 3.26 SEM micrograph for the untreated and the enzyme-treated cotton knit fabric in 500 ×(20 μm), a) Grey b) D8 c) BL21, and d) CS

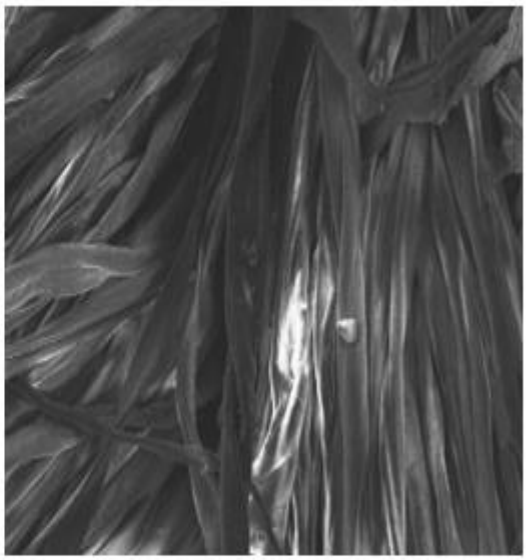
Grey



D8



BL21



CS

Figure: 3.27 SEM micrograph for the untreated and the enzyme-treated cotton knit fabric in 1000 ×(10 μm), a) Grey b) D8 c) BL21 and d) CS

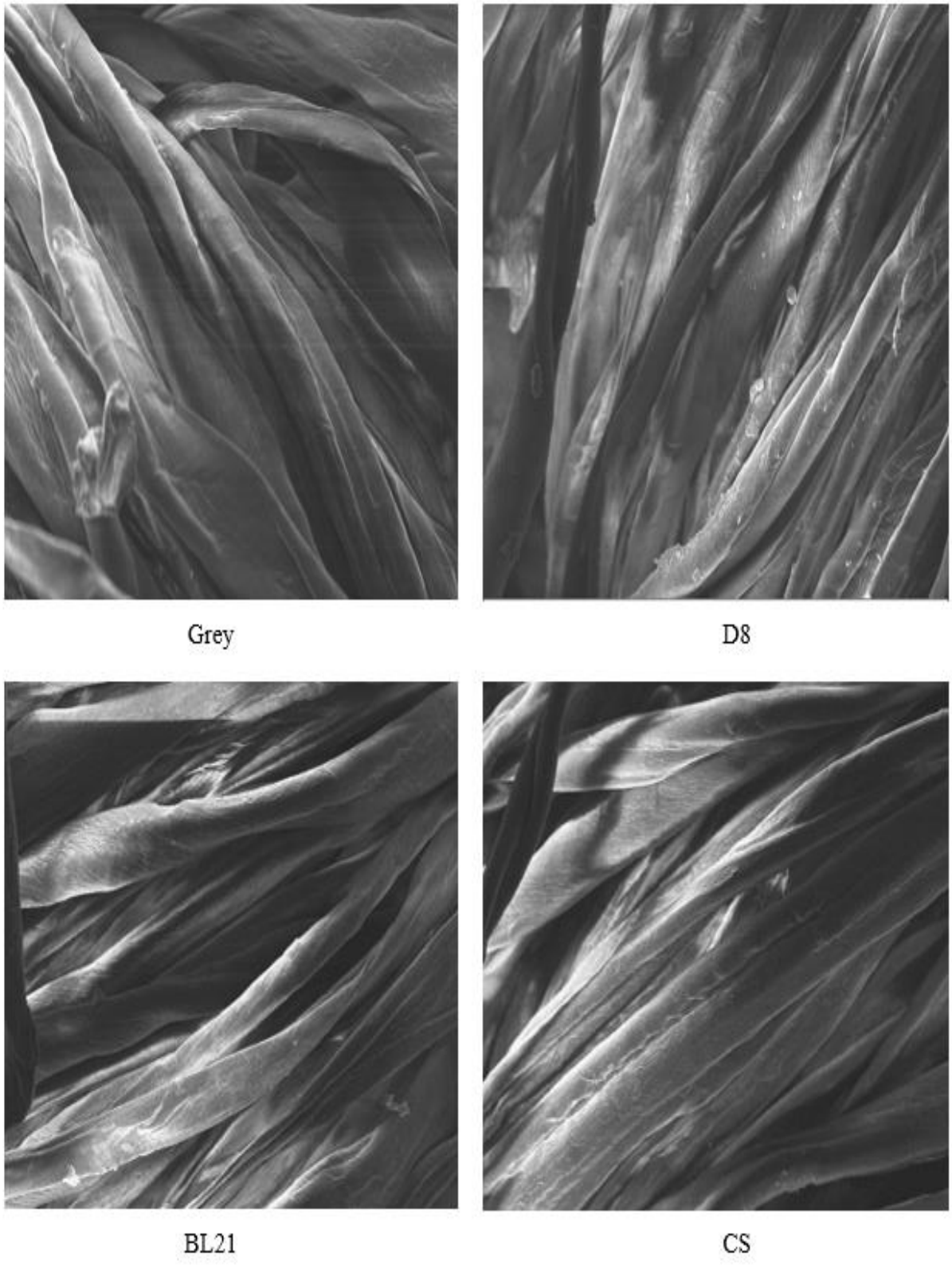


Figure: 3.28 SEM micrograph for the untreated and the enzyme-treated cotton knit fabric in 2000 ×(20 μm), a) Grey b) D8 c) BL21 and d) CS

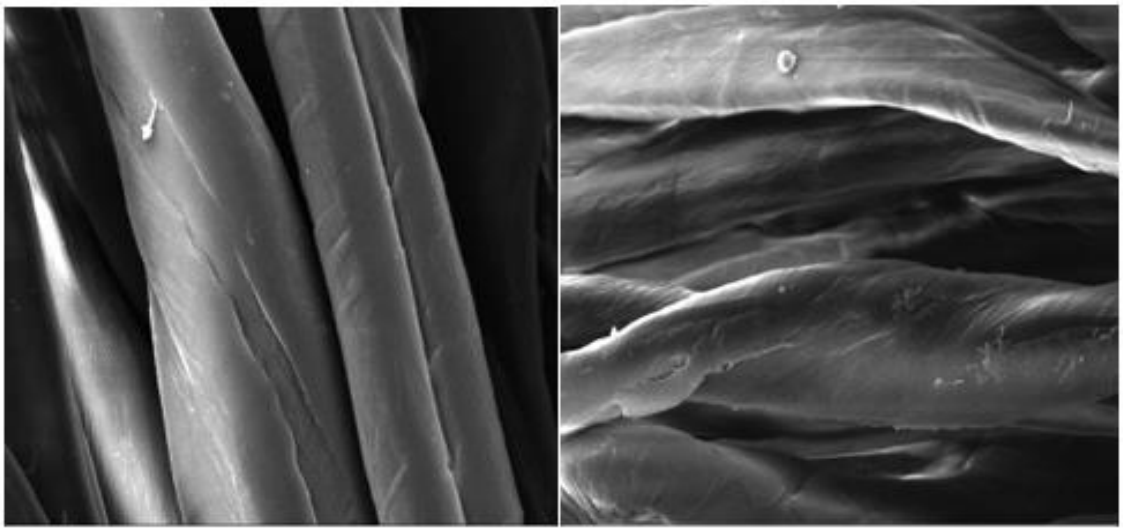


Figure: 3.29 SEM image for the CSP (3µm), and CSBP (2µm) of cotton knit fabric in 5000 ×

3.4.6 Scanning electron microscopy (SEM) analysis for the bio-scoured fabric

The microscopic examination allows us to visually inspect and analyze the effects of different treatments on the fabric surface at a micro-level. This information is crucial for understanding the structural changes, evaluating the effectiveness of the process, and optimizing the treatment conditions for better results in textile processing. In the context of bio scouring, which involves the use of enzymes to remove impurities from fibres, the microscopic images reveal that the enzyme-treated fibres have a smoother surface compared to chemically treated fibres. The enzymatic treatment is gentler on the fibres and causes less damage[2].Additionally, the smoother appearance of enzyme-treated fibres may indicate potential benefits such as improved fabric feel and reduced fibre breakage, contributing to the overall quality of the treated textiles. The smooth waxy layer was present which was shown in untreated grey fabric. The primary wall of the cotton fibre was unveiled because of pectinolytic activity during the bio-scouring process showed scratches on the surface in Figure 3.26, Figure 3.27and Figure 3.28 [85] caused some cracks in the chemical scouring process in Figure 3.29 [105]. Enzymatic activity was much more visible in the case of recombinant enzyme from DH5α bacterial species than recombinant enzyme from BL21 bacterial species and CS enzyme from natural source had less impact. The SEM micrographs of cotton knit fabric for CSP had cracks and swelling and CSBP showed the rougher and swelled surface in Figure 3.29.

3.5 Chemical Scoured and Bio-scoured Bleached Fabric

To increase the whiteness effect in the bio-scoured fabric, initially, sequential oxidative and reductive bleaching was applied for the chemical-scoured fabric to check the feasibility of implementation for reductive and oxidative bleaching on bio-scoured fabric.

3.5.1 Bleaching effect on the chemical-scoured fabric

In textile industry bleaching with hydrogen peroxide as a bleaching agent is a common practice before coloration of fabric. Generally, an amount of 3 to 4 g/L hydrogen peroxide is used in bleaching for regular colouration and for very light colouration double bleach is applied to the material. Here attempt was made for oxidizing the agent with the reducing agent to improve the whiteness index.

3.5.1.1 Effect of hydrogen peroxide and thiourea in a bleach bath

A comparison of the whiteness index of the bleached fabrics with the different concentrations of hydrogen peroxide incorporated in different bleach baths and converting these oxidative baths of 4 g/L (P2a), 6 g/L (P2b), 8 g/L (P2c), and 10 g/L (P2d) hydrogen peroxide to reductive baths with 6 g/L thiourea according to process code P3 for demineralized and grey fabric respectively shown in Figure A44 and Figure 3.30. In every case, reductive bleach improved the whiteness index (W.I.) of the cotton demineralized fabric but in the case of grey fabric, W.I. was not improved uniformly in each case for reductive bath. It is the evidence of decolouration of cellulose with reductive bleaching after oxidative bleaching[49].

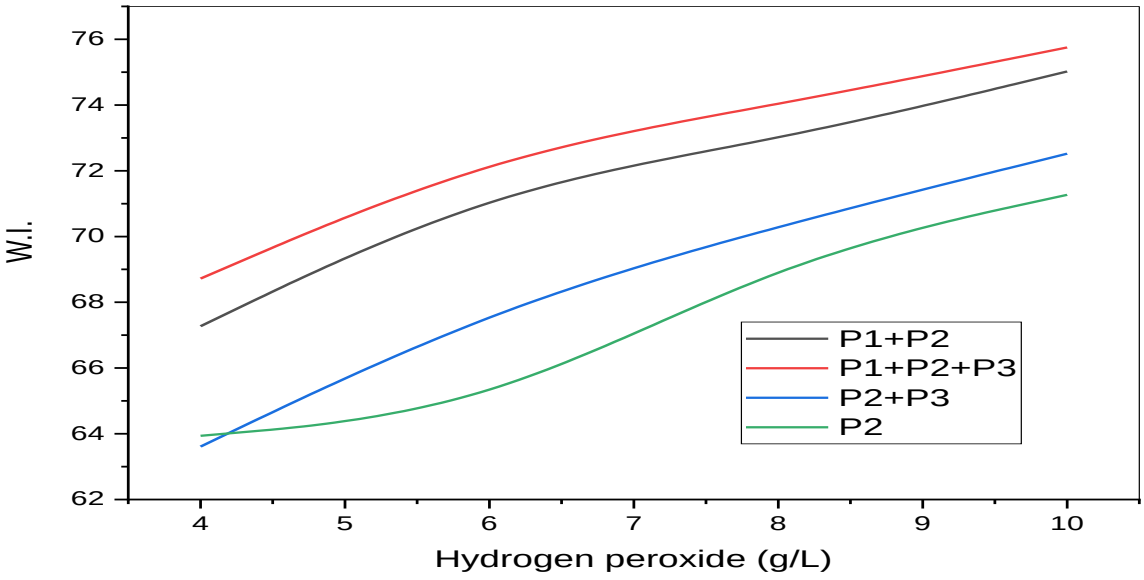


Figure: 3.30 W.I. for the bleaching with different concentrations of hydrogen peroxide and bleaching with thiourea at the same hydrogen peroxide bath

3.5.1.2 Effect of acid addition for the bleaching with thiourea after conventional bleaching with hydrogen peroxide

Depending on the pH and the molar ratio of the reactant, hydrogen peroxide and thiourea produce intermediate products thiourea dioxide during the reactions to convert thiourea. The thiourea dioxide intermediate; then hydrolyzes to yield sulfinate anion and urea if the initial reaction of thiourea with hydrogen peroxide is allowed to take place in an acidic or neutral medium (pH = 4.0-7.0). Because thiourea dioxide hydrolyzes in solution, there is a rapid change in ORP from a positive value to a high negative value. The sulfinate ion causes the negative redox potential, and hence the sulfinate ion is responsible for reductive bleaching. Hydrogen peroxide should be twice in molar ratio with thiourea to convert thiourea dioxide for reaction. Once thiourea is converted to thiourea dioxide at pH 4.5 (showed positive redox potential +186 mV), pH adjustment to 7.1 causes hydrolysis to the highly reductive sulfinate ion (showed highly negative redox potential -637 mV since sulfinate is a strong reducing agent), and causes reductive bleaching[106]. After conventional scouring bleaching (according to process P2d) with a demineralized sample (from process P1) the bath was followed by the recipe according to process P6.5, P4e, P4f, P4g, P4h. The addition of acetic acid at the thiourea reductive bleach bath increased the whiteness and got 78.57 from 75.02 and further addition of sodium borohydride of 1 g/L, 3 g/L, and 5 g/L made the whiteness 79.04, 79.39, 79.64 and 79.54 respectively for demineralized sample. ORP values were +88, -839, -877, -893, and -902 mV for P6.5, P4e, P4f, P4g, and P4h processes respectively.

3.5.1.3 Effect of the addition of sodium borohydride for the bleaching with thiourea after conventional bleaching

After conventional scouring bleaching (according to process P2d) with a demineralized sample (from process P1) the bath was followed by the recipe according to process P3, P4a, P4b, P4c, P4d. The addition of sodium borohydride of 1 g/L, 3 g/L, and 5 g/L in the thiourea bleach bath without pH adjustment made the whiteness 77.44, 77.38, 78, and 77.19 respectively. ORP values were 2, -270, -862, -888, -897, and -917 for P2d, P3, P4a, P4b, P4c, and P4d respectively. The amount of sodium borohydride matter on the redox potential of the bleaching bath increased proportionally with a concentration in the bath but a further increase with a high amount of 7g/L did not increase whiteness.

3.5.1.4 Comparative analysis of the demineralized and the grey samples

The outer layer of the cotton fiber is composed of different noncellulosic substances like waxes, lipids, pectin, protein, organic acid, and unidentified compounds[107]. Waxy materials and pectin are responsible for the hydrophobic properties of raw cotton. The efficiency of the scouring process is directly related to the success of the subsequent wet-processing operations[108]. Raw cotton can exhibit widely different contents of alkaline earth metals (Ca and Mg) and heavy metal ions (Fe, Cu, Mn). Metals, however, cannot be adequately removed by conventional alkaline processes since, in an alkaline medium, sequestering agents cannot quantitatively separate the minerals of a complex structure containing heavy metals. Moreover, in the alkaline pH region, cellulose swells rapidly and strongly, thus impairing the transport of crystalline minerals from the core to the periphery of the fiber. Demineralization with organic or inorganic acid is more effective as compared to the alkaline treatment process [109]. Iron(II), Co(E), and Mn(II) are very effective catalysts for the homolytic decomposition of hydrogen peroxide in low concentrations although they can retard oxidation at high concentrations[57]. Iron in grey fabric acts as a catalyst for unfavourable bleaching[110]. Magnesium compounds retard cellulose oxidation that is catalyzed by transition metals [57]. Under alkaline conditions or in the presence of transition metals, hydrogen peroxide starts to decompose[111].

Table 3.8 Comparative analysis for using a demineralized and grey sample in 10 g/L hydrogens peroxide oxidative bath and sequential reductive bath

Sample	After conventional oxidative bleaching		Bursting strength	After conventional bleaching +Thiourea Addition		Bursting strength	After conventional bleaching +Thiourea +$NaBH_4$ (5g/L.) addition		Bursting strength	After conventional bleaching +Thiourea + acid addition		Bursting strength	After conventional bleaching +Thiourea + acid +$NaBH_4$ (5g/L.) addition		Bursting strength
	pH	mV	KPa	pH	mV	KPa	pH	mV	KPa	pH	mV	KPa	pH	mV	KPa
Demineralized	**11.14**	**2**	**216.4**	**11.32**	**-270**	**216.4**	**-3.54**	**-897**	**208.5**	**6.33**	**+112**	**230**	**-1.04**	**-899**	**215**
Grey	**11.04**	**39**	**231.3**	**9.72**	**-204**	**221.5**	**-1.54**	**-897**	**210.7**	**6.36**	**-204**	**237.9**	**-1.31**	**-895**	**231.7**

Comparative analysis of the demineralized and grey samples was done by using the process P1+P2d, P2d, P1+P2d+P3, P2d+P3, P1+P2d+P6.5, P2d+P6.5, P1+P2d+P3+, P2d+P3P2d+ P4c, P1+P2d+ P4c and P2d+ P4g, P1+P2d+ P4g. From Figure 3.31, whiteness was not improved in acid addition for sequential oxidative and reductive bleaching for the grey samples but it reduced bursting strength for demineralized fabric than grey fabric (Table 3.8). From Figure A45, the

W.I. of grey fabric was improved without acid addition in the bath of sequential oxidative and reductive bleaching. For fabric demineralization, the sequestering agent gives hydrogen ion which acts on grey fabric by forming an earth metal complex with the sequestering agent and making high bleaching performance with acid addition or without acid addition in the sequential oxidative and reductive bleaching.

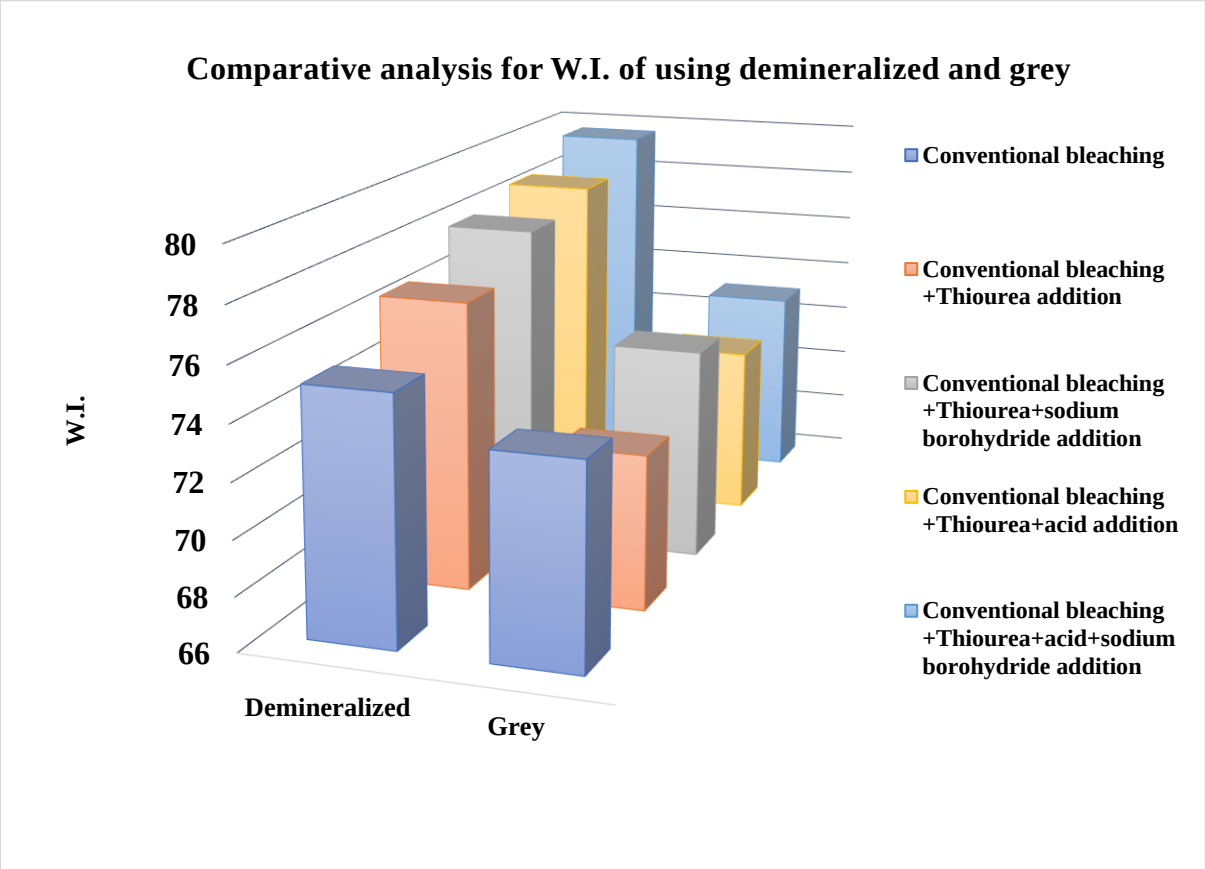


Figure: 3.31 Whiteness for the bleaching demineralized and grey fabric for different processes in 10 g/L hydrogens peroxide oxidative bath and sequential reductive bath

3.5.1.5 FTIR analysis of chemically oxidized and bleached demineralized and grey samples

The characteristic bands of different bleached samples are illustrated in Figure 3.32. These curves represent more weakening bands in the case of sequential oxidative and reductive bleaching than only oxidative bleaching of the characteristic bands of hydroxyl (3288 cm^{-1}), aliphatic CH stretch (2923 and 2885 cm^{-1}), ester carbonyl (1732 cm^{-1}) of aliphatic cutin and wax of cotton cellulose. The weakened and moved-out bands in the region of 1740 ~ 1200 cm^{-1} refer to the loss or some components of cotton impurities that were removed from the cotton ensuring a better pre-treatment effect in conventional scouring bleaching. Further reductive bleaching weakened the region of hydroxyl (3288 cm^{-1}), aliphatic CH stretch (2923 and 2885 cm^{-1})), (500 ~ 1600 cm^{-1}), indicating that the maximum number of constituents of cotton impurities was

eliminated from the cotton and thus increasing more whiteness Furthermore,1650 ~ 1600 cm^{-1}(quinone and OH) which indicates the reduction of the carbonyl group in pigment.

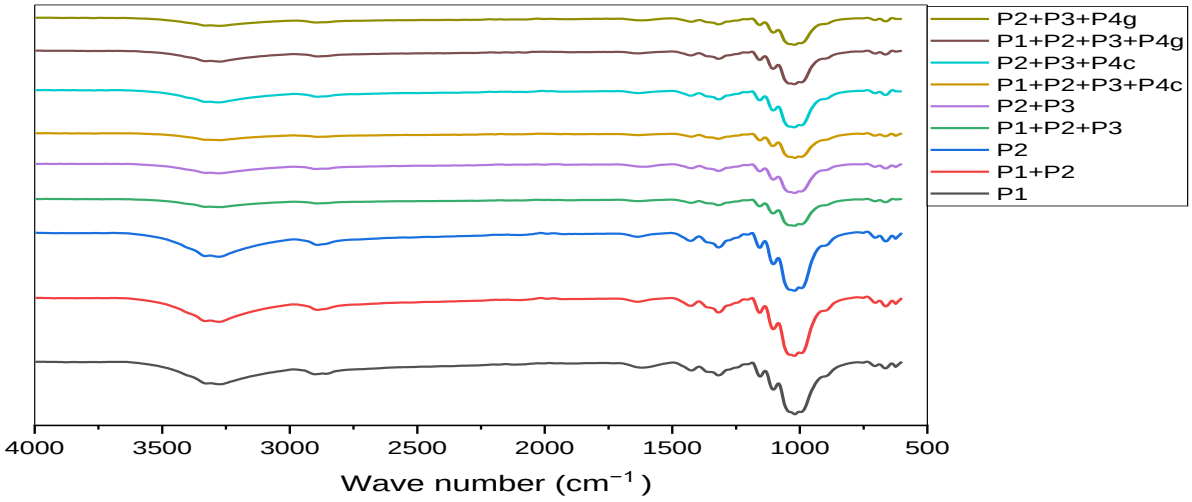


Figure: 3.32 Characteristic bands for the bleaching demineralized and grey fabric for different processes

3.5.1.6 Result of lightness value of the dyed samples of chemical scoured bleached fabric

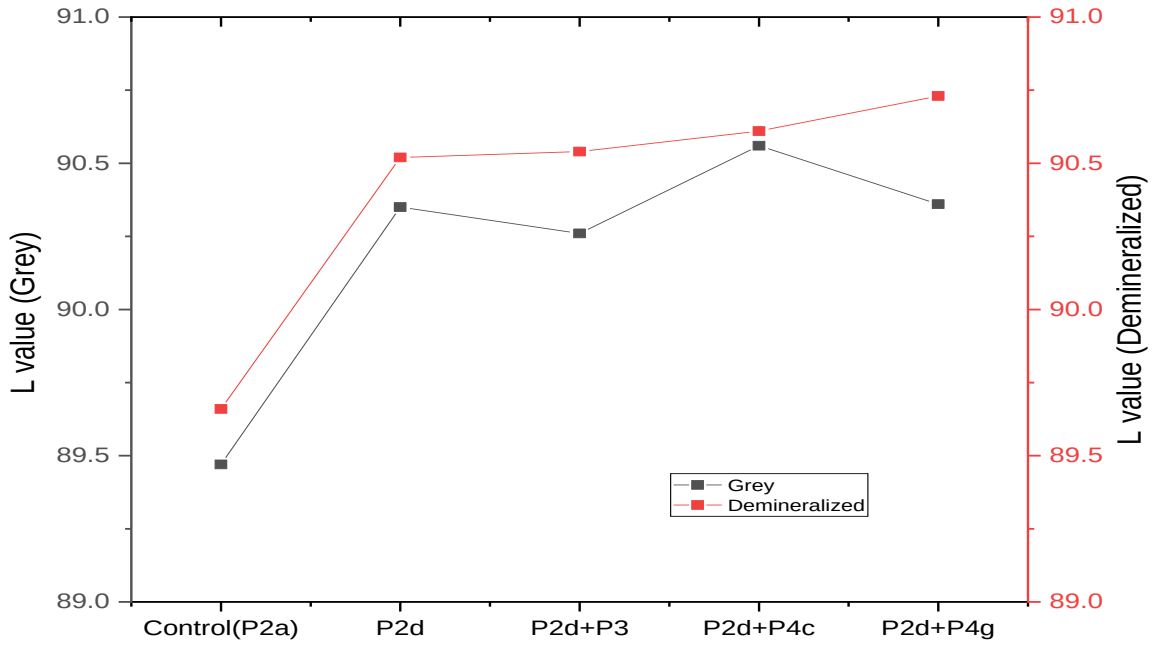


Figure: 3.33 Comparison of lightness for the dyed samples with 0.5% yellow 4GL after bleaching in different processes

After bleaching in different processes samples were dyed and we used the spectrophotometer to measure the lightness (L) of the dyed samples. It was found the higher lightness value (Figure

3.33) and chroma value (Figure 3.34) from dyed fabric which was treated with thiourea and sodium borohydride at different processing conditions by comparing the 4 g/L hydrogen peroxide treated control dyed sample. Lightness value was improved from 89.47 to 90.35, 90.26, 90.56, 90.36 after dyeing P2d, P2d+ P3, P2d+ P4c, and P2d+ P4g processed grey samples respectively, and from 89.66 to 90.52, 90.54, 90.61, 90.73 after dyeing P1+ P2d, P1+ P2d+ P3, P1+ P2d+ P4c, P1+ P2d+ P4g processed demineralized fabric respectively. It is evidence of improvement in the whiteness of the samples for the respective process. More white fabric shows more lightness value after measuring colour value in a spectrophotometer. In the case of chromaticity, it has been shown that chromaticity improved from 60.22 to 61.53, 61.53, 61.55, 61.64 after dyeing P2d, P2d+ P3, P2d+ P4c, and P2d+ P4g processed grey samples respectively, and from 60.38 to 61.85, 61.89, 61.91 62.15 after dyeing P1+ P2d, P1+ P2d+ P3, P1+ P2d+ P4c, P1+ P2d+ P4g processed demineralized fabric respectively which also the evidence of improving the whiteness of bleached fabric as because more whiten ground make high chroma [112].

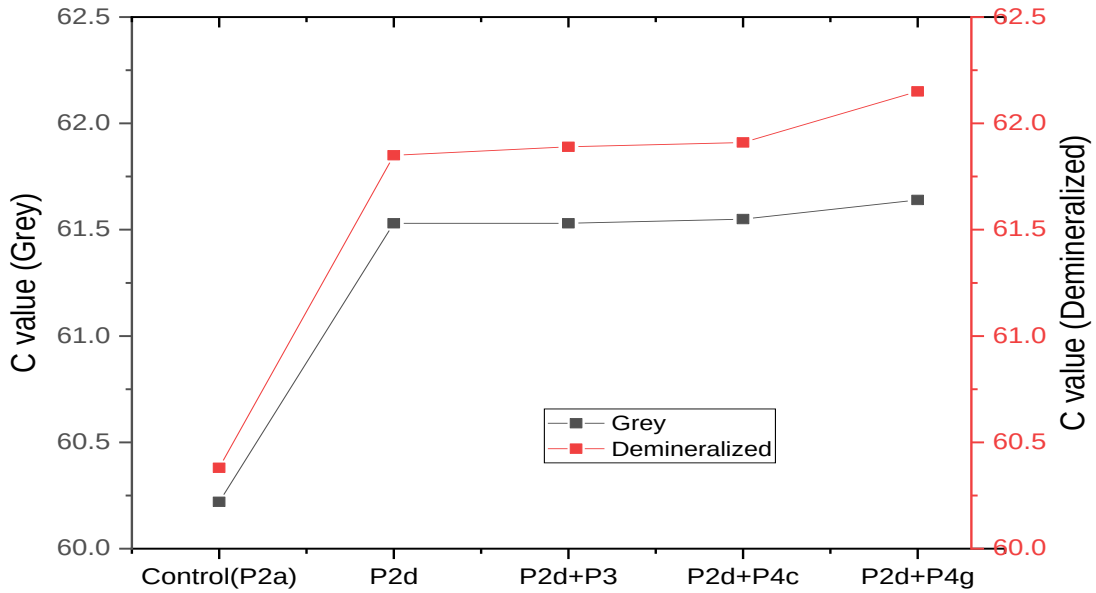


Figure: 3.34 Comparison of chromaticity for the dyed samples with 0.5% yellow 4GL after bleaching in different processes

3.5.2 Bleaching effect on the enzyme-treated fabric

3.5.2.1 Effect of hydrogen peroxide concentration

Significant whiteness difference was not observed among bleaching with CS, BL, and D8 bio-scoured fabric but whiteness was increased with the different concentrations of hydrogen peroxide incorporated in different oxidative baths of 4g/L, 6g/L, 8g/L, and 10 g/L hydrogen peroxide (30%) concentration, this increment was not significantly changed after 10 g/L of hydrogen peroxide. Figure 3.35 shows the amount of hydrogen peroxide incorporated in different bleach baths and the corresponding whiteness index. Decolouration of chromophore in cotton was the reason for increasing whiteness and recombinant enzyme from DH5α species treated scoured sample indicated more whitening effect.

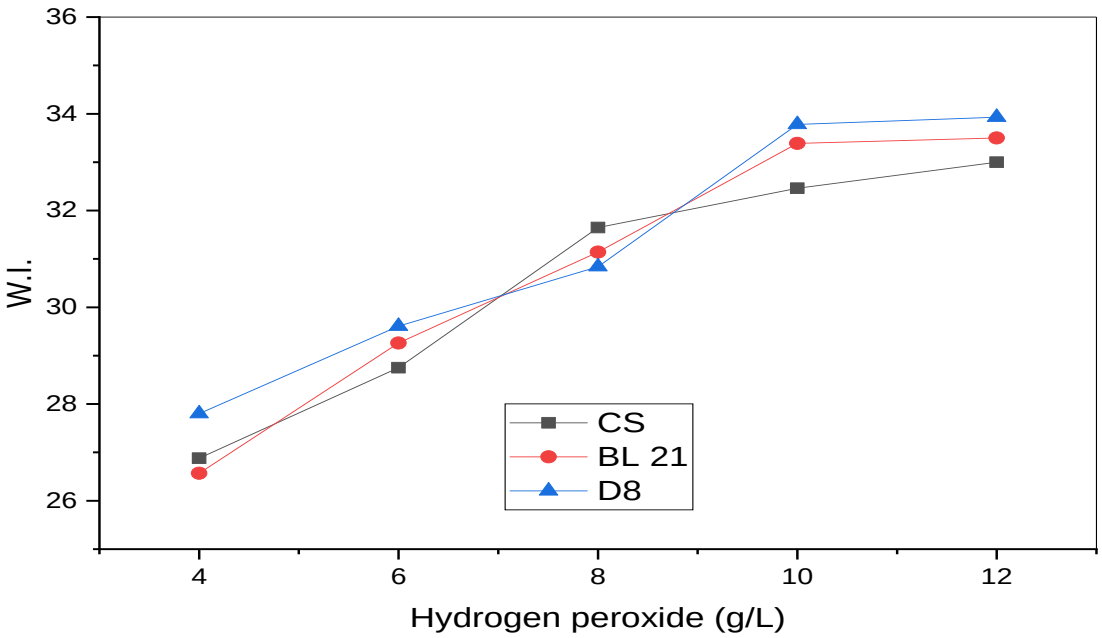


Figure: 3.35 Effect of different concentrations of hydrogen peroxide on the enzyme-treated fabric

3.5.2.2 Effect of hydrogen peroxide and sodium borohydride in a bleach bath

Bleaching with 30% (10 g/L) hydrogen peroxide made the whiteness 36.90, 37.12, 36.88 and with the addition of sodium borohydride, they became 46.44, 47.47, 48.20 for CS, BL, and D8 bio-scoured fabric respectively which is shown in Figure 3.36. It increased more whiteness when 50% (10 g/L) hydrogen peroxide was applied instead of 30% hydrogen peroxide and whiteness further improved with the addition of sodium borohydride activator in the oxidative hydrogen peroxide bleaching bath to convert the reductive bath after completing the oxidative bleaching.

Bleaching with 50% (10 g/L) hydrogen peroxide showed the whiteness 51.50, 50.38, 50.1 and with the addition of sodium borohydride, they become 61.15, 58.14, 59.72. for CS, BL, and D8 bio-scoured fabric respectively in Figure 3.37. Bleaching of conventional chemical-scoured fabric was trailed for 50% (10 g/L) hydrogen peroxide and the whiteness index was approximately 76. In industrial practice, 4 g/L hydrogen peroxide is generally used for chemical bleaching[48]. CSBP4 gives a whiteness index of 64 which is nearer to our bio-scoured bleaching sample. For special cases, a double bleaching process is followed to get extra whiteness, in this respect we can follow CSBP10. The choice between 30% and 50% hydrogen peroxide depends on the specific requirements and characteristics of the fabric. The application of sodium borohydride enhances whiteness, suggesting its effectiveness as an activator in oxidative bleaching.

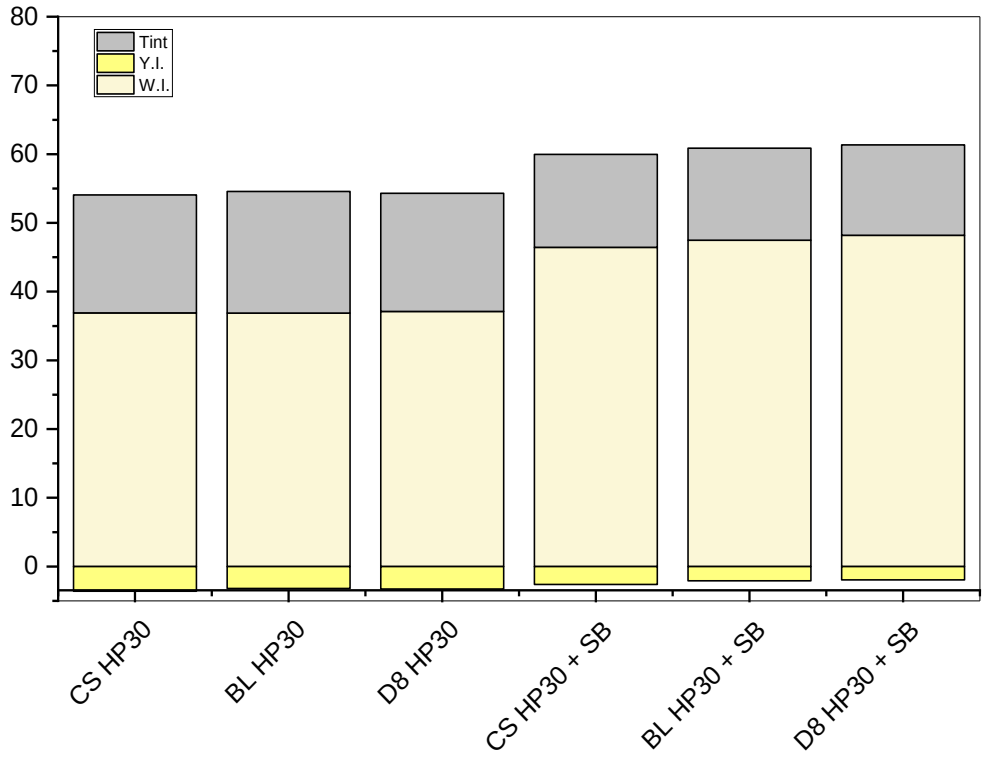


Figure: 3.36 Effect of hydrogen peroxide (30%) and $NaBH_4$ on the enzyme-treated fabric

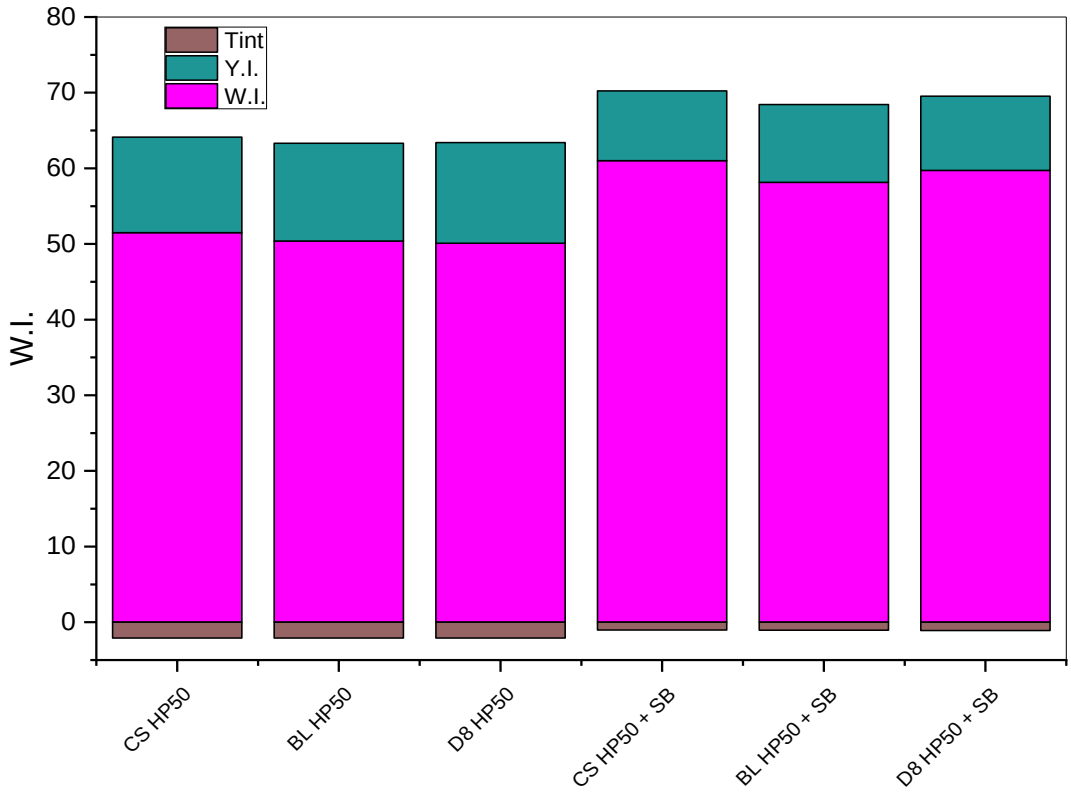


Figure: 3.37 Effect of hydrogen peroxide (50%) and $NaBH_4$ on the enzyme-treated fabric

3.2.2.3 Bursting strength of the bio-scoured and bleached fabric

The presence of non-cellulosic substances in scoured fibres provides some protection against cellulase attack, preserving the hydrophobic nature. However, when applied to clean, unprotected cellulose fibres after scouring, the enzyme can lead to substantial degradation of cellulose chains, affecting the overall structure of the fibres[102].Enzyme-treated CS, BL, and D8 samples were bleached with 50% hydrogen peroxide bleaching agent and the oxidative bleaching process caused some cellulose degradation which caused the lowering of fabric strength from the bio-scoured fabric. Further addition of sodium borohydride also reduces fabric strength (Figure 3.38) due to chemical structural changes in the polymer system. It may degrade and break down the bond in the cellulose structure. Bursting strength was lower for D8 enzyme than BL21 and CS. Deterioration of strength was higher in the D8 recombinant enzyme with sodium borohydride application into the oxidative bath than the only oxidative bleaching.

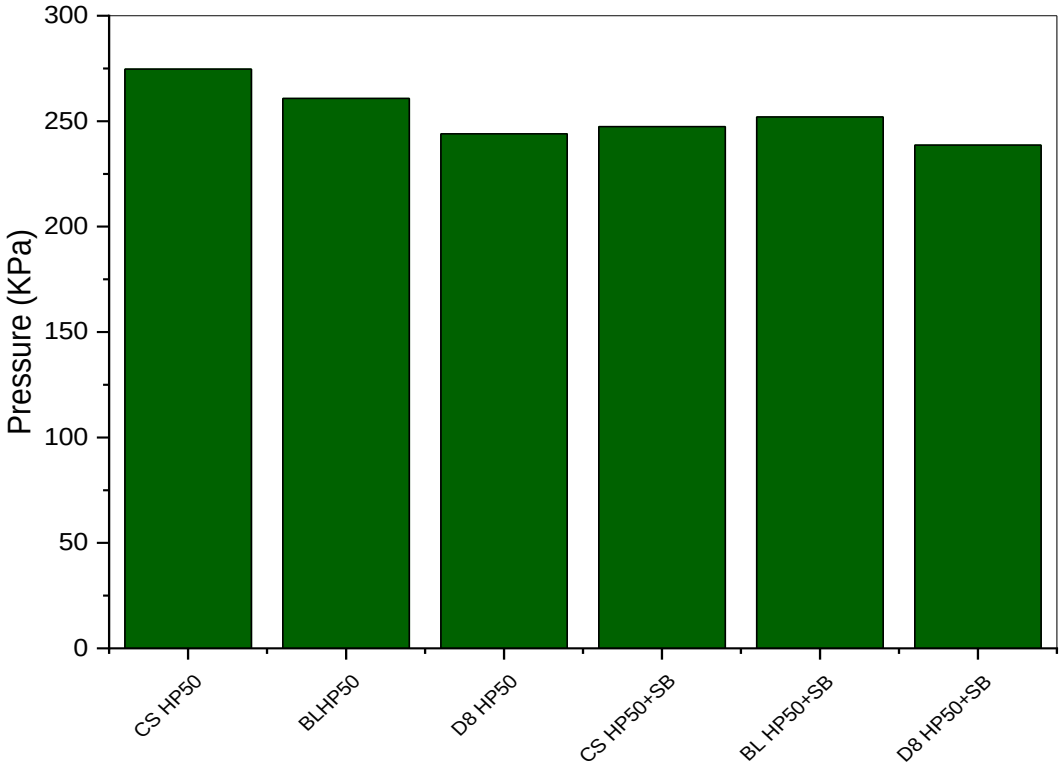


Figure 3.38 Bursting strength of the different bio-scoured and bleached fabric

3.5.2.4 Scanning Electron Microscopy for the bio-scoured and bleached fabric

While sodium hydroxide and hydrogen peroxide play roles in the bleaching process, the combination of the two is not necessarily known for causing the highest level of deterioration on a molecular level. The effectiveness of the bleaching process depends on various factors, including concentration, temperature, and duration of treatment. Bio scouring and bleaching are two important processes in the treatment of cotton fibres to enhance their quality and prepare them for various applications[113].Oxidative bleaching process of CS HP50, BL HP50, and D8 HP50 causes more cellulose degradation from the bio-scoured fabric and micro creases appear on the fabric structure due to oxidative bleaching. Further addition of sodium borohydride also caused rougher in the fabric surface due to the reductive bleaching action in the polymer system. D8 enzyme-treated fabric showed prominence in concern of crease and roughness of surface among all the treated fabrics (Figure 3.39).

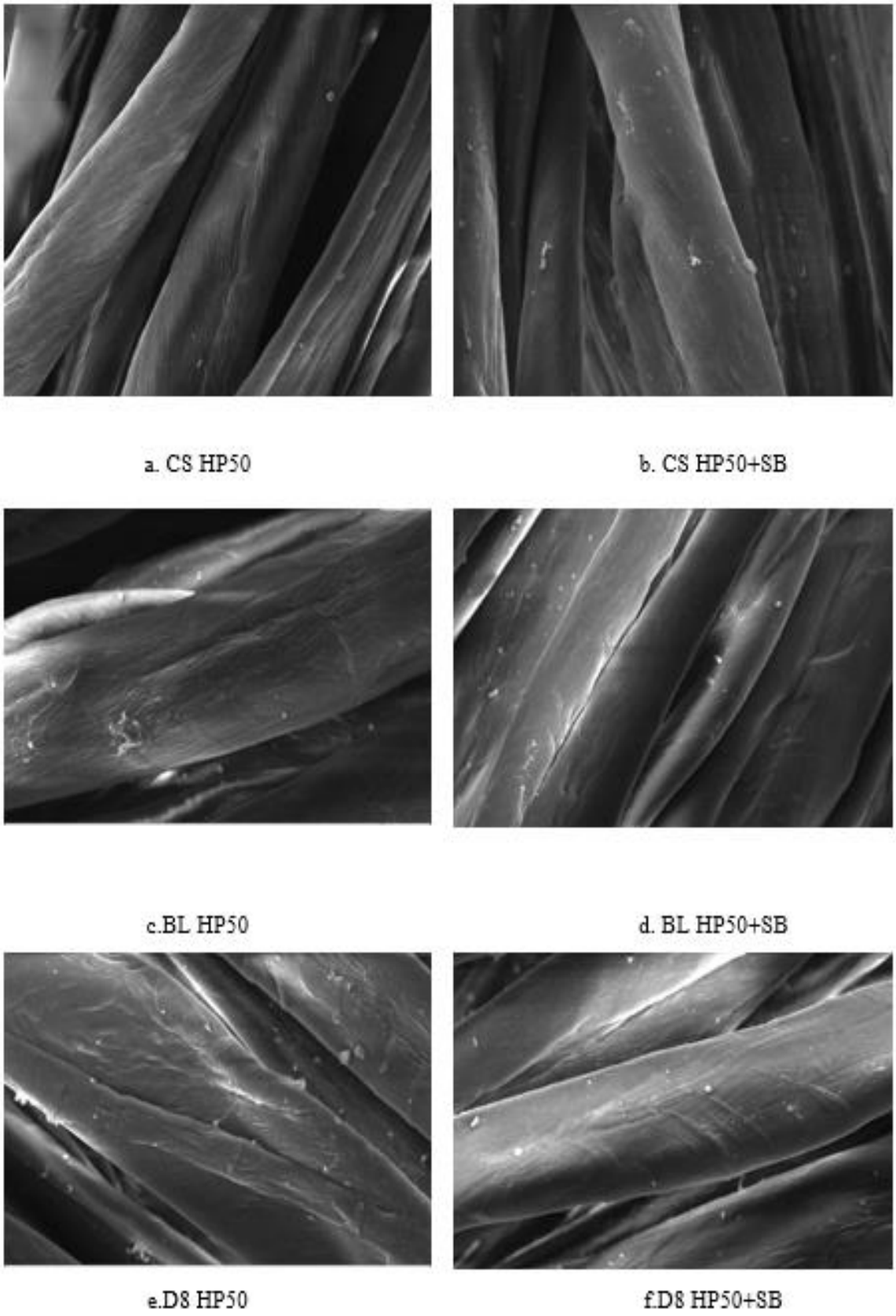


Figure: 3.39 SEM micrograph for the bio-scoured and bleached cotton knit fabric in 5000 ×(2μm), a) HP50 b) HP50+SB c) BL HP50 d) BL HP50+SB e) D8 HP50+SB f) D8 HP50+SB

3.5.2.5 Assessment of CIE whiteness (L*) of the bio-scoured and bleached fabric

In the case of the pretreated fabric, investigation is commonly required on the whiteness test. Full white fabric or sharp whiteness values are not the requirement for dark colour shade matching. The resultant CIE L value denotes the lightness grading of the dyed fabric. Thus, the value is enough for the interpretation of optimum whiteness of the dyed textile. Samples treated with hydrogen peroxide and sodium borohydride exhibit values nearer to the control samples. In usual practice, bio-scoured fabric is not directly used for light colouration. Bio-scouring cannot remove black dot marks in the fabric and some patches are also present in the bio-scoured fabric, for this reason, some unevenness of colouration may arise in dyeing with bio-scoured fabric (Figure A42) which cannot be visible in dark colouration (Figure A43). After dyeing, the chemically scoured bleached dyed sample showed a lightness value of 25.61 for the dark navy and 89.48 for the light-yellow colour and the lightness values were 24.51, 24.33, 24.41 for the dark navy colour, and 87.84, 87.22, and 87.75 for the light-yellow colour of bio-scoured natural, BL21, and DH5α bleached fabric with only hydrogen peroxide, on the other hand, the lightness

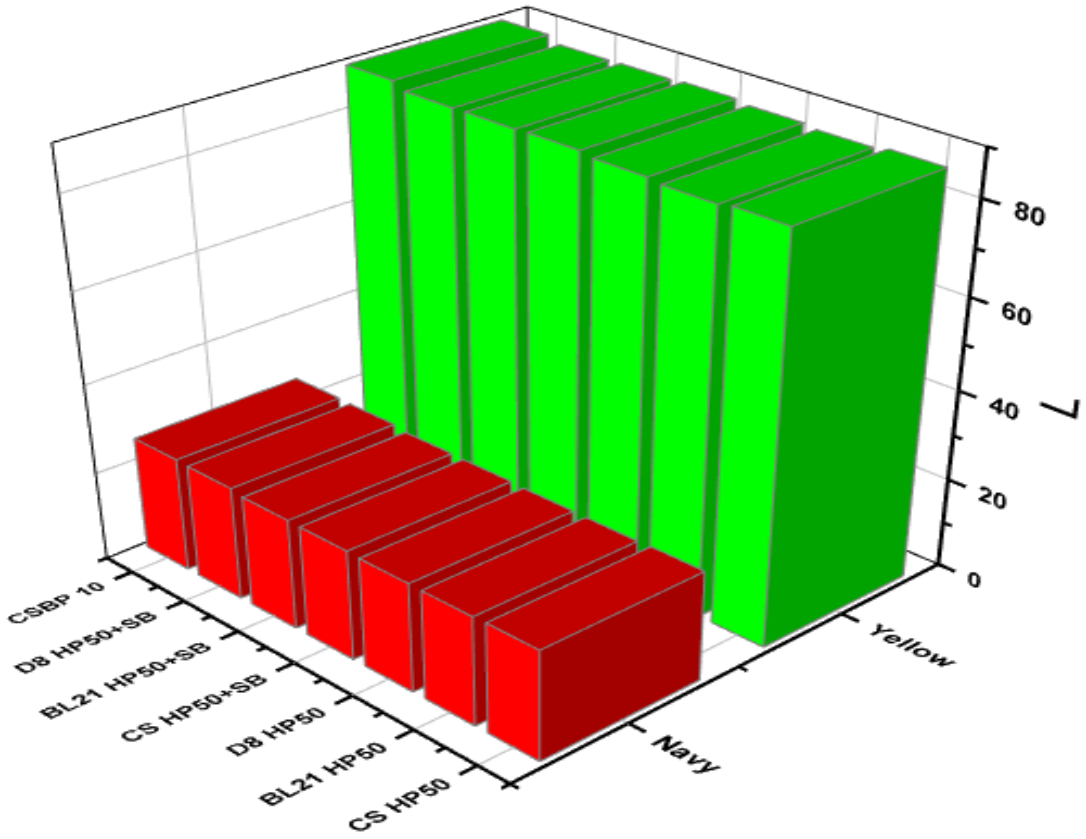


Figure: 3.40 L value of the dyed sample for bio-scoured and bleached fabric of HP50, HP50+SB, BL HP50, BL HP50+SB, D8 HP50+SB, D8 HP50+SB

values were 24.63, 24.75, and 26.40 for the dark navy colour, and 88.37, 88.14, and 88.09 for the light-yellow colour of bio-scoured natural, BL21, and DH5α bleached fabric with hydrogen peroxide and sodium borohydride (Figure A34, A35, A36, A37, A38, A39, A40, A41). The application of sodium borohydride with hydrogen peroxide improved the lightness value more than only an oxidative bath. They showed an almost similar lightness value with the comparison of chemically bleached dyed samples for the same shade (Figure 3.40). No black spot or shade variation was visible in any sample (Figure A 46 and Figure A 47). So, applying oxidative and reductive bleaching with this bio-scoured sample can be used for both light and deep shade dyeing.

3.5.2.6 Fastness properties to wash and rubbing of the reactive dyed of bio-scoured and bleached fabric

The colour fastness of a textile dyed sample can be defined as a textile's colour's resistance to fading[114]. Colour fastness is the property of colourant and it is directly related to the binding force between the dye and the fibre[115].The colour fastness has an impact on the processing method and choice of processing ingredients [48].The fastness properties to washing as well as rubbing of reactive dyed samples were excellent in the case of yellow 4GL(Table 3.9) (in every case the rating was 4 to 4/5, on a scale of 5 from Figure A20, A21, A22, A23, A24, A25 and A26) and Navy RGB colour has excellent wash fastness with moderate wet rubbing fastness from Figure A27, A28, A29, A30, A31, A32 and A33 (Table 3.10) which was similar for the conventionally pretreated dyed samples. Cotton fibre interaction with reactive dye may not be interrupted by bio scouring and combining hydrogen peroxide with sodium borohydride bleaching treatment i.e. dye fibre interaction with dye remained the same as the chemically pretreated dyed sample. In this work, low-temperature bio-scouring was the key aspect. It is believed that fastness properties mainly depend on the precision of the dyeing process[116]. The reactive dyeing condition remained the same for all of the cases, and thus, the bio-scouring conditions had less scope to affect the fastness properties of the dyed samples which were assessed.

Table 3.9 The colourfastness of the different dyed samples with Yellow 4GL

Sample (dyed with 0.5%Yellow 4GL)	Wash fastness (1-5)							Rubbing fastness (1 - 5)	
	Colour change	Colour staining						Dry	Wet
		Acetate	Cotton	Nylon	Polyester	Acrylic	Wool		
CS HP50	4 -5	4 -5	4 -5	4 -5	4 -5	4 -5	4 -5	5	4
BL21 HP50	4 -5	4 -5	4 -5	4 -5	4 -5	4 -5	4-5	5	4
D8 HP50	4 -5	4 -5	4 -5	4 -5	4 -5	4 -5	4-5	5	4
CS HP50+SB	4 -5	4 -5	4 -5	4 -5	4 -5	4 -5	4-5	5	4
BL21 HP50+SB	4 -5	4 -5	4	4 -5	4 -5	4 -5	4-5	5	4
D8 HP50+SB	4 -5	4 -5	4-5	4 -5	4 -5	4 -5	4-5	5	4
CSBP10	4	4 -5	4 -5	4 -5	4 -5	4 -5	4-5	5	4

Table 3.10 The colourfastness of the different dyed samples with Navy blue RGB

Sample (dyed with 2%Navy blue RGB)	Wash fastness (1-5)							Rubbing fastness (1 -5)	
	Colour change	Colour staining						Dry	Wet
		Acetate	Cotton	Nylon	Polyester	Acrylic	Wool		
CS HP50	4 -5	4 -5	3	4 -5	4 -5	4 -5	4	5	3
BL21 HP50	4 -5	4 -5	4	4 -5	4 -5	4 -5	4-5	5	3
D8 HP50	4 -5	4 -5	4	4 -5	4 -5	4 -5	4-5	5	3
CS HP50+SB	4 -5	4 -5	4	4 -5	4 -5	4 -5	4-5	5	3
BL21 HP50+SB	4 -5	4 -5	4	4 -5	4 -5	4 -5	4-5	5	3
D8 HP50+SB	4 -5	4 -5	4-5	4 -5	4 -5	4 -5	4-5	5	3
CSBP10	4 -5	4	4	4 -5	4 -5	4 -5	4-5	5	3

Chapter 4

Conclusion

Chapter 4

Conclusion

The main concern of research was to develop a novel wet process technique with a low-temperature assisted bio-scouring process and improvement of the whiteness effect of bio-scoured fabric with dyeing acceptance. A recombinant pectinase enzyme was used to achieve this goal as well as with a natural pectinase enzyme. Ten gene fragments were synthesized commercially for recombinant enzyme production and ligated in a PCR machine. The gene length confirmation was carried out by gel electrophoresis test where the ligated gene length was between 1000 to 1500 base pairs which is in the expected region (1143 bps), and pGLO plasmid was successfully isolated and analyzed using gel electrophoresis where DNA band lay between 5000 and 6000 bps. Successful transformation of the fungal pectinase gene into bacterial plasmid DNA was achieved. Two types of bacterial species, DH5α and BL21, were used for gene transformation, and bacterial growth was assessed in ampicillin-containing LB agar media from the positive response with the white colour of GFP for the transformed gene in pGLO plasmid under UV ray. The molecular weight of the pectinase enzyme of different colonies and pGLO plasmid was investigated with the SDS PAGE run and matched with their reference values of approximately 37 and 26 respectively. The bacterial sequence with known *Escherichia coli* was confirmed for recombinant enzyme and *Burkholderia cepacia*, a previously unknown bacterial species, exhibited pectinolytic activity for natural pectinase. Qualitative analysis for recombinant and natural pectinase has proved the pectinolytic activity by breaking the pectin substances and losing their stained colour for the iodine and Congo red test. A quantitative DNS test was carried out by observing the change in the yellow DNS solution to reddish-orange which indicates the ability to produce reducing ends of polygalacturonic acid and the amount of released reducing ends was 1.4 and 1.1 for species DH5α and BL21 respectively. Bio-scouring of cotton knit fabric was performed with both recombinant and natural pectinase enzymes. Both enzymes operated at 37°C for bio scouring, representing an energy-saving process compared to commercial enzymes. In the wicking test, transformed bacteria DH5α and BL21 extract-treated fabric had travelled more than untransformed ones, and the wicking rate of recombinant DH5α, BL21, and natural enzymes in cotton knit fabric scouring was found 85 mm, 65 mm, and 55 mm after ten minutes. On the contrary, it was taken 3.4 sec, 4 sec, 4.5 sec, more than 5 minutes, 5 minutes, and 3.5 minutes for the treated fabric with D8 enzyme, CS enzyme, BL enzyme, without enzyme, untransformed bacterial solution and BS enzyme respectively to reach 4 cm height. The grey fabric was

completely untreated and could not cross more than 1 cm. So, the successful production and application of recombinant and natural pectinase enzymes in cotton bio-scouring has been achieved which was one of the objectives. FTIR analysis, SEM image, and bursting strength also proved the removal of pectin is evidence of enzyme activity. Enzymatic activity was much more visible in the case of recombinant enzyme from DH5α bacterial species than recombinant enzyme from BL21 bacterial species and CS enzyme from natural source. Bio-scoured fabric contains more tint than chemical-scoured and bleached fabric. Chemical scouring and combined scouring bleaching processes eliminate much more tint due to the alkali and alkali with hydrogen peroxide at higher temperatures respectively. For that reason, chemical scouring and combined scouring bleaching processed samples showed less K/S values as compared to bio-scoured samples. The colour strength was 14.5, 14.7, 14.8, and 14.3 after dyeing with recombinant DH5α, BL21, and natural and commercial enzyme-treated fabric respectively. These low-temperature bio-scoured fabrics were successfully dyed for dark colour and were closer to colour strength by comparing commercial enzymes. So, the newly developed enzyme has the dyeing acceptance for dark colouration.

Sequential oxidative and reductive bleaching was applied for the chemical-scoured fabric to check the feasibility of increasing whiteness. It obtained an excellent whitening effect than the conventional bleaching process. For reductive bleaching, sodium borohydride with thiourea application showed an increment of negative redox potential (-897 mV) from only with thiourea (-204 mV) and caused more whitening after oxidative bleaching (+39 mV). Findings for oxidative bleaching with hydrogen peroxide of 10g/L restrict further increment of whitening and up to 5 g/L sodium borohydride increases whiteness after oxidative bleaching. Finally, it was limited to 3 g/L sodium borohydride for bio-scoured fabric to restrict foam formation with easy handling.

In the chemical scouring and chemical bleaching, the whiteness index was improved from approximately 64 to a superior whiteness index of 80 in acid addition and 78 in without acid addition conditions by using thiourea with sodium borohydride in the reductive bleaching process after oxidative bleaching with hydrogen peroxide. Bursting strength was reduced for the sequential bleaching process. The characteristic bands of different bleached represent more weakening bands in the case of sequential oxidative and reductive bleaching than only oxidative bleaching which indicates improvement of whiteness of the fabric. The dyed sample using this

highly bleached fabric showed higher lightness and chromaticity value for demineralized than grey fabric compared to conventional processed demineralized and grey samples.

In the bio scouring and chemical bleaching, bleaching with 30% (10 g/L) hydrogen peroxide made the whiteness 36.90, 37.12, 36.88 and with the addition of sodium borohydride, they became 46.44, 47.47, 48.20 and bleaching with 50% (10 g/L) hydrogen peroxide showed the whiteness 51.50, 50.38, 50.1 and with the addition of sodium borohydride, they became 61.15, 58.14, 59.72 with the pectinase enzyme from the source of natural, recombinant BL21, and recombinant DH5α treated bio-scoured fabric respectively. It increased more whiteness when 50% (10 g/L) hydrogen peroxide was applied instead of 30% hydrogen peroxide and whiteness further improved with the addition of sodium borohydride activator in the oxidative hydrogen peroxide bleaching bath to convert the reductive bath after completing the oxidative bleaching. Bleaching of conventional chemical-scoured fabric was trailed for 50% (10 g/L) hydrogen peroxide and the whiteness index was approximately 75. The conventional chemical-scoured fabric with 50% (4 g/L) hydrogen peroxide, industrial practice, gives a whiteness index of 64 which is nearer to our bleached sample from bio-scoured fabric. For special cases, a double bleaching process is followed in the industry to get extra whiteness, in this respect we can follow CSBP10. The addition of sodium borohydride in the oxidative bath enhances whiteness, suggesting its effectiveness as an activator in oxidative bleaching. The oxidative bleaching process causes more cellulose degradation from the bio-scoured fabric and micro creases appear on the fabric structure due to oxidative bleaching. Further addition of sodium borohydride also caused a mild roughness in the fabric structure in both bleaching processes. After dyeing with dark navy and light-yellow colour, it showed an almost similar lightness value of enzyme treated and chemical bleached dyed fabric with the comparison of the chemical scoured and chemical bleached dyed fabric. The value was 24.63, 24.75, 26.40, 25.61 for dark navy colour and 88.37, 88.14, 88.09, 89.48 for the light-yellow shade of the sample from bleaching with 50% H_2O_2 and sodium borohydride with natural enzyme, 50% H_2O_2 and sodium borohydride with recombinant BL21 enzyme, 50% H_2O_2 and sodium borohydride with recombinant DH5α enzyme treated bio-scoured fabric and chemical scoured bleached fabric with 10 g/L H_2O_2 respectively. Black spot and shade variation was not visible in any sample. So, the bio-scoured sample with oxidative and reductive bleaching can be used for light colour dyeing as well as dark colour.

In summary, the thesis explores the production, characterization, and application of natural and recombinant pectinase enzymes for bio-scouring in the textile industry. The study includes the comparison of recombinant and natural pectinase enzyme activity in low-temperature bio-scouring process, enzyme-treated fabric properties, and the performance of bleaching on chemical-scoured and bio-scoured fabric, highlighting the development of novel energy-efficient textile processing.

4.1 Future Recommendations

a) All the clones of the transformed bacterial plates need to be repeatedly applied to produce bacteria for the pectinase enzyme production, so the entire capacity of all clones in the transformed bacterial plate can be assessed with the increasing or decreasing pectinolytic activity from the achieved clones.

b) Ligated genes can be transformed into another type of bacterial species so that new qualitative and quantitative pectinolytic activity can be achieved for the production of the pectinase enzyme.

c) Enzymatic bleaching with laccase enzyme after bio-scouring with the developed pectinase enzymes can change the conventional textile wet preparatory process in an eco-friendly way and natural dye can be useful for colouration to make a whole echo-friendly process.

d) All types of cotton can undergo trials for the novel developed wet process with their dyeing performance.

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Appendix

Appendix

List of Figures

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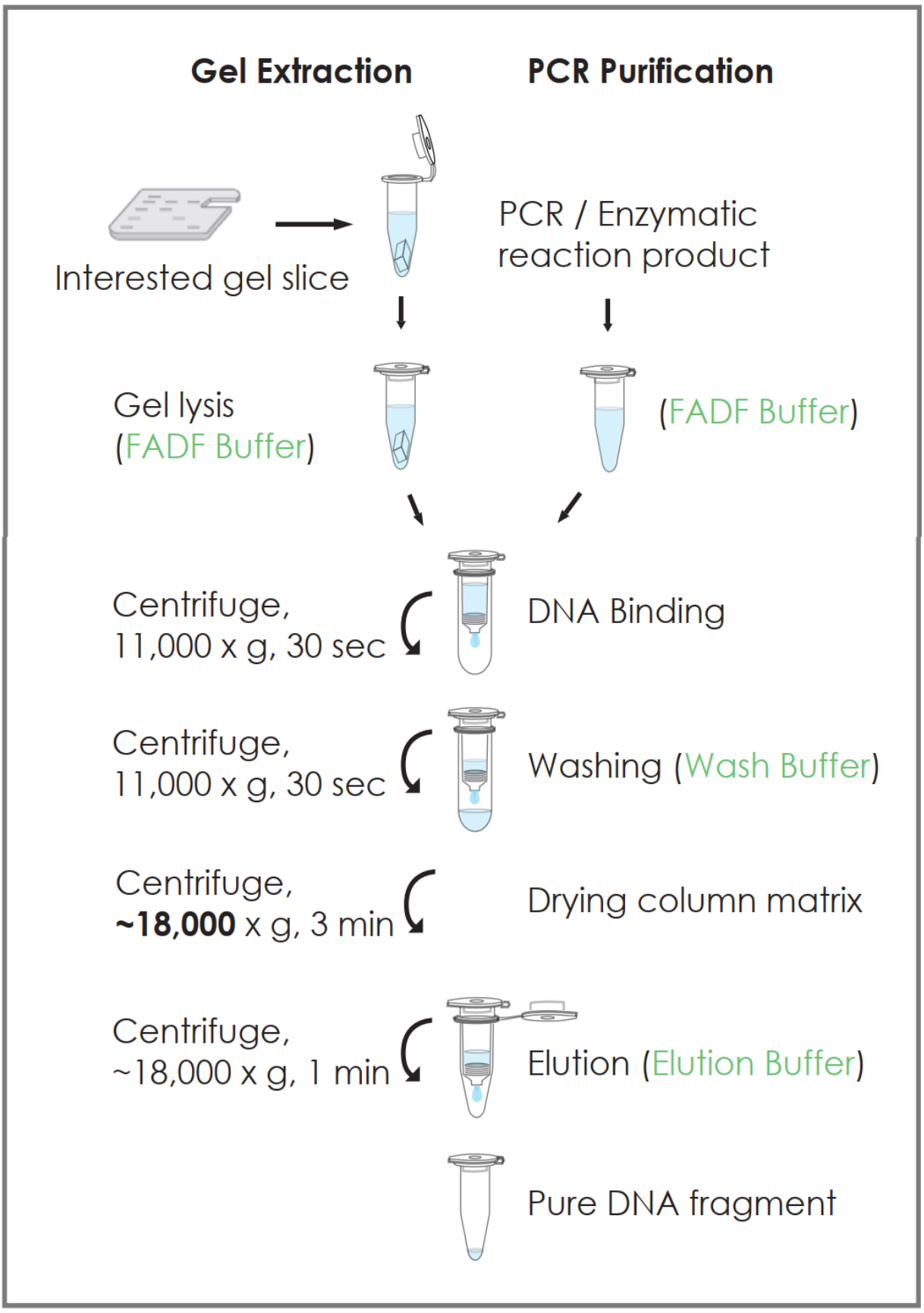


Figure A1 Gel/PCR product purification procedure using the FavorPrep^{TM} mini kit

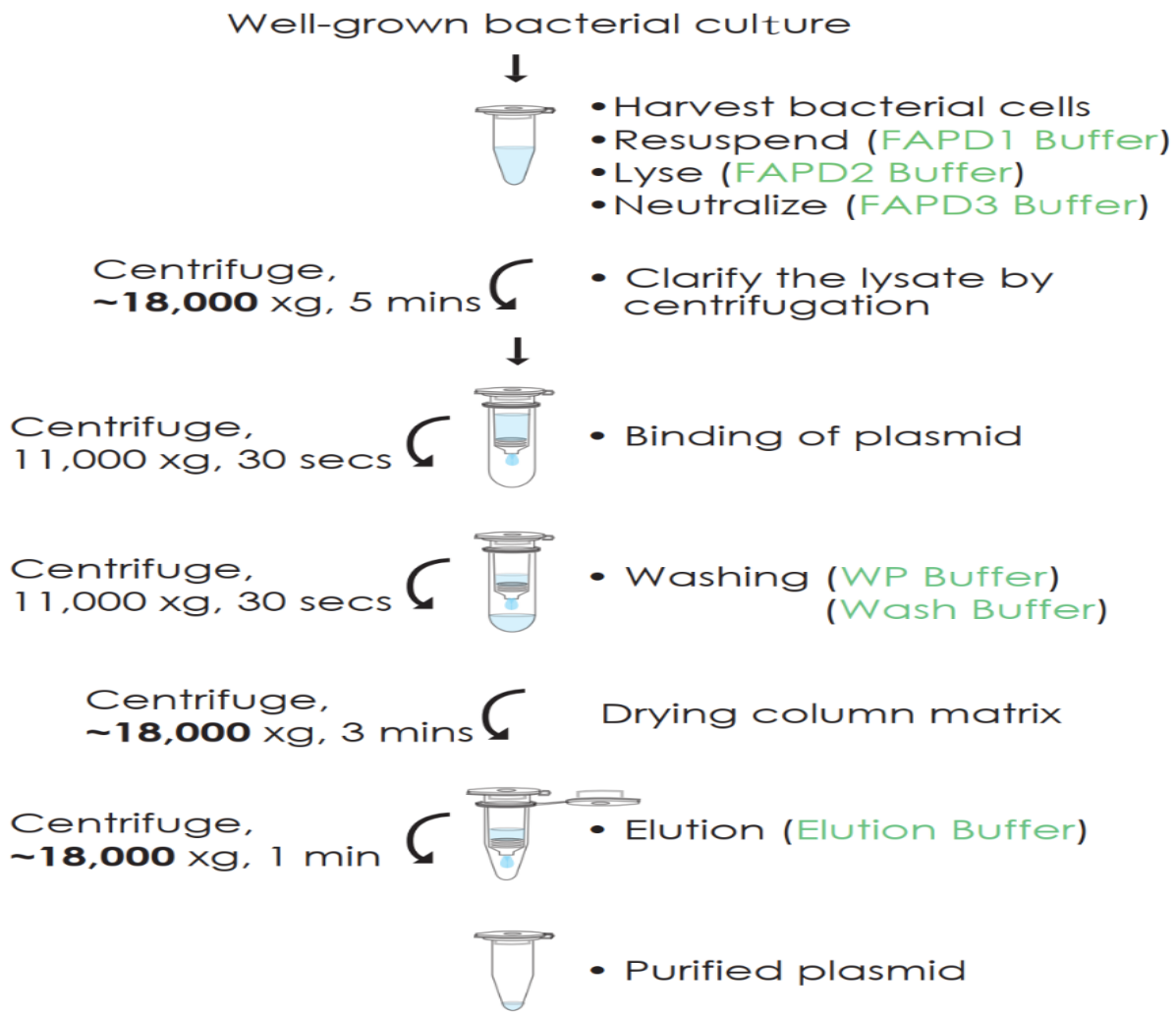
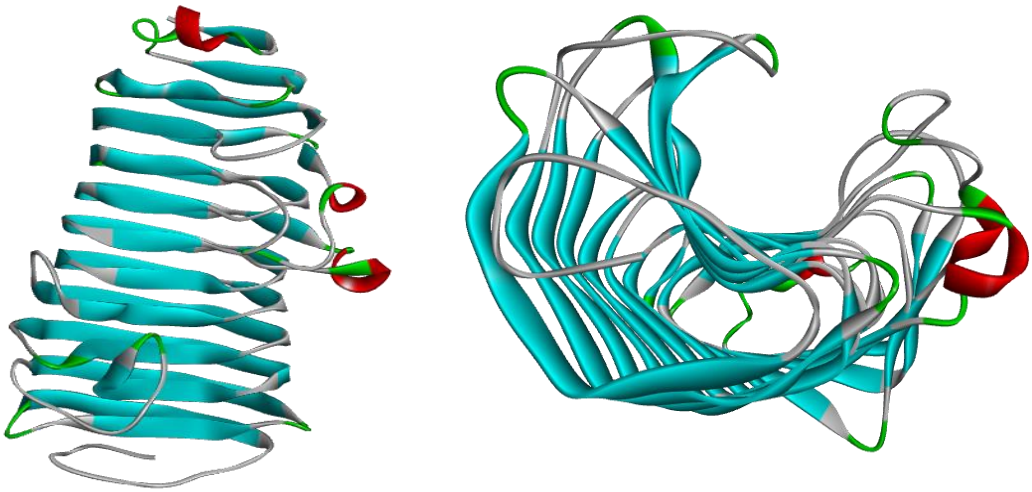


Figure: A2 Plasmid isolation procedure using FavorPrep Plasmid isolation kit. Image taken from FavorPrep™ kit protocols



(a) (b)

Figure: A3 Predicted PHYRE2 protein structure. (a) Viewed with N-terminal at the top and C-terminal at the bottom. (b) Viewed from the point of C-terminal

In the Figure A3(a) shows that a beta-sheet pattern is predominantly responsible for the structure. In Figure A3(b), the structure shows a groove on the upper-right side. the default settings in the PHYRE2 website were kept to make the predicted model, which was visualized using the Discovery Studio software.

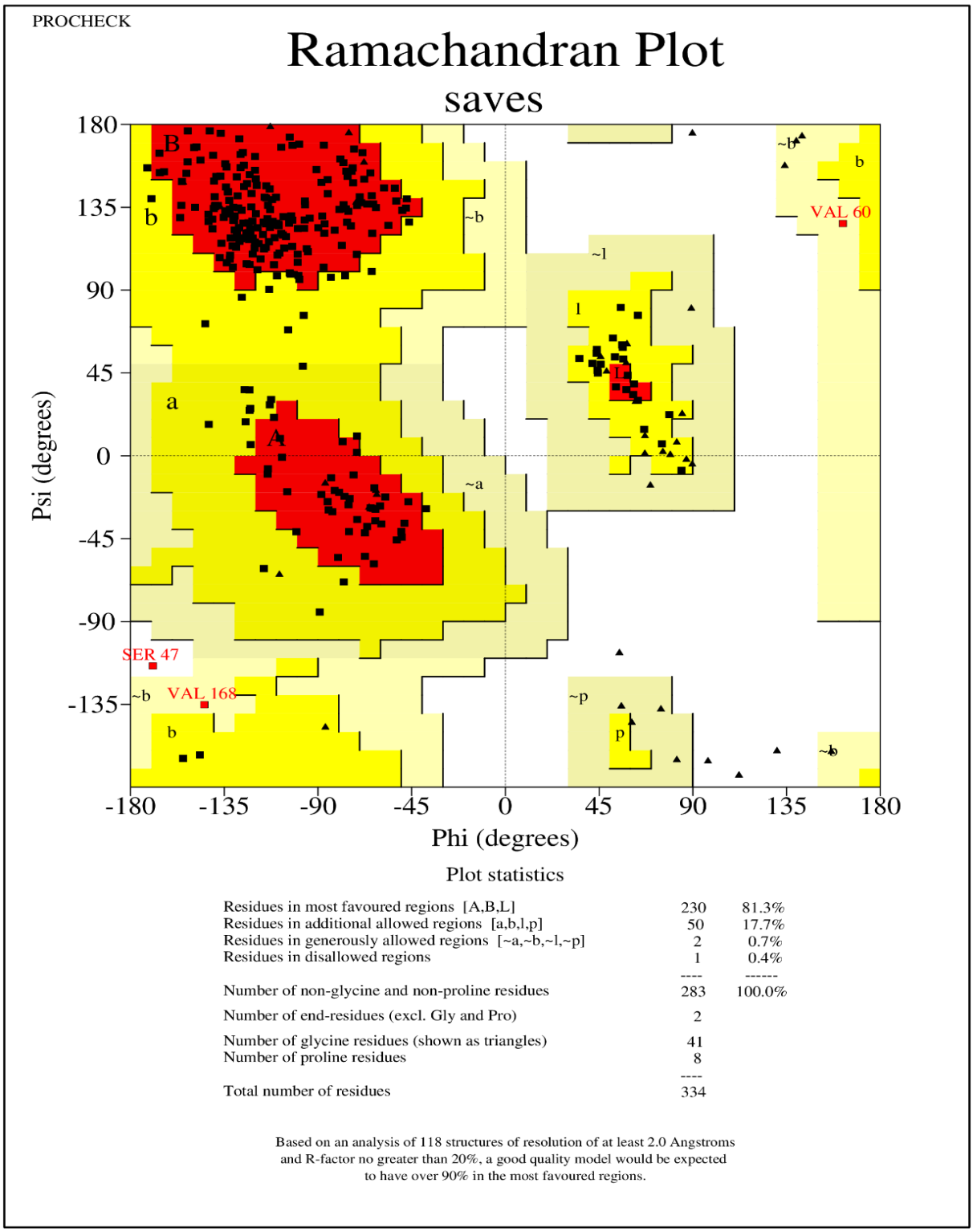
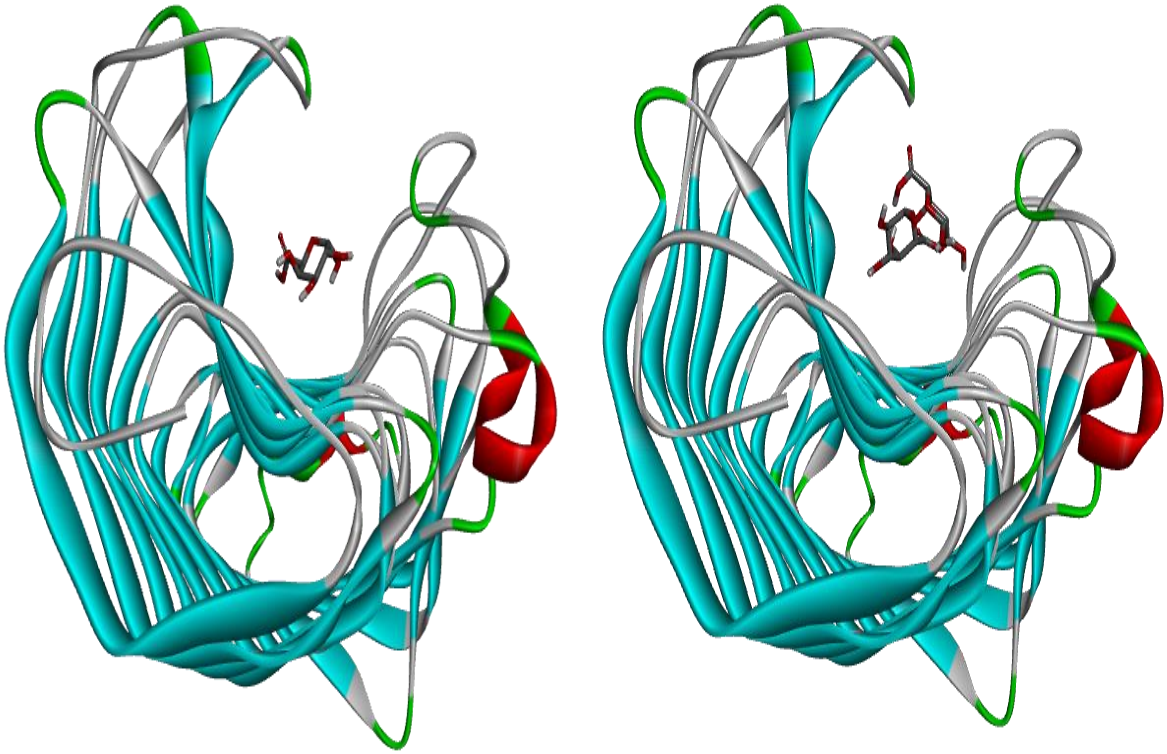


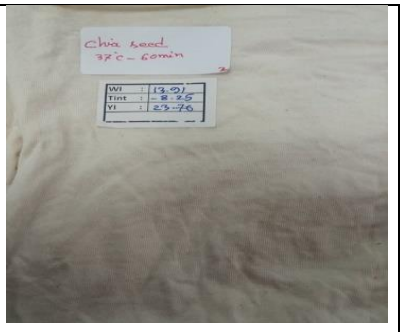
Figure: A4 Ramachandran plot for PHYRE2 output of predicted protein structure



(a) (b)

Figure A5 : Docked (a) Alpha-D-galacturonic acid, (b) Digalacturonic acid to the PHYRE2 protein prediction structure

Figure A5 exhibits, after performing molecular docking analysis using AutoDock Vina, the output file contained the docked site of the ligand (Alpha-D-galacturonic acid or Digalacturonic acid), which can then be visualized after opening in the Discovery Studio software in conjunction with the predicted protein .The monomer alpha-D-glacturonic acid showed an affinity of -6.0 kcal/mol and the digalacturonic acid showed an affinity of -6.9 kcal/mol. This can indicate that more groups of the actual substrate, polygalacturonic acid, may interact with higher affinity due to the presence of many groups filling the grooves of the protein.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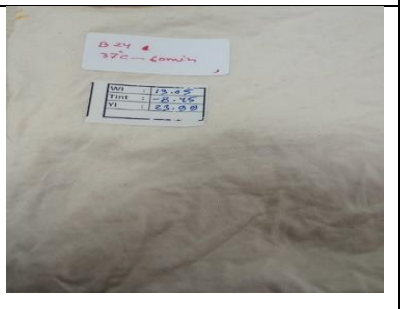
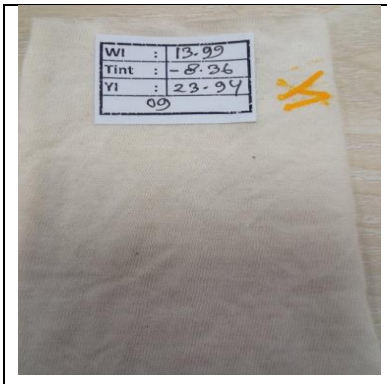
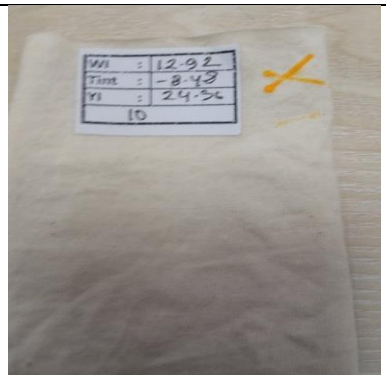
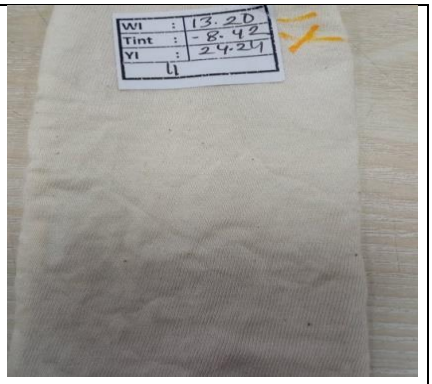
d	E	F
		
g	H	I
		
j	K	L

Figure: A6 Untreated and enzyme-treated cotton knit fabric a) Grey b) WE37 c) CS1 d) CS1 e) CS2 f) CS3 g) CS4 h) CS1 i) CS 2 j) UT 1 k) UT 2 l) BL 1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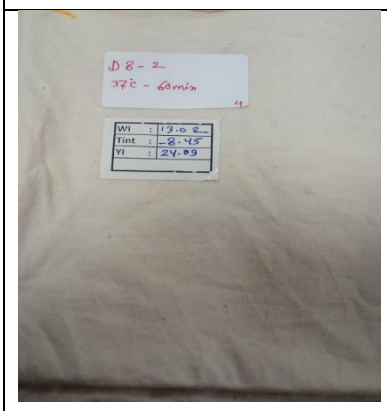		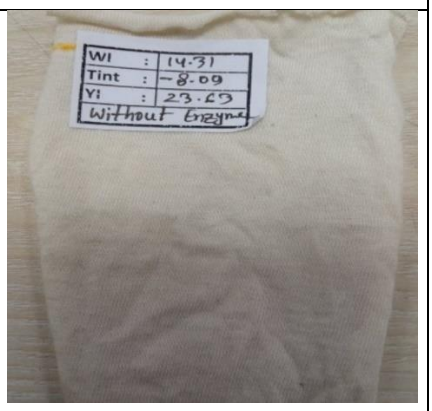
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	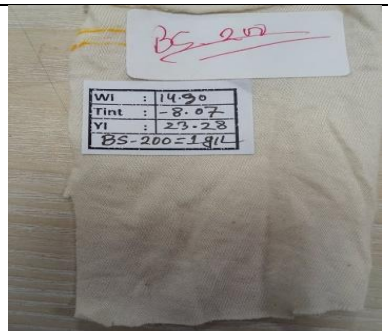	
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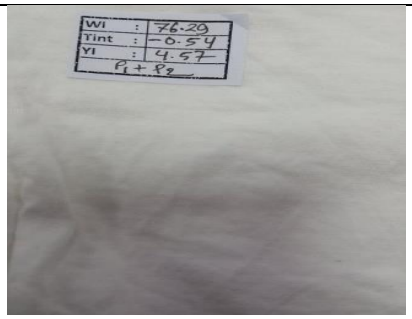
		
w	X	y

Figure: A7 Enzyme-treated cotton knit fabric n) BL 1 o) BL 2 p) D8 1 q) D8 2 r) WE 37 s) WE 60 t) WE 60 u) BS 60 v) BS 37 w) CSP x) CSP y) CSBP 10

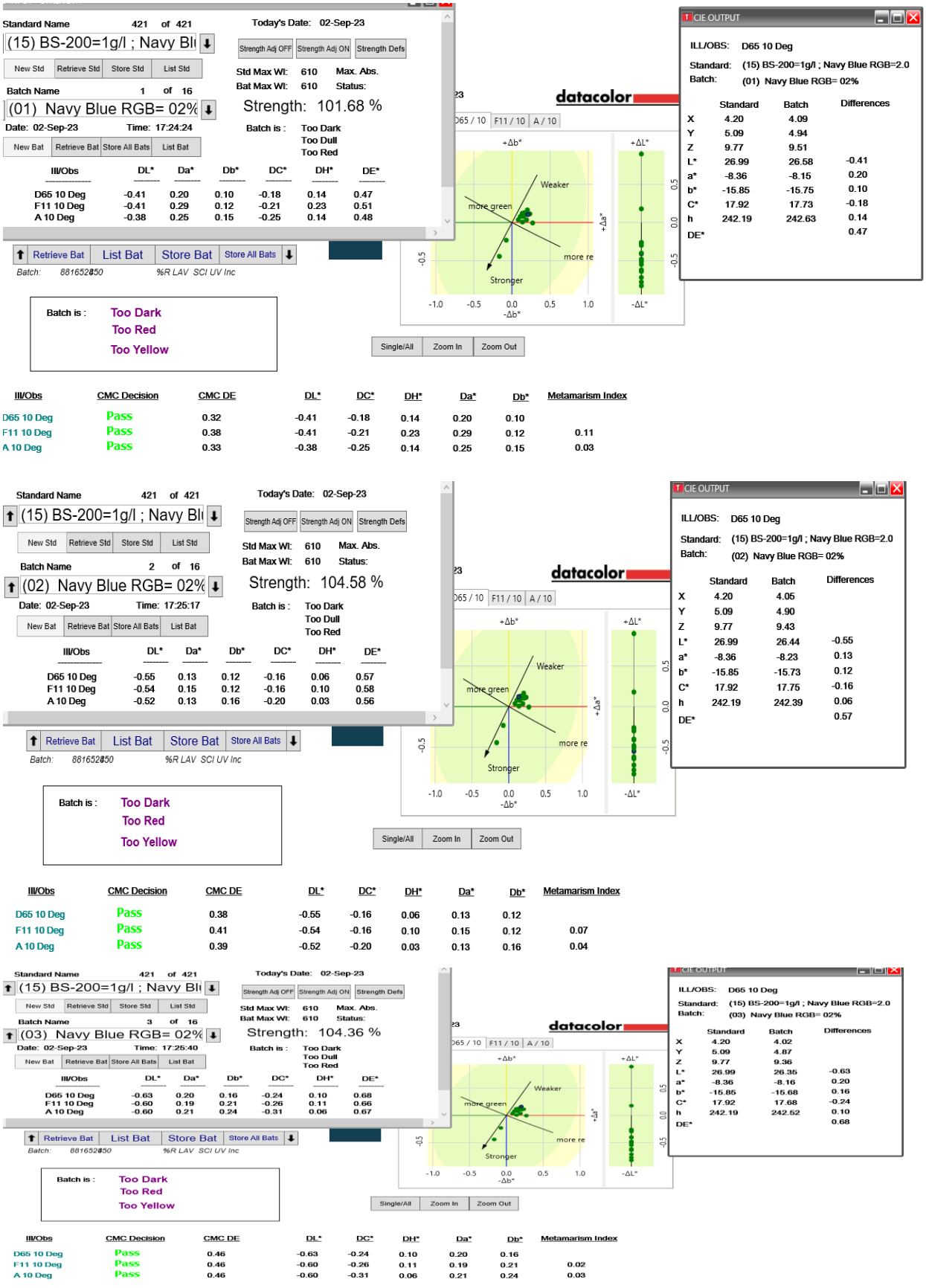


Figure: A8 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of CS1 enzyme

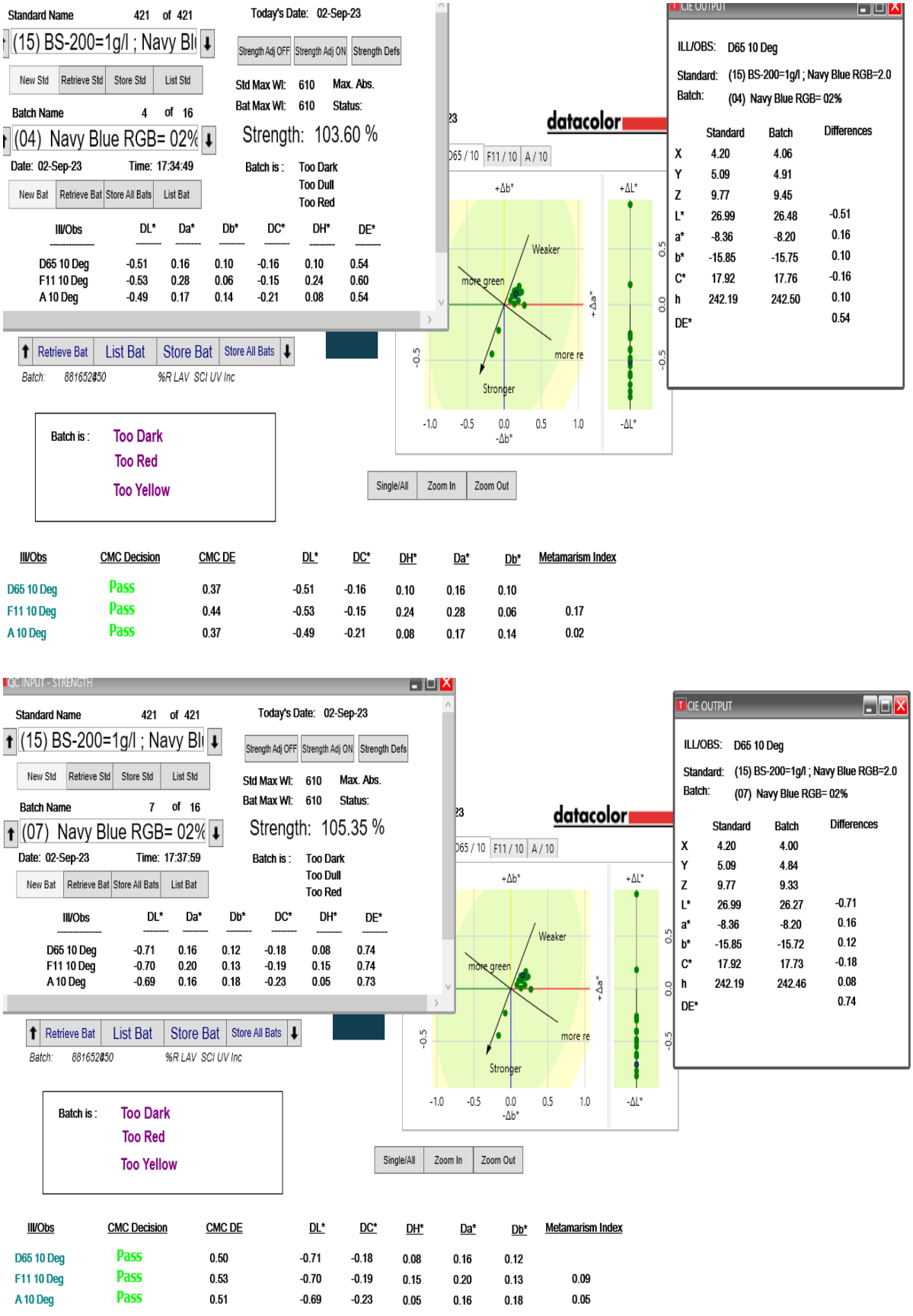


Figure: 9 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of CS 2 enzyme

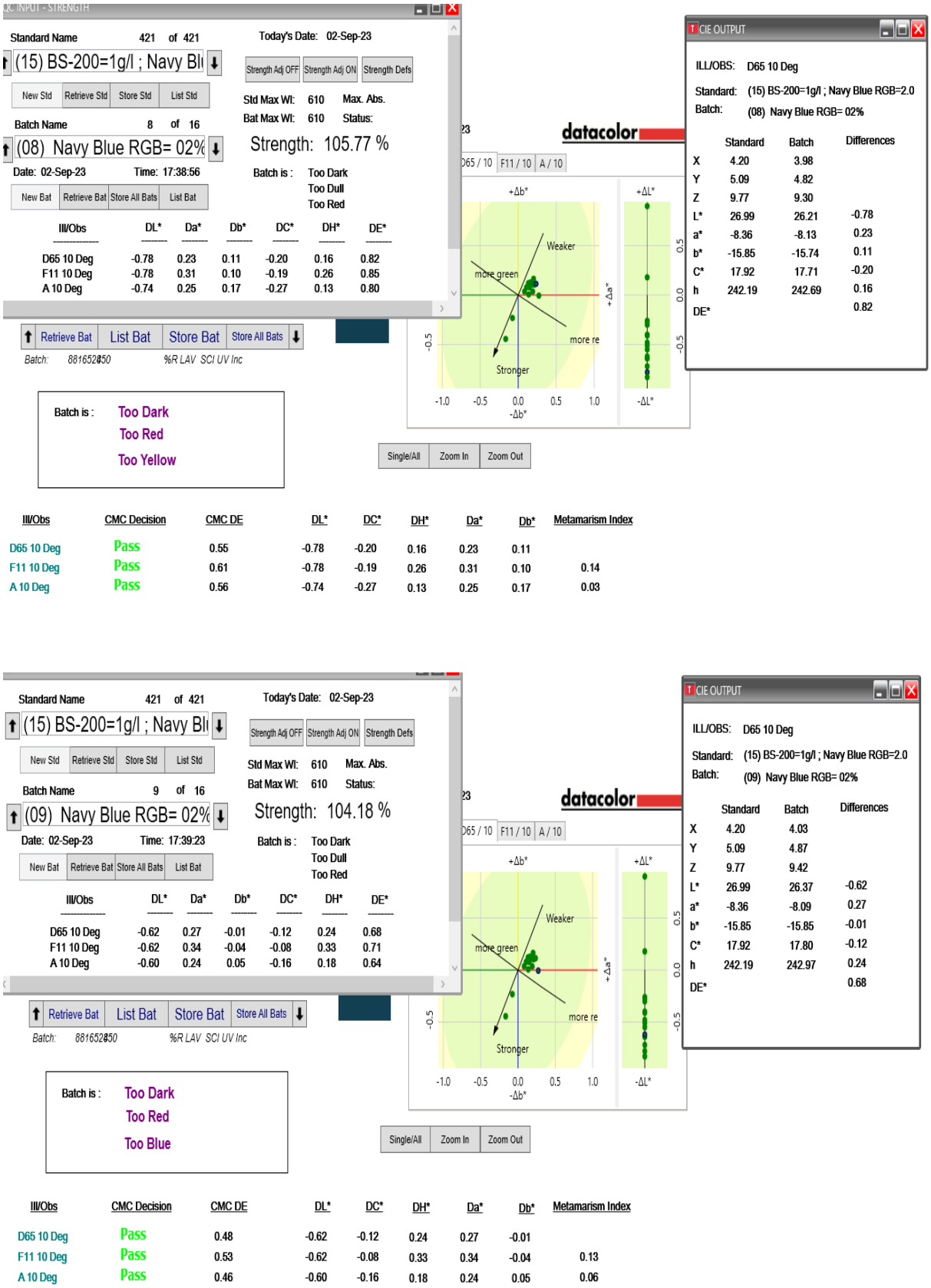


Figure: A10 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of BL enzyme

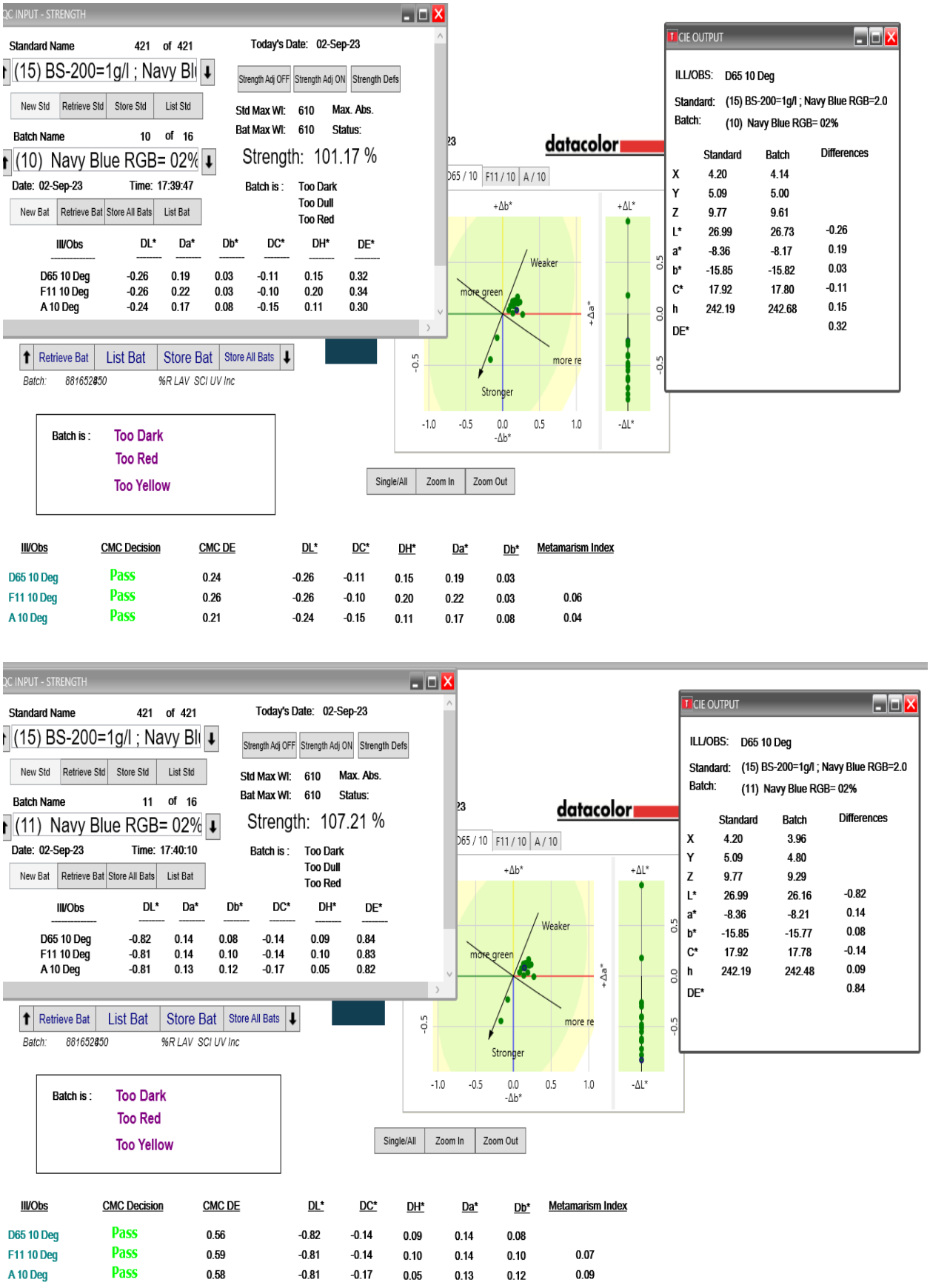


Figure: A11 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of D8 enzyme

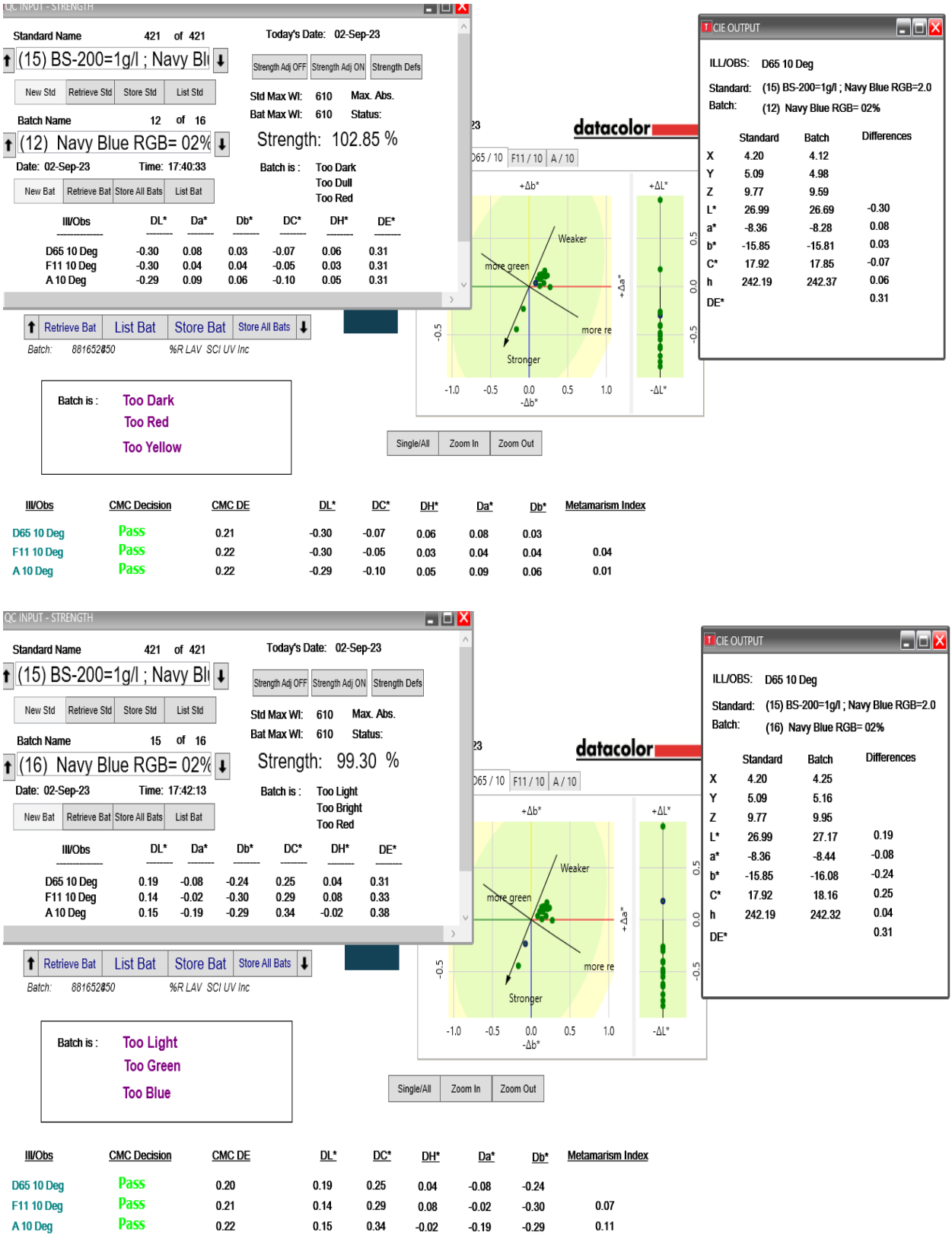


Figure: A12 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of CS 3 enzyme and CSP

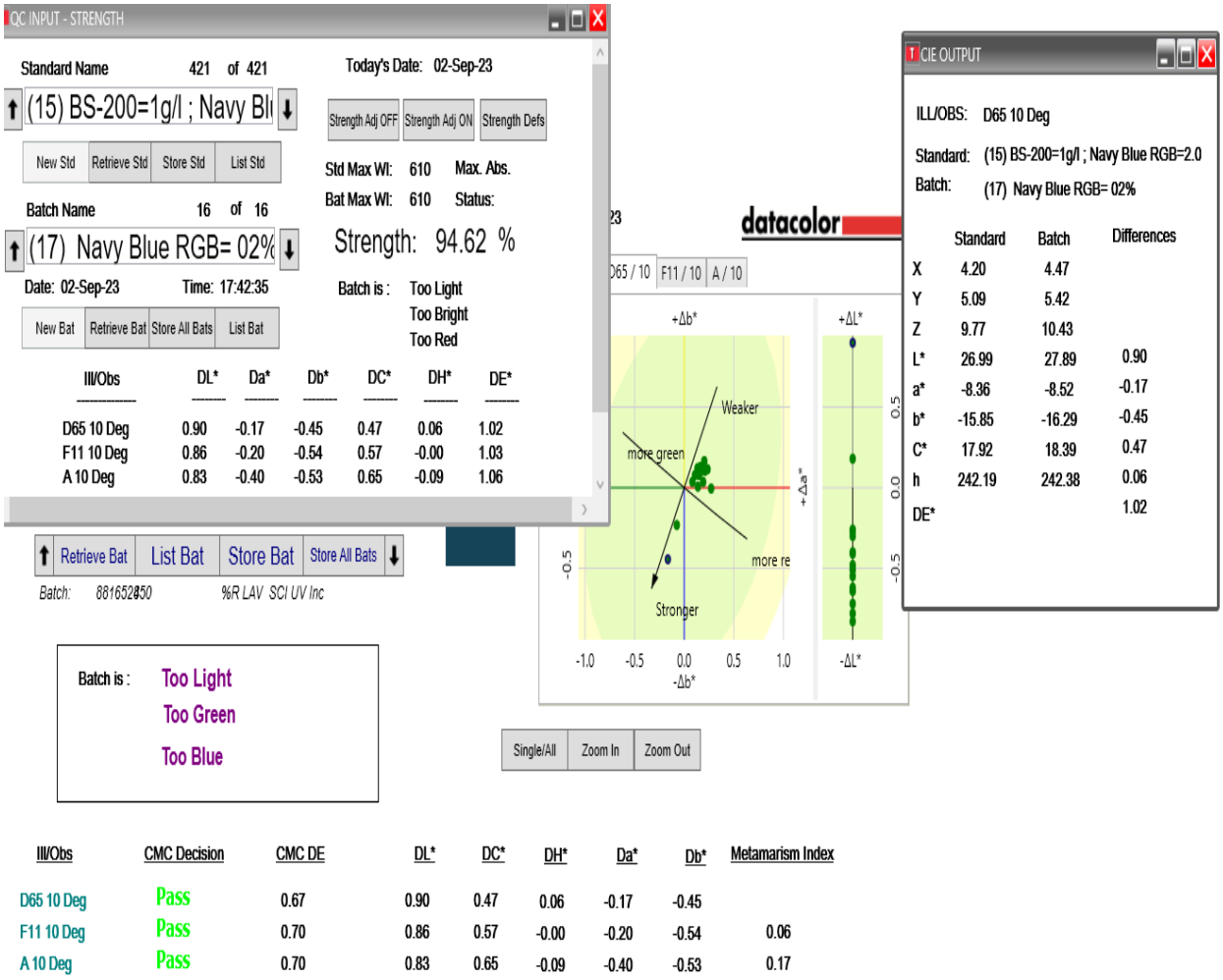
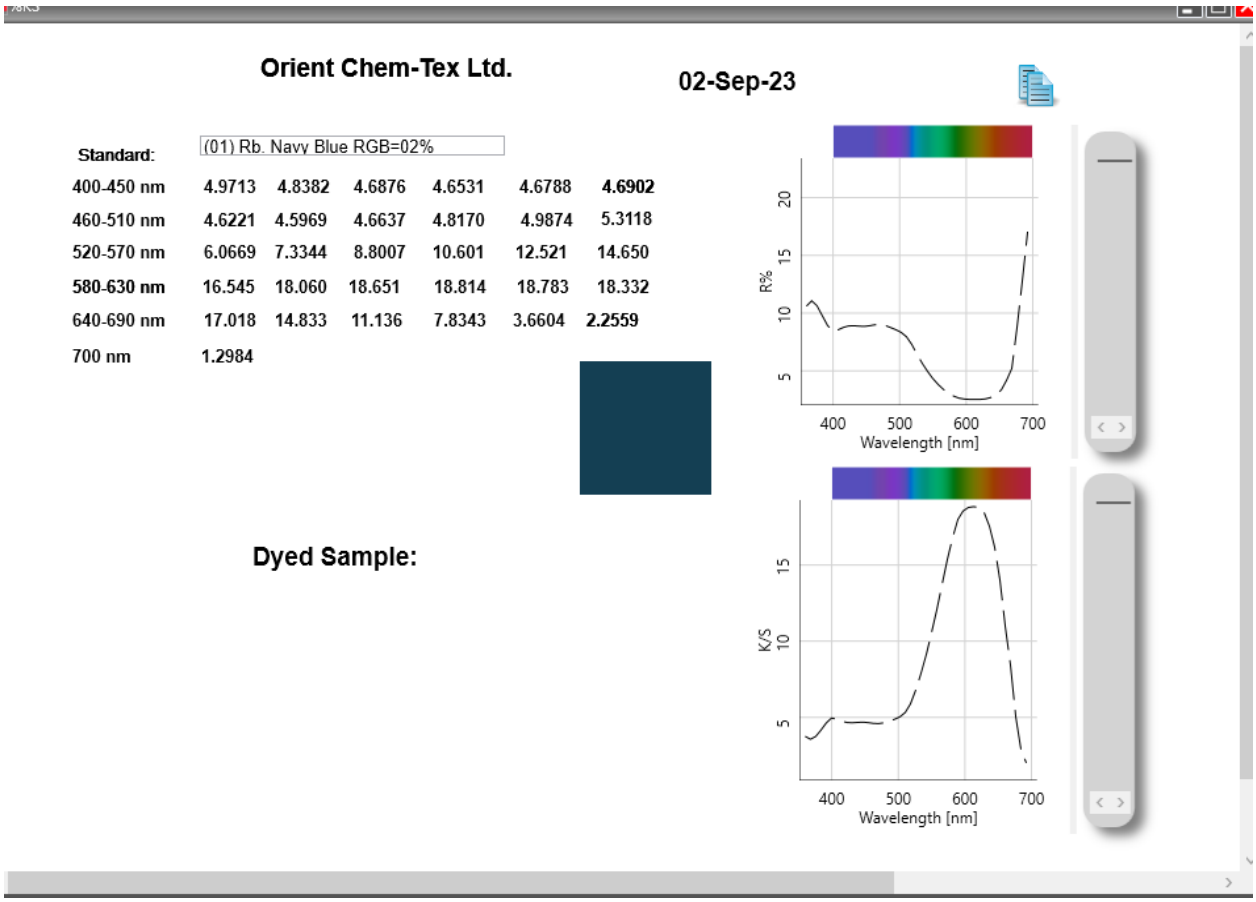


Figure: A13 Colour attributes for the dyed sample with Navy blue RGB of bio-scoured of BS 60 enzyme and CSBP



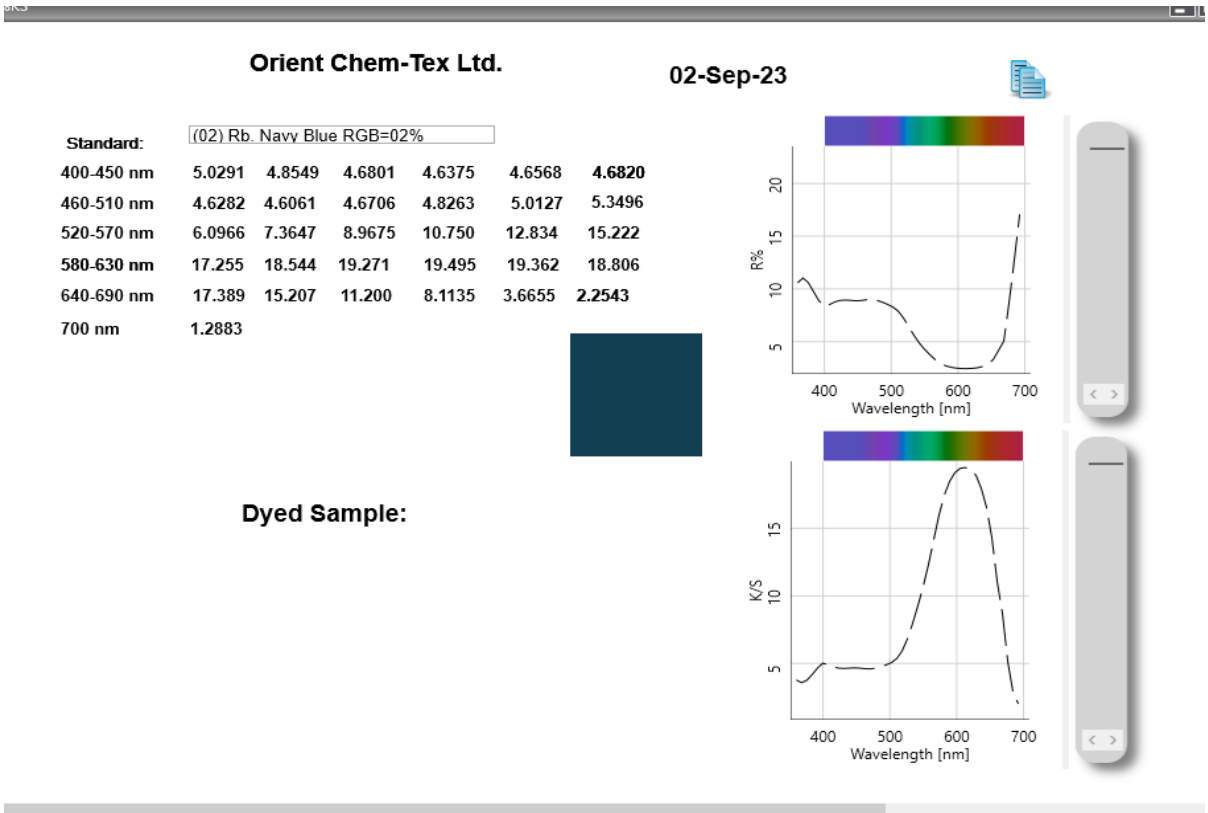
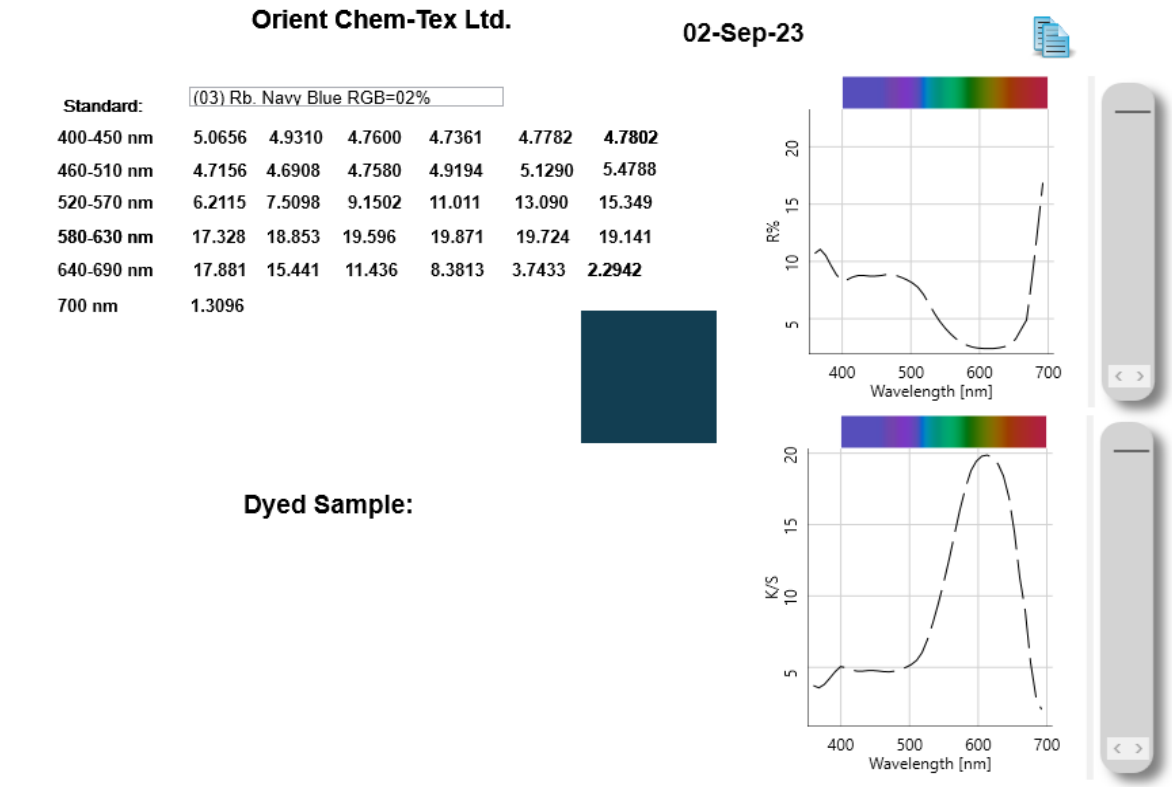


Figure: A14 K/S value for the dyed sample with Navy blue RGB of bio-scoured of CS1and CS2 enzyme



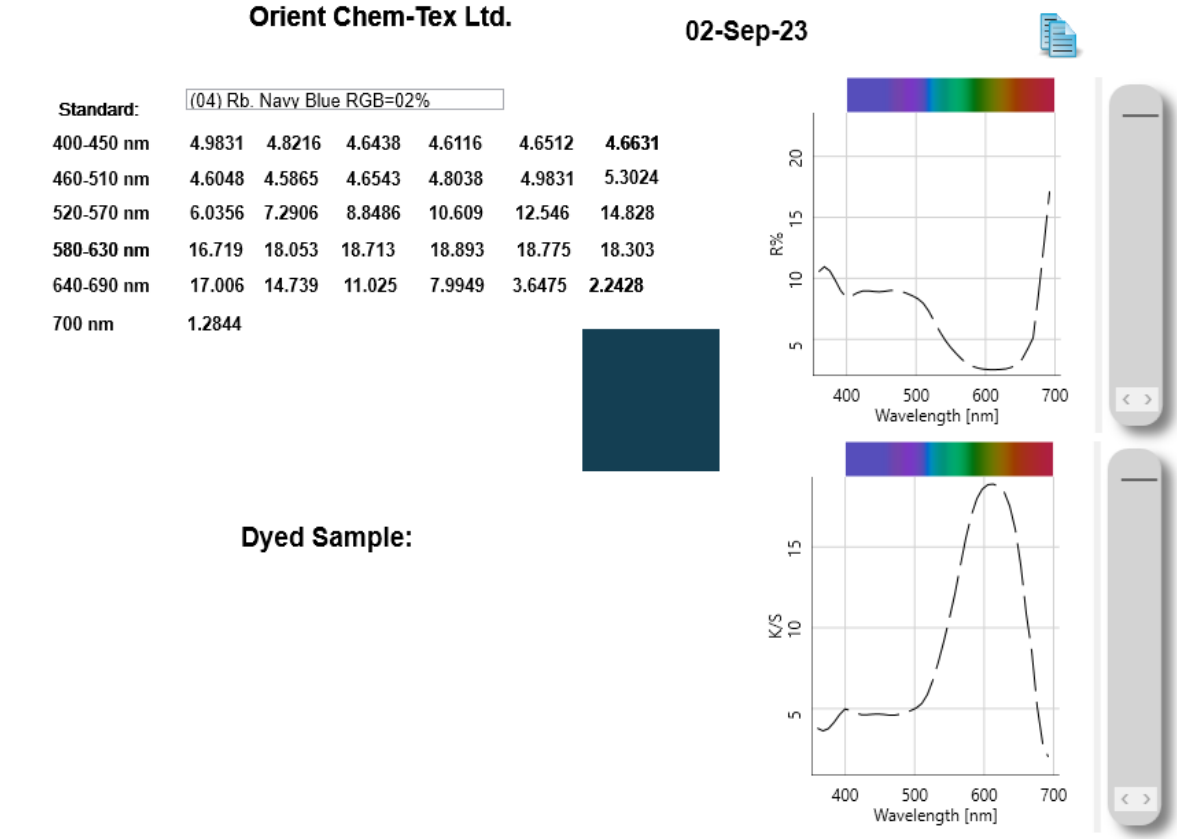
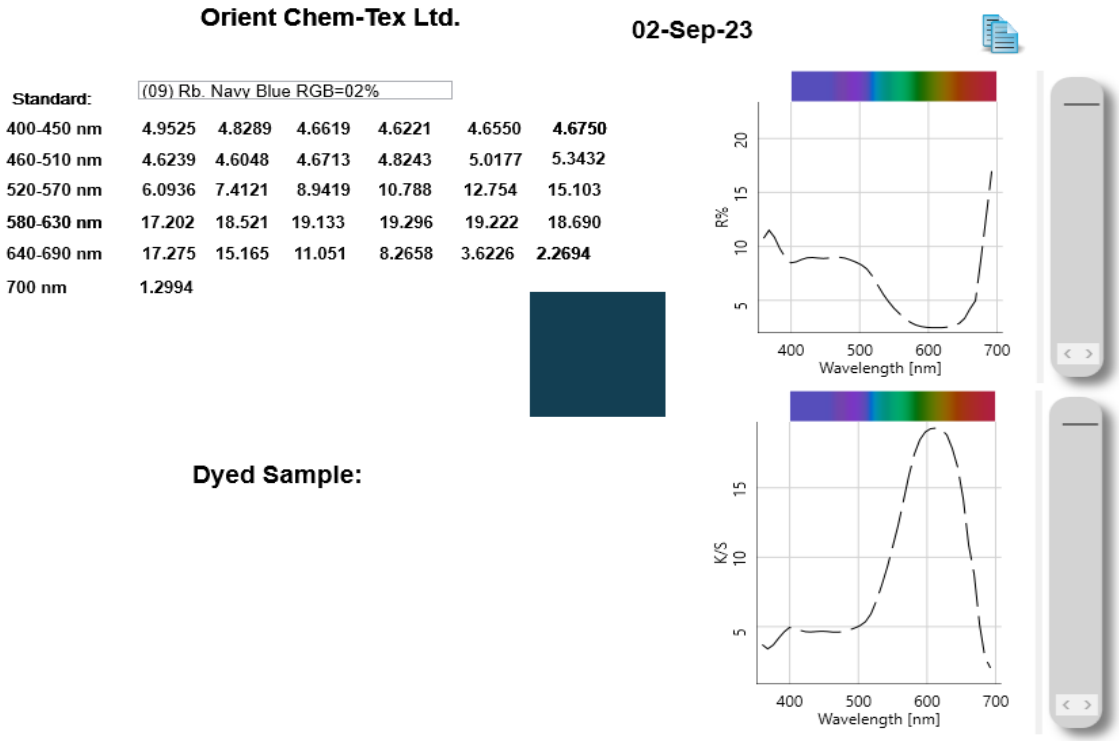


Figure: A15 K/S value for the dyed sample with Navy blue RGB of bio-scoured of CS3and CS4 enzyme



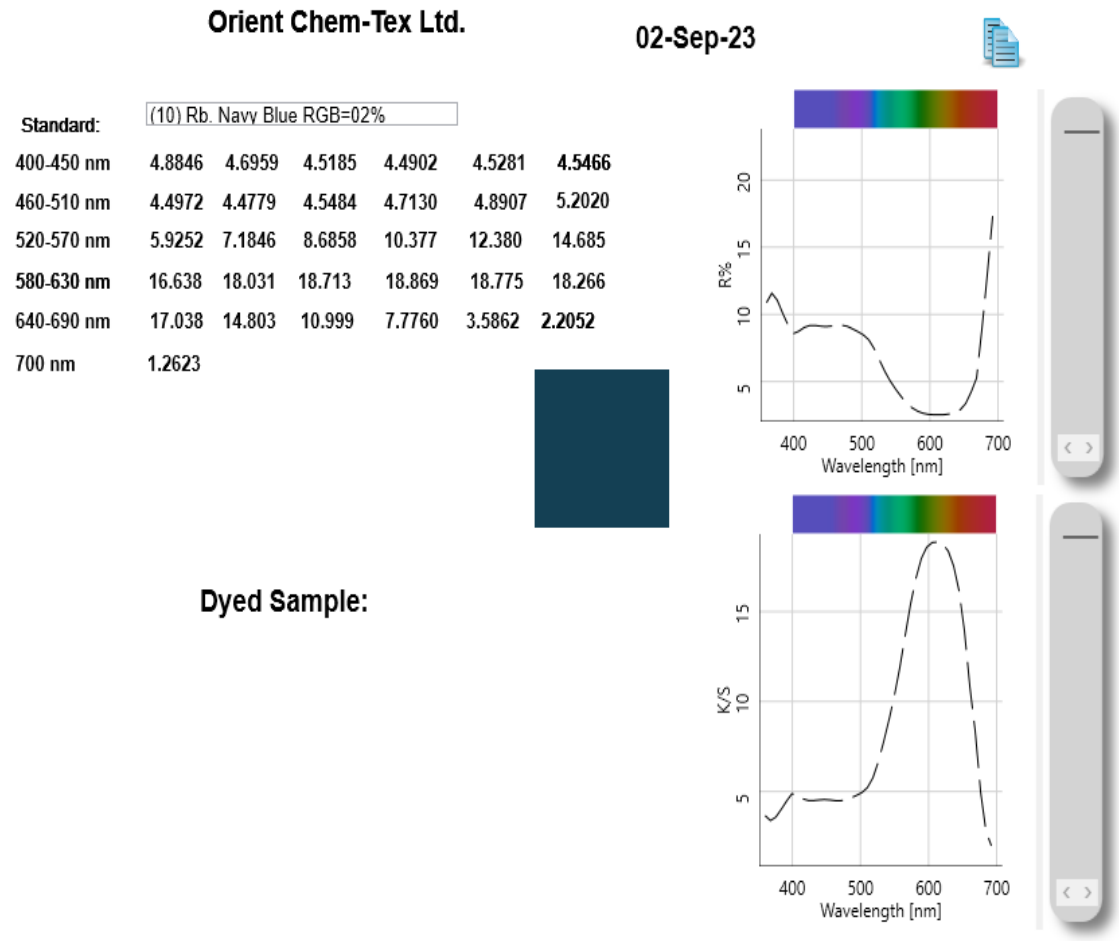
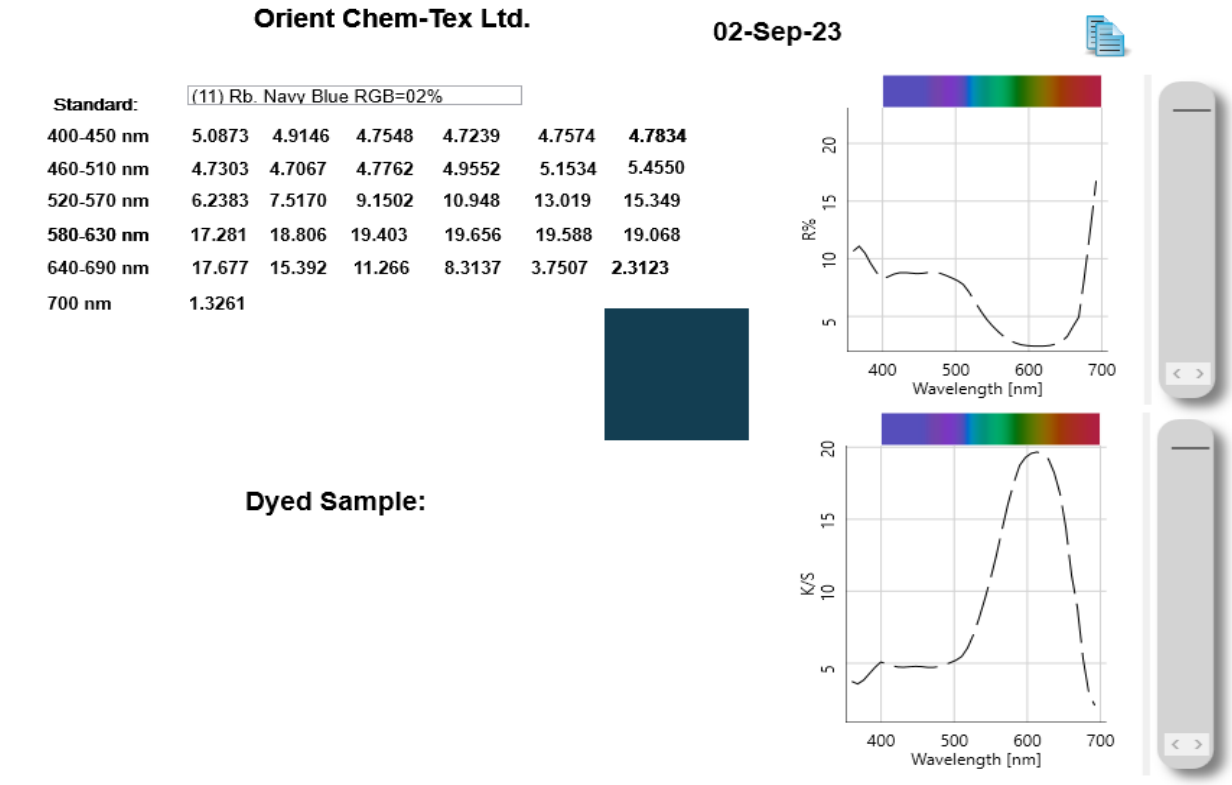


Figure: A16 K/S value for dyed sample with Navy blue RGB of bio-scoured of BL enzyme



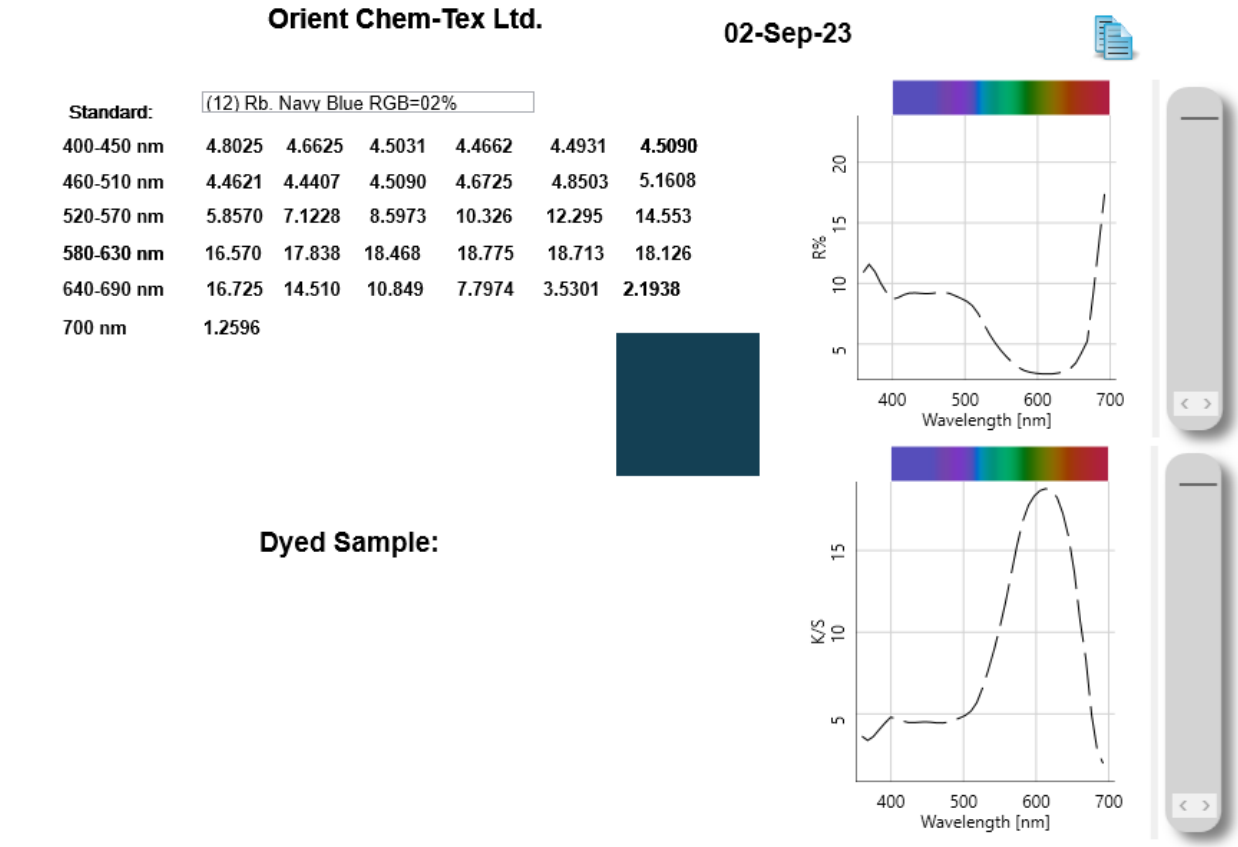


Figure: A17 K/S value for the dyed sample with Navy blue RGB of bio-scoured of D8 enzyme

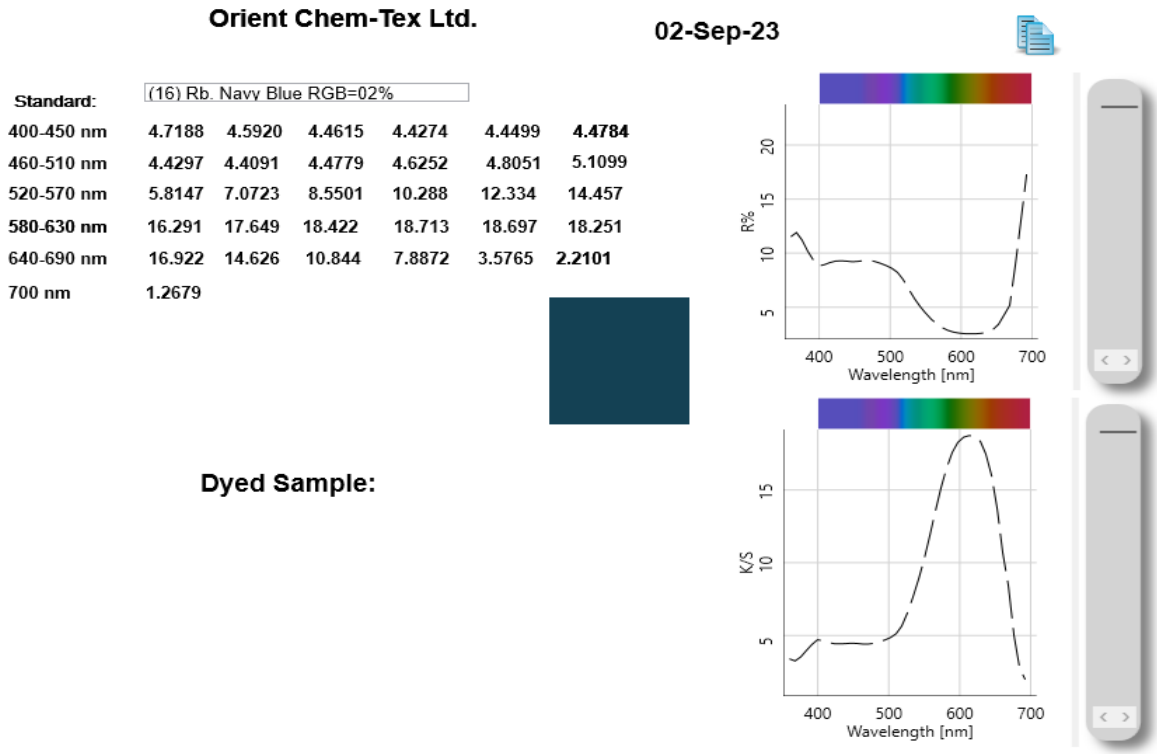


Figure: A18 K/S value for the dyed sample with Navy blue RGB of bio-scoured of CSP sample

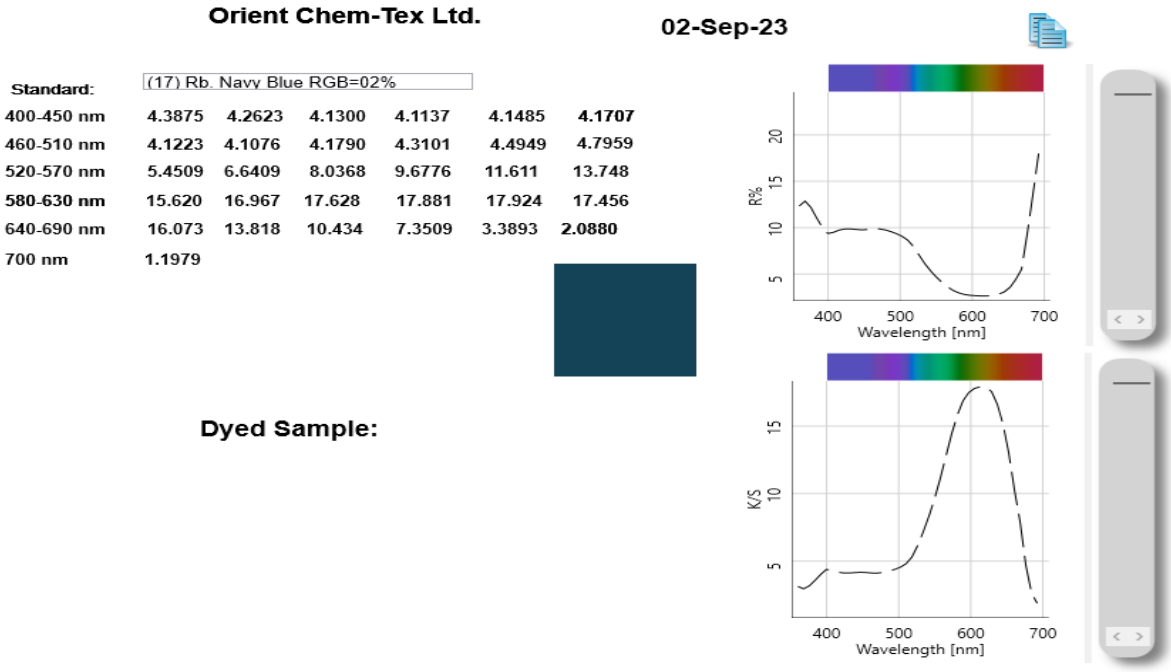
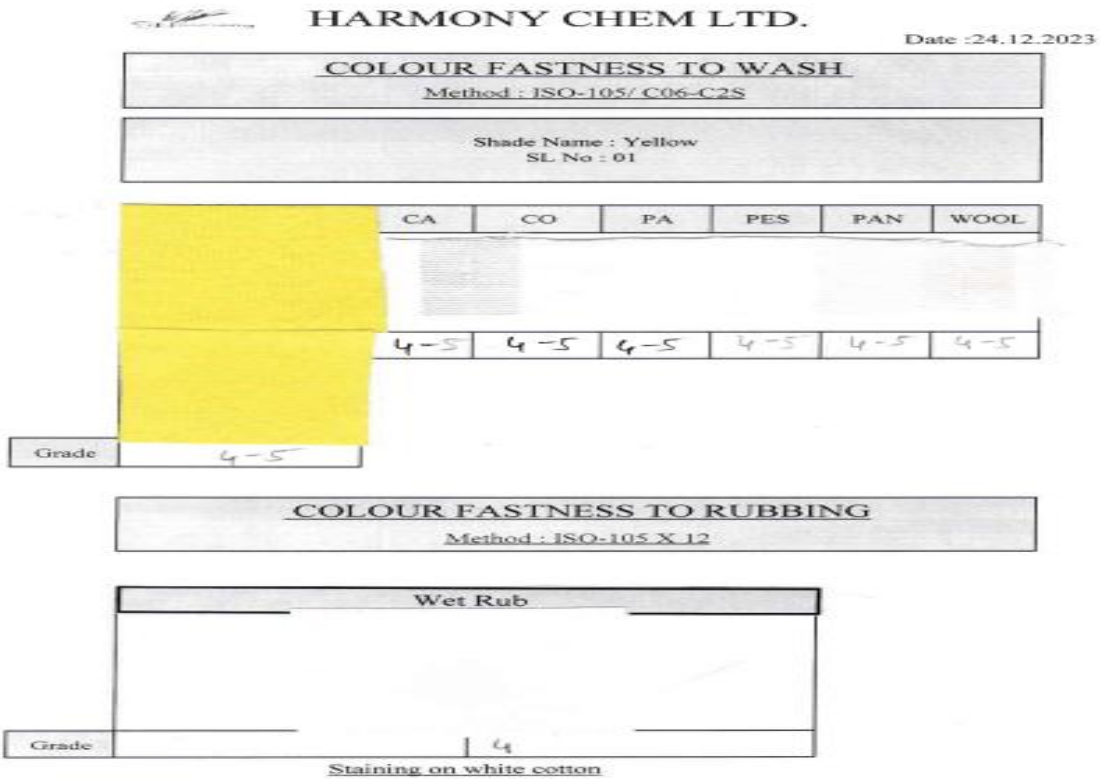


Figure: A19 K/S value for the dyed sample with Navy blue RGB of bio-scoured of CSBP sample


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 01

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4-5					

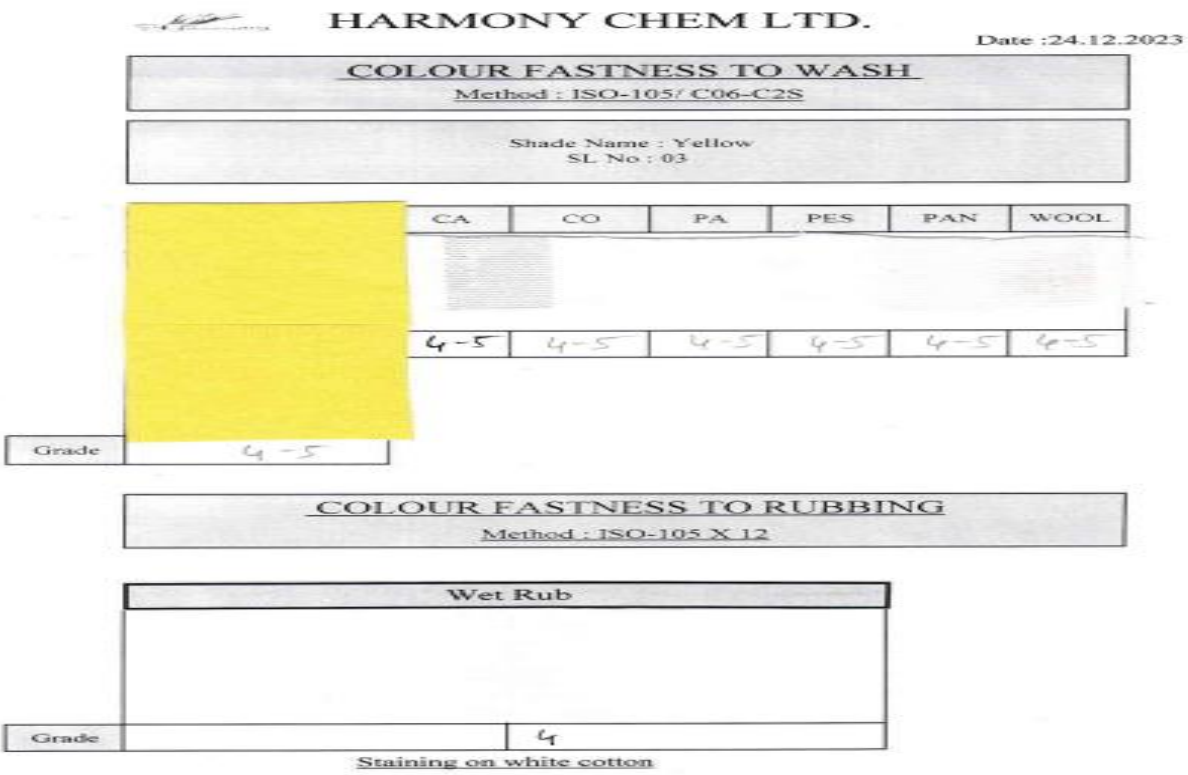
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A20 Colour fastness report for the dyed sample with Yellow 4GL of CS HB50


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 03

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4-5					

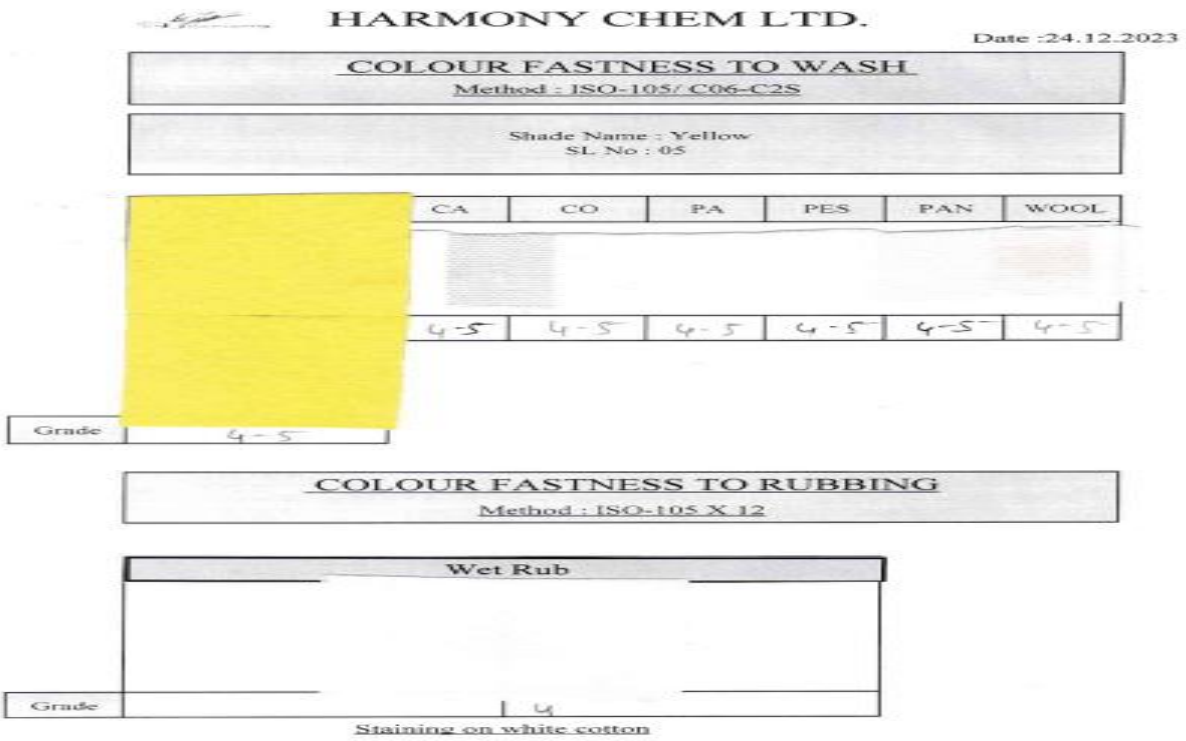
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A21 Colour fastness report for the dyed sample with Yellow 4GL of CS HB50+SB



HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 05

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4-5					

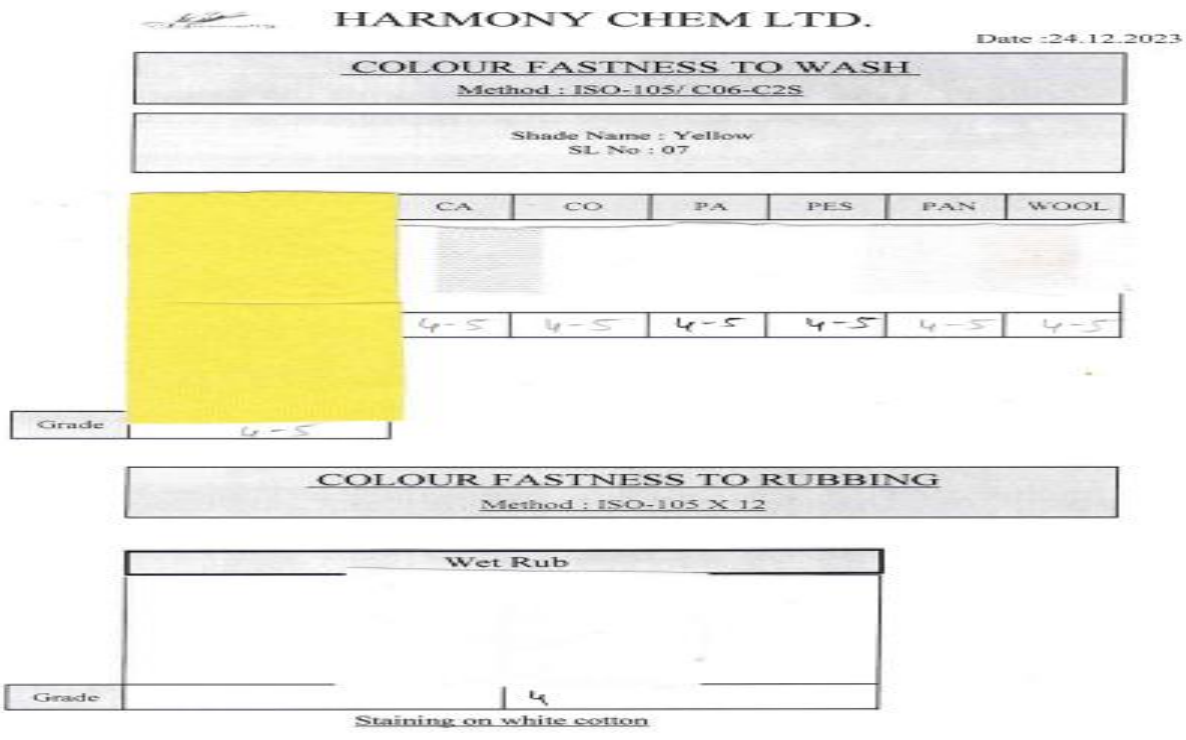
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A22 Colour fastness report for the dyed sample with Yellow 4GL of BL HB50



HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 07

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4-5					

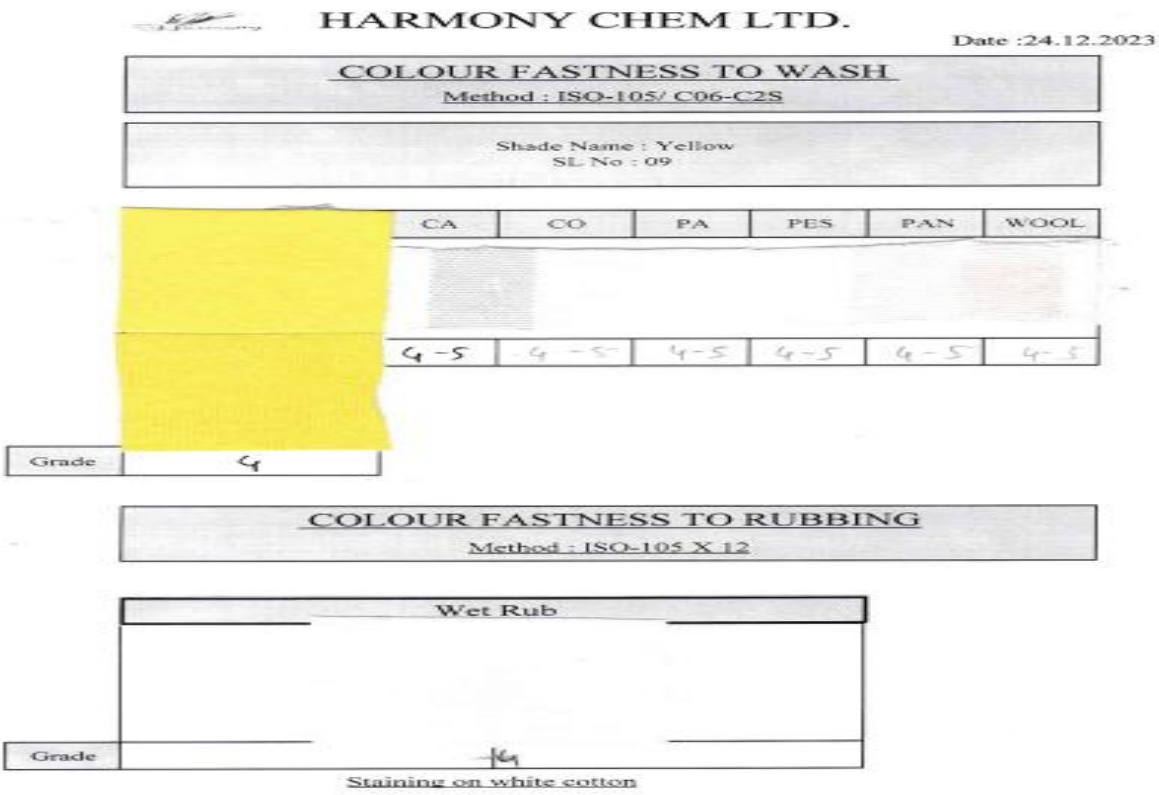
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A 23 Colour fastness report for the dyed sample with Yellow 4GL of BL HB50+SB


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 09

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4					

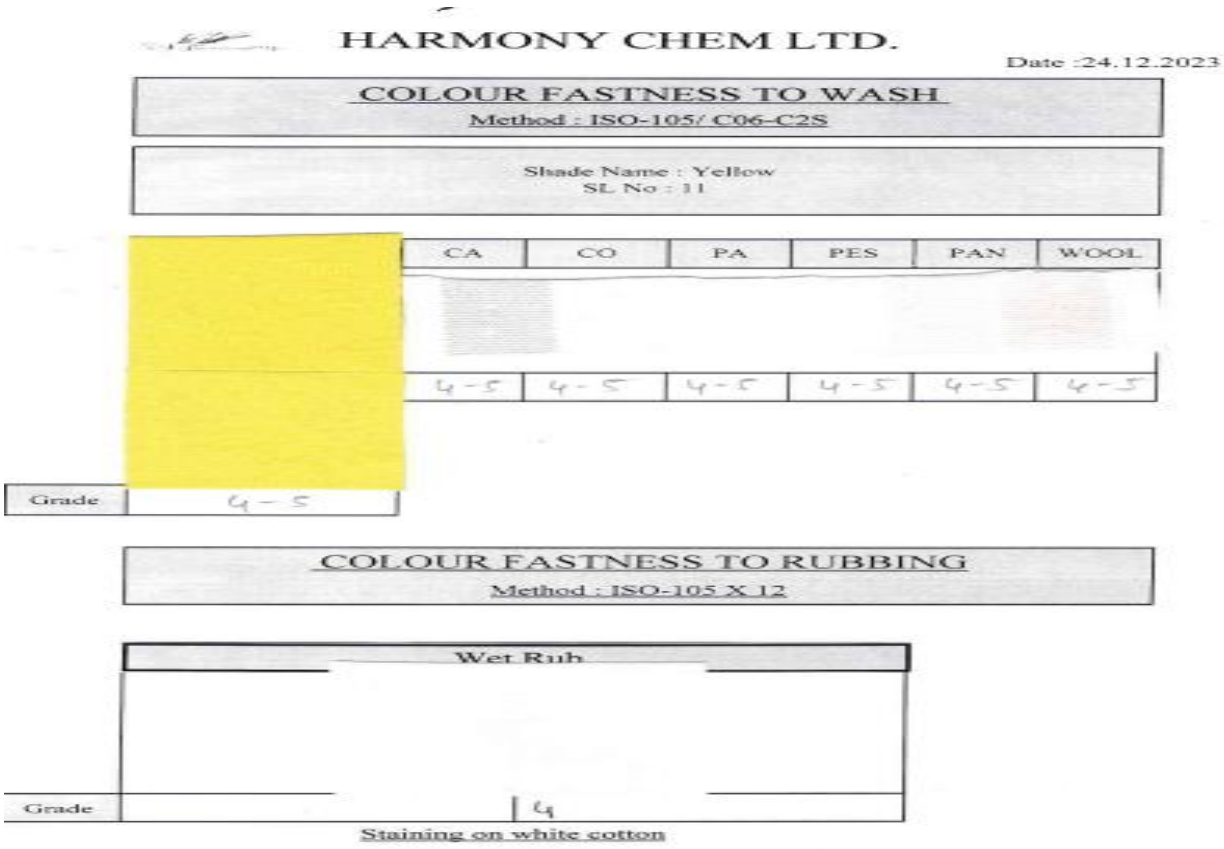
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A24 Colour fastness report for the dyed sample with Yellow 4GL of D8 HB50


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 11

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4-5					

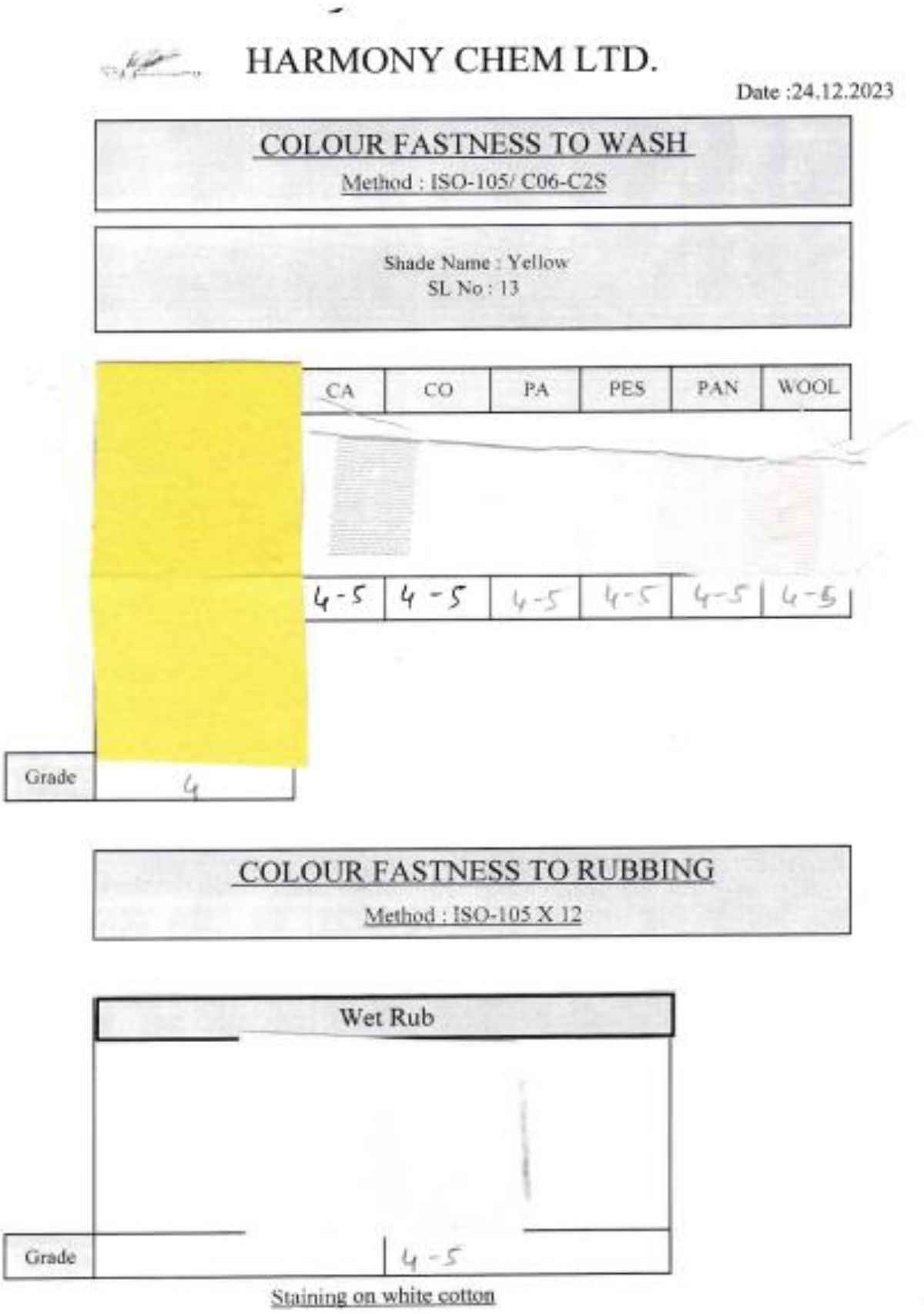
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4

Staining on white cotton

Figure: A 25 Colour fastness report for the dyed sample with Yellow 4GL of D8 HB50+SB



HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Yellow
SL No : 13

	CA	CO	PA	PES	PAN	WOOL
	4-5	4-5	4-5	4-5	4-5	4-5
Grade	4					

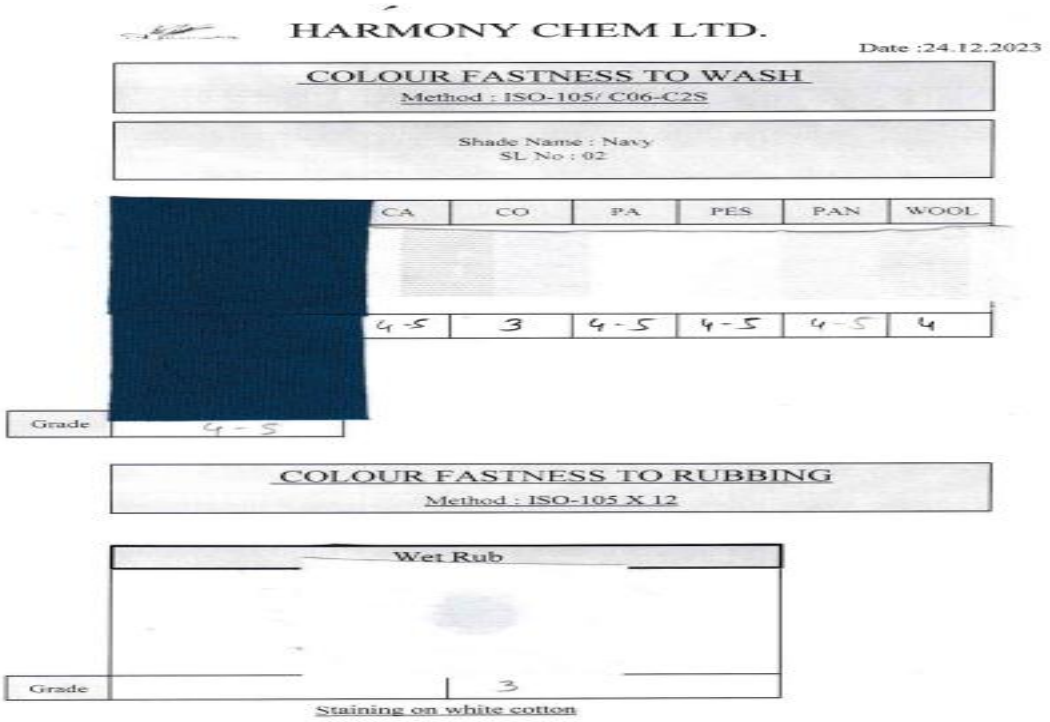
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	4-5

Staining on white cotton

Figure: A26 Colour fastness report for the dyed sample with Yellow 4GL of CSBP10.


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 02

	CA	CO	PA	PES	PAN	WOOL
	4-5	3	4-5	4-5	4-5	4
Grade	4-5					

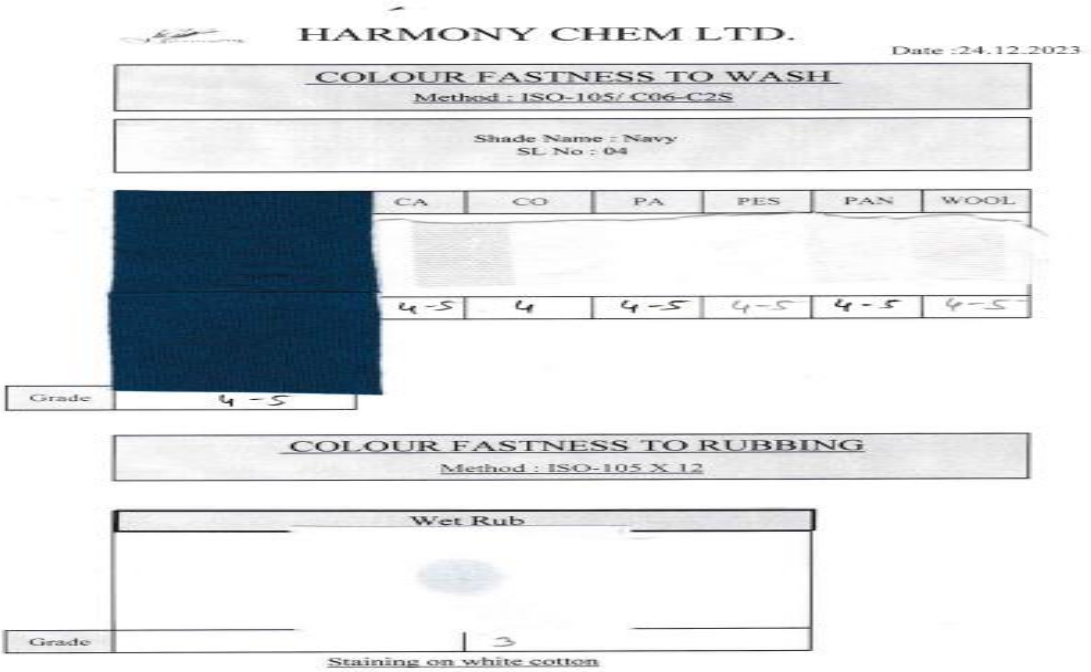
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A27 Colour fastness report for the dyed sample with Navy blue RGB of CS HB50


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 04

	CA	CO	PA	PES	PAN	WOOL
	4-5	4	4-5	4-5	4-5	4-5
Grade	4-5					

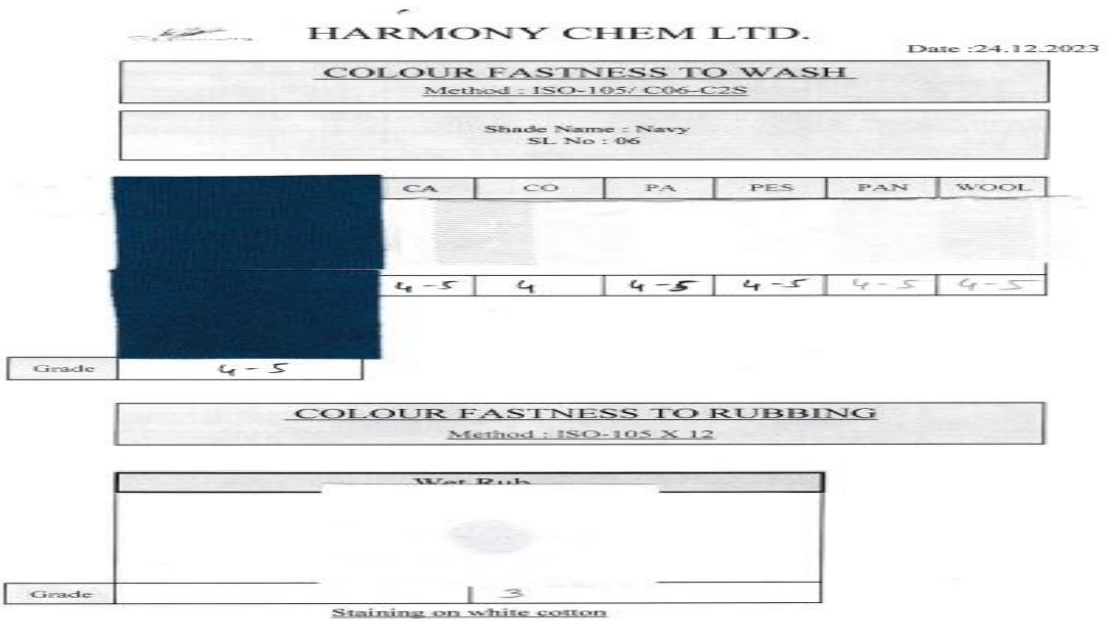
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A28 Colour fastness report for the dyed sample with Navy blue RGB of CS HB50+SB


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 06

	CA	CO	PA	PES	PAN	WOOL
	4-5	4	4-5	4-5	4-5	4-5
Grade	4-5					

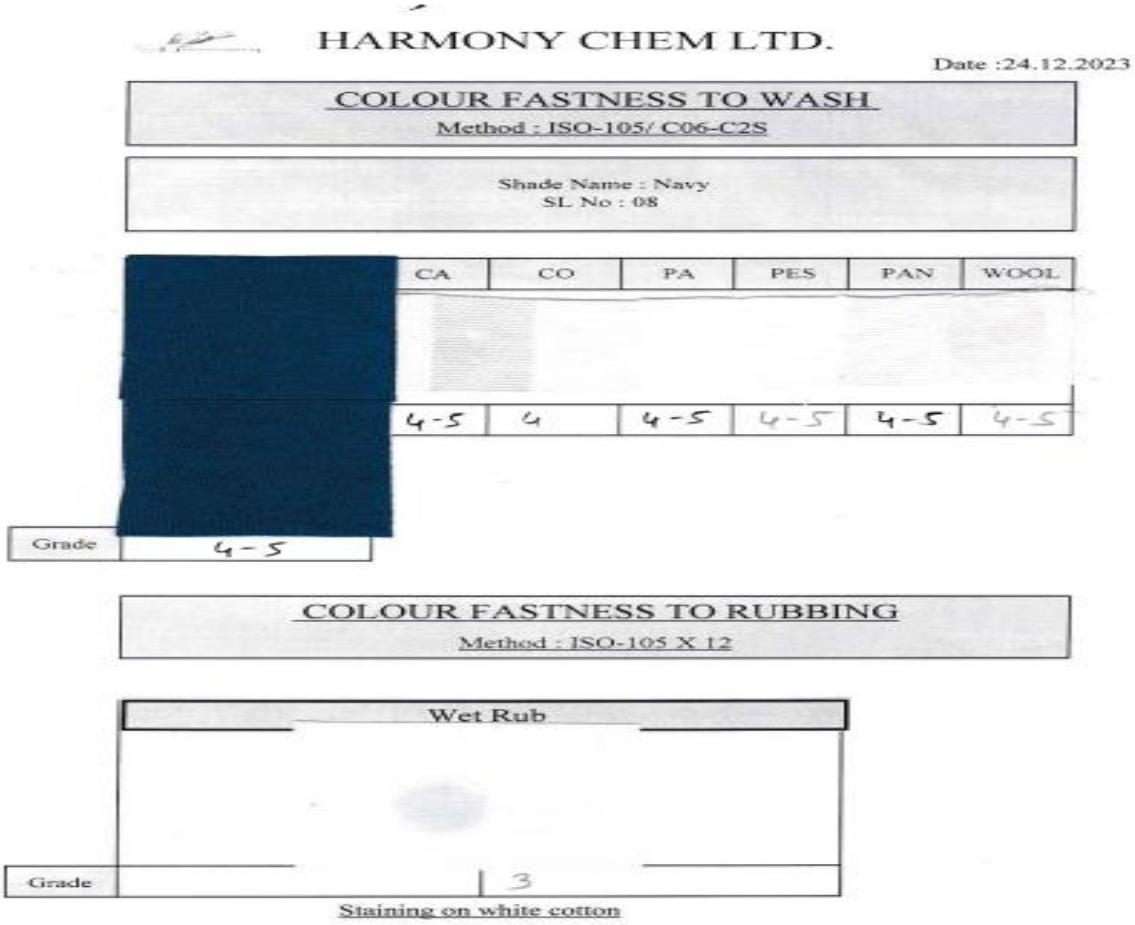
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A29 Colour fastness report for the dyed sample with Navy blue RGB of CS HB50+SB


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 08

	CA	CO	PA	PES	PAN	WOOL
	4-5	4	4-5	4-5	4-5	4-5
Grade	4-5					

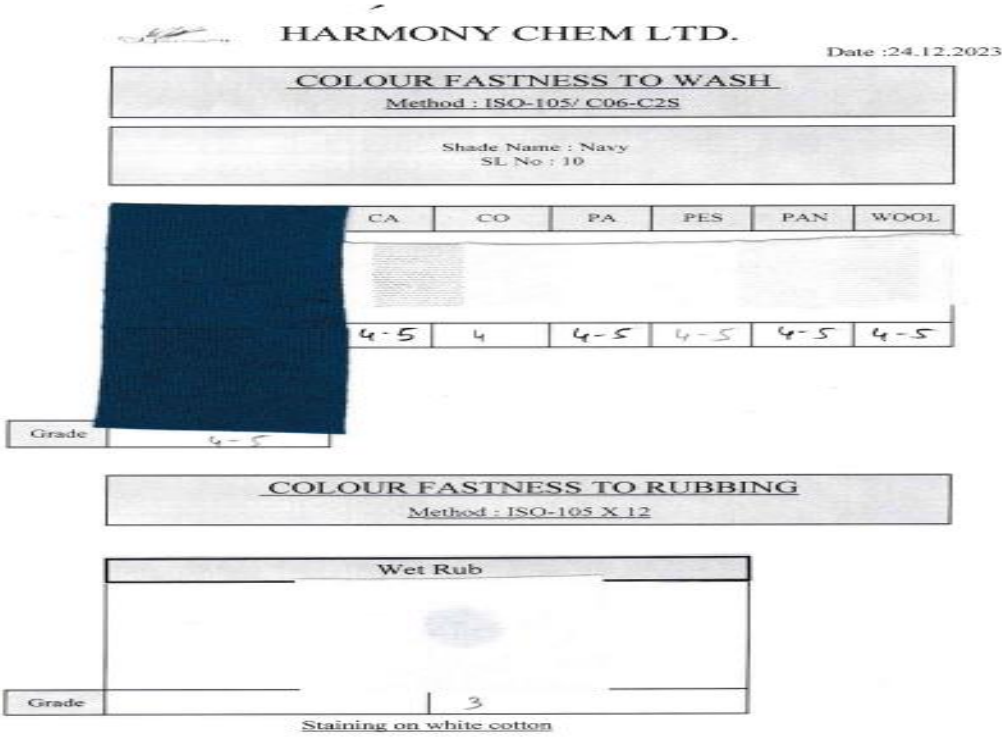
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A30 Colour fastness report for the dyed sample with Navy blue RGB of BL HB50+SB


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 10

	CA	CO	PA	PES	PAN	WOOL
Grade	4-5	4	4-5	4-5	4-5	4-5

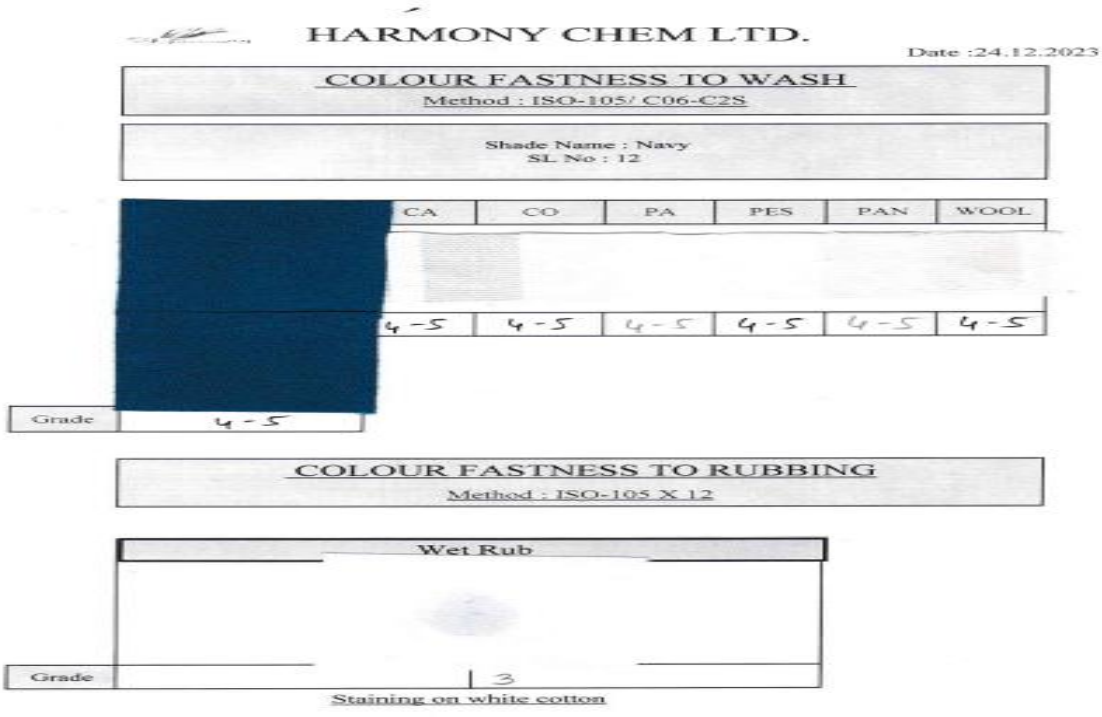
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A31 Colour fastness report for the dyed sample with Navy blue RGB of D8 HB50


HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 12

	CA	CO	PA	PES	PAN	WOOL
Grade	4-5	4-5	4-5	4-5	4-5	4-5

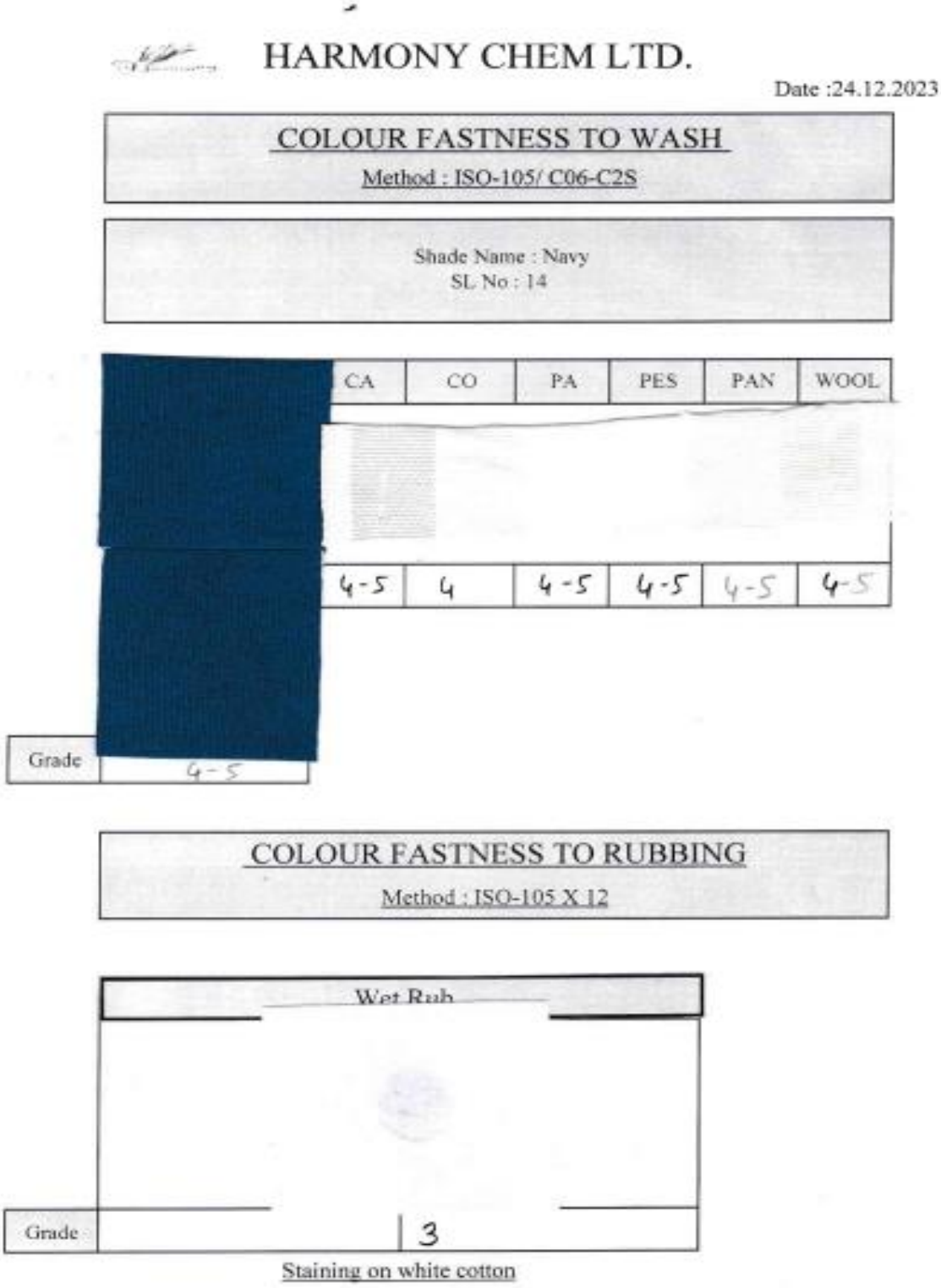
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A 32 Colour fastness report for the dyed sample with Navy blue RGB of D8 HB50+SB



HARMONY CHEM LTD.

Date :24.12.2023

COLOUR FASTNESS TO WASH

Method : ISO-105/ C06-C2S

Shade Name : Navy
SL No : 14

	CA	CO	PA	PES	PAN	WOOL
	4-5	4	4-5	4-5	4-5	4-5
Grade	4-5					

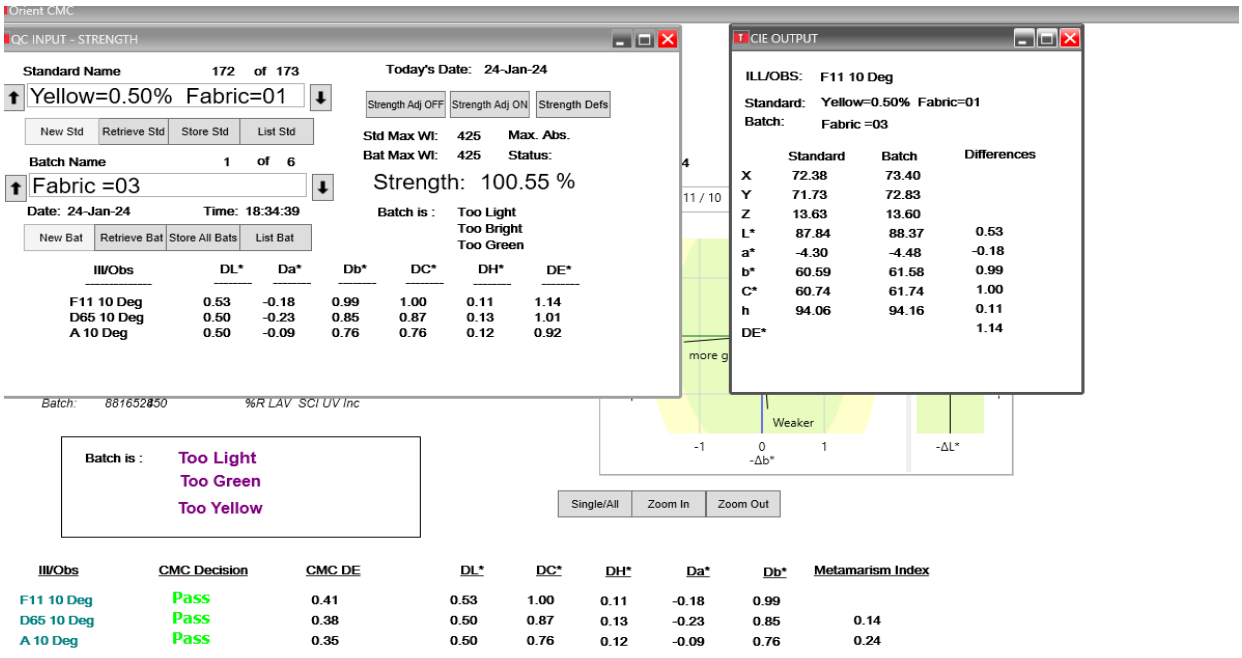
COLOUR FASTNESS TO RUBBING

Method : ISO-105 X 12

	Wet Rub
Grade	3

Staining on white cotton

Figure: A33 Colour fastness report for the dyed sample with Navy blue RGB of CSBP



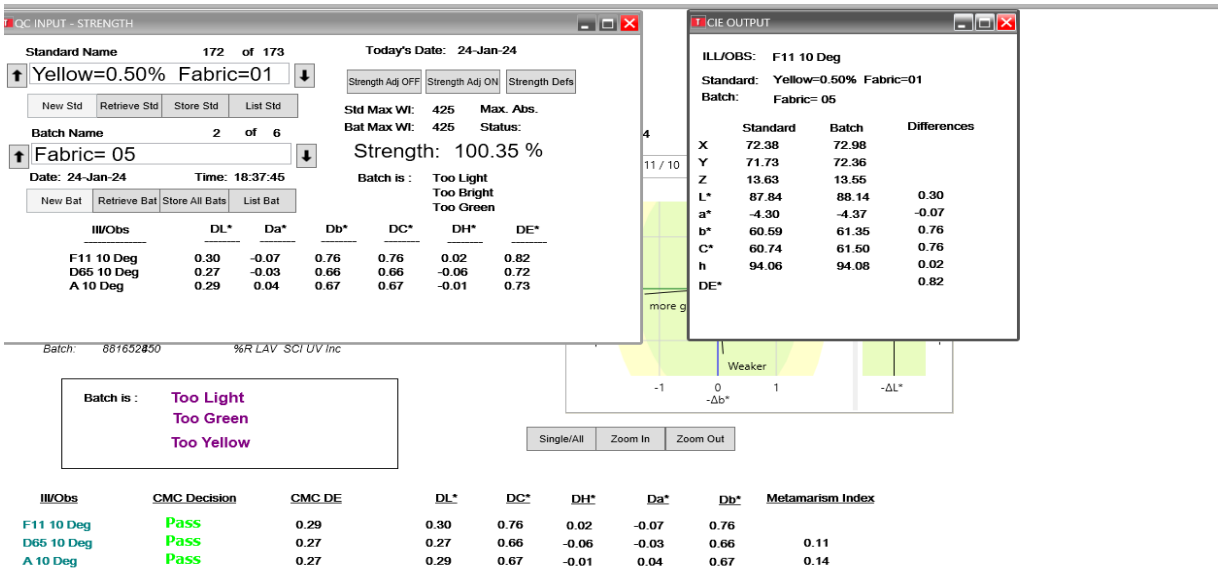


Figure: A 34 L value for the dyed sample with yellow 4GL of bio-scoured and bleached fabric of CS HP50 CS HP50+SB and BL HP50+SB.

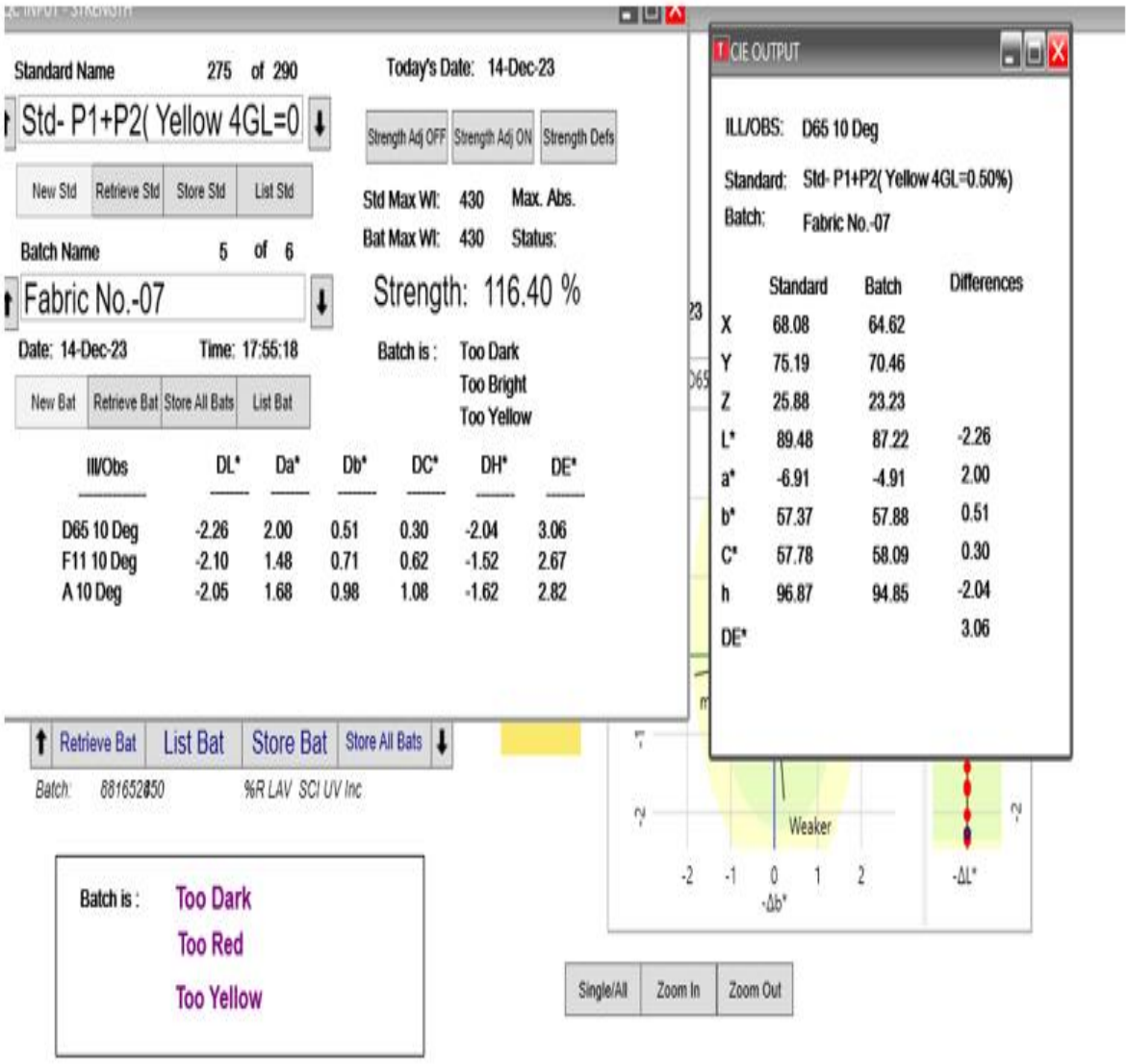


Figure: A35 L value for the dyed sample with yellow 4GL of bio-scoured and bleached fabric of CSBP10 and BL HP50

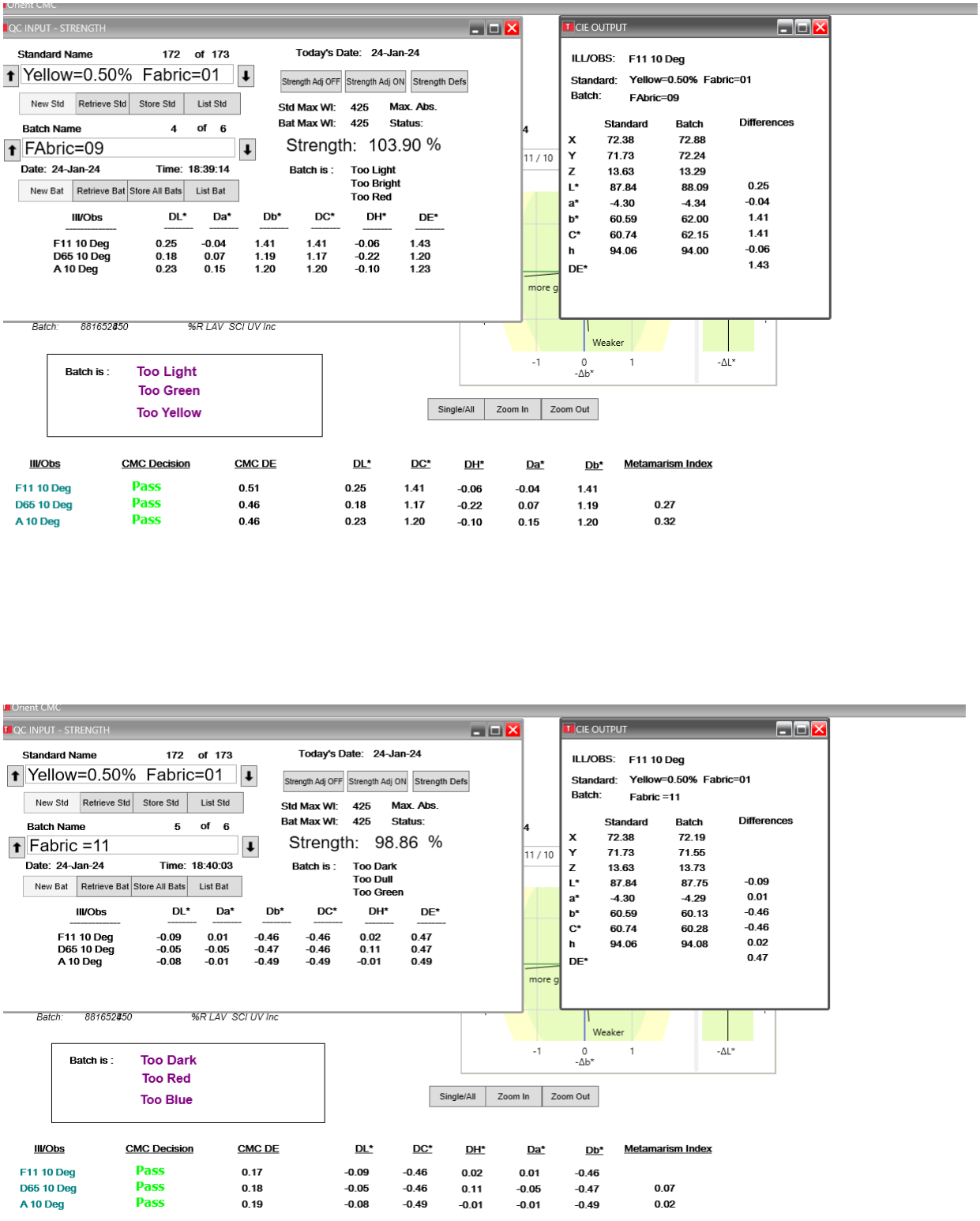


Figure: A 36 L value the for the dyed sample with yellow 4GL of bio-scoured and bleached fabric D8 HP50 +SB and D8 HP50

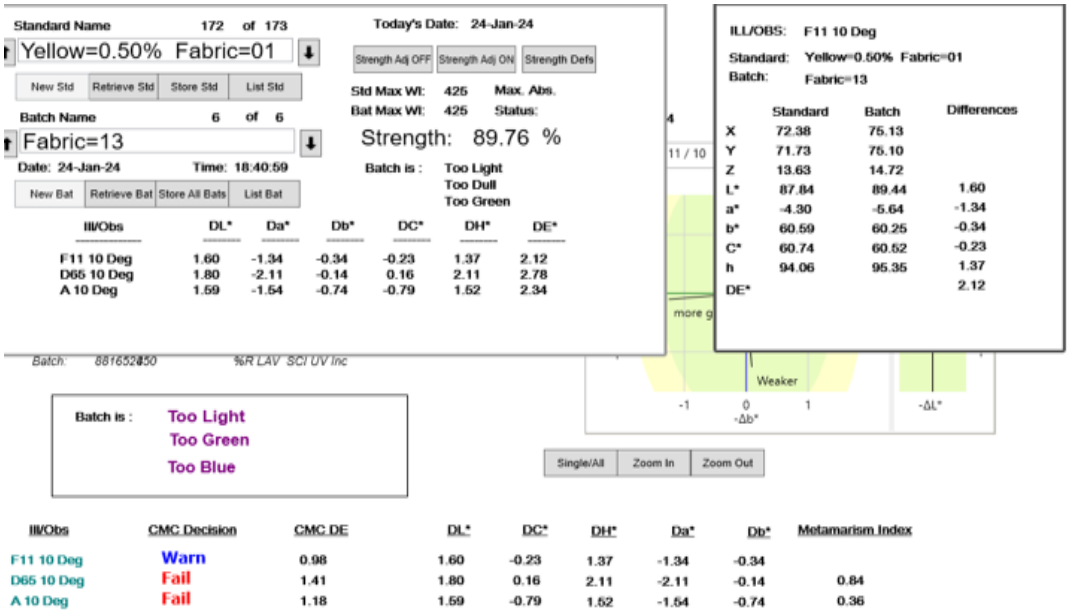


Figure A37 L value for the dyed sample with yellow 4GL of bio-scoured and bleached fabric of CS HP50 and CSBP10

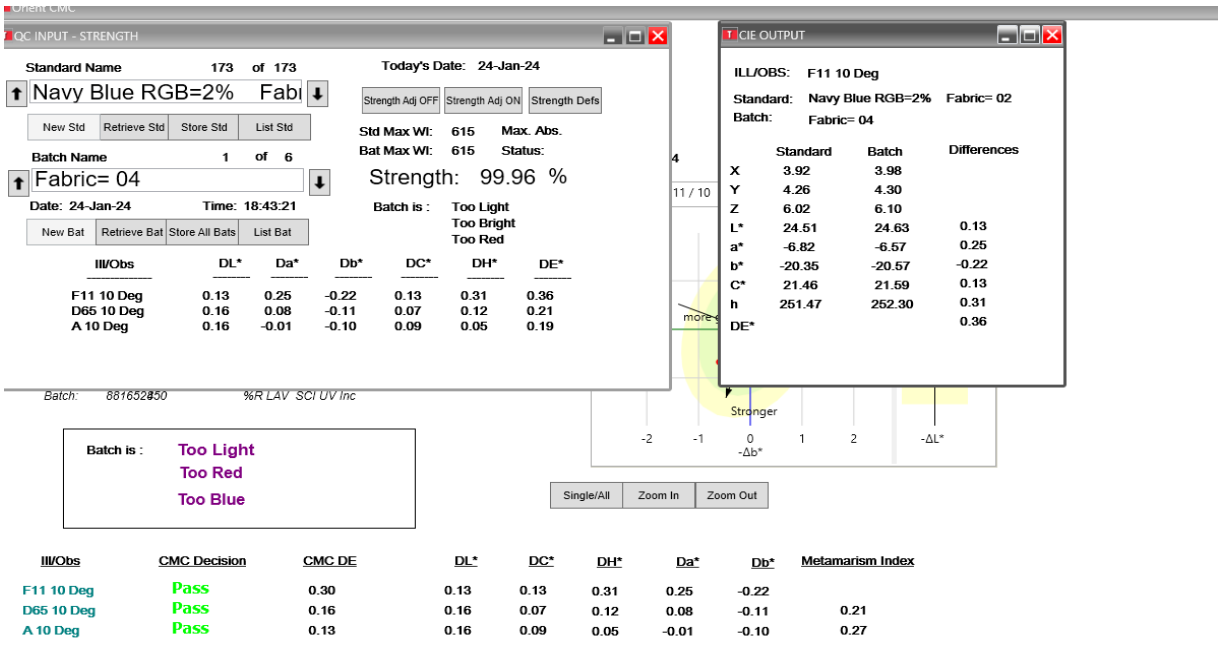
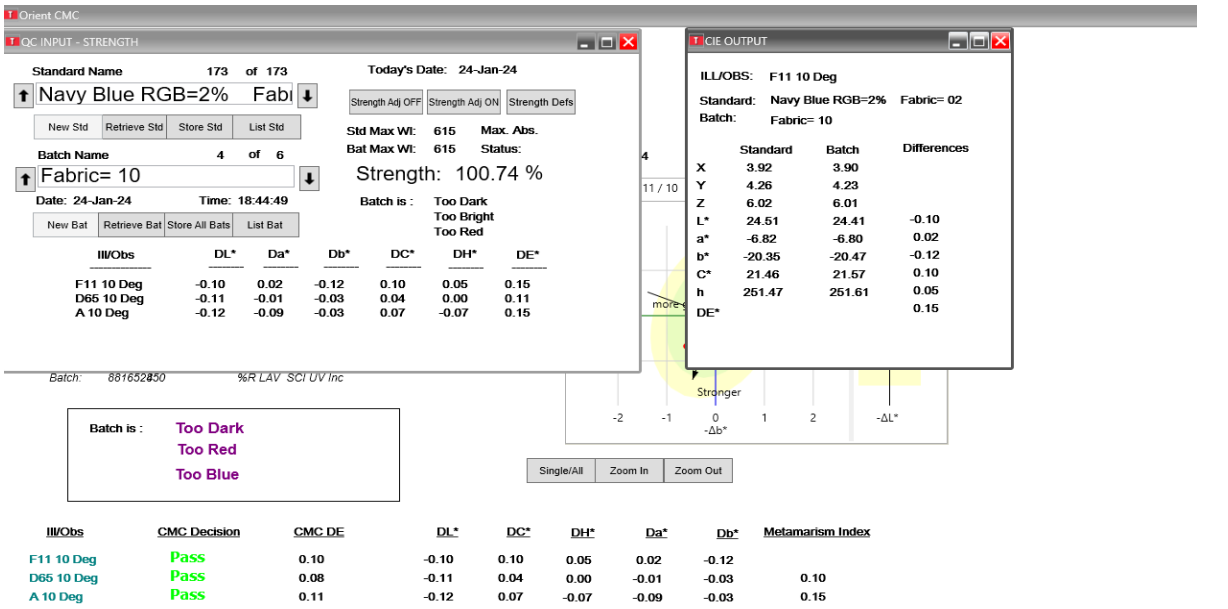


Figure: A38 L value for the dyed sample with Navy blue RGB of bio-scoured and bleached fabric of CS HP50 and CS HP50+SB

Figure: A39 L value for the dyed sample with Navy blue RGB of bio-scoured and bleached fabric of BL HP50 and BL HP50+SB



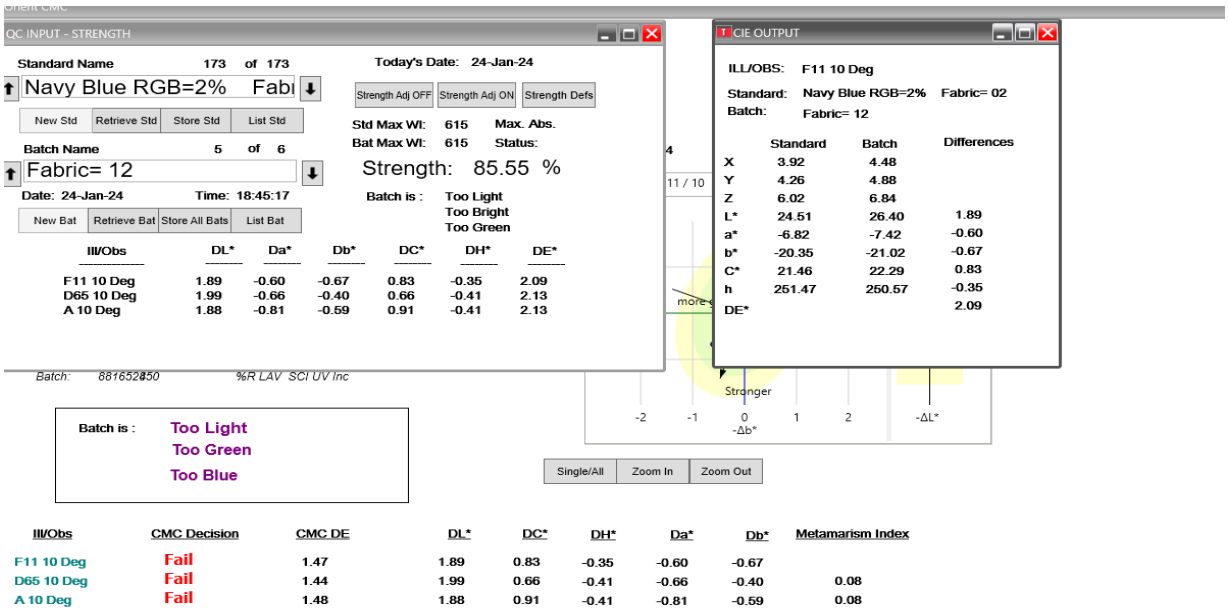


Figure: A40 L value for the dyed sample with Navy blue RGB of bio-scoured and bleached fabric of D8 HP50 and D8 HP50+SB

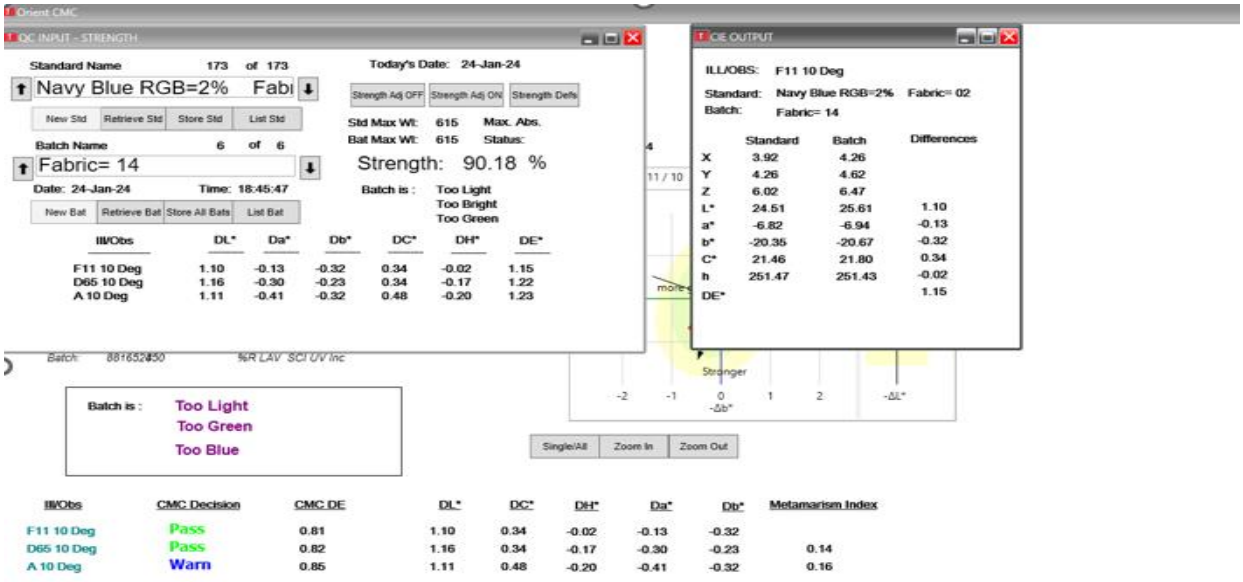


Figure: A41 L value for the dyed sample with Navy blue RGB of the bio-scoured and bleached fabric of CS HP50 and CSBP10

Table A1 The top ten BLAST hits for the 16 rRNA gene sequence of the isolate

Scientific name	Percent identity	Max score	Total score	Query cover	Alignment length	E-value	Accession
Burkholderia cepacia ATCC 25416	99.87	1404	1404	100%	1124	0	NR_118057.1
Burkholderia seminalis	99.74	1400	1400	100%	1498	0	NR_042635.1
Burkholderia paludis	99.74	1399	1399	100%	1497	0	NR_178850.1
Burkholderia cepacia ATCC 25416	99.74	1399	1399	100%	1462	0	NR_114491.1
Burkholderia contaminans	99.74	1399	1399	100%	1485	0	NR_104978.1
Burkholderia anthina	99.74	1399	1399	100%	1485	0	NR_104975.1
Burkholderia anthina	99.74	1399	1399	100%	1124	0	NR_118053.1
Burkholderia cepacia	99.61	1397	1397	100%	1458	0	NR_113645.1
Burkholderia territorii	99.61	1397	1397	100%	1443	0	NR_136496.1
Burkholderia aenigmatica	99.48	1393	1393	100%	1485	0	NR_174230.1

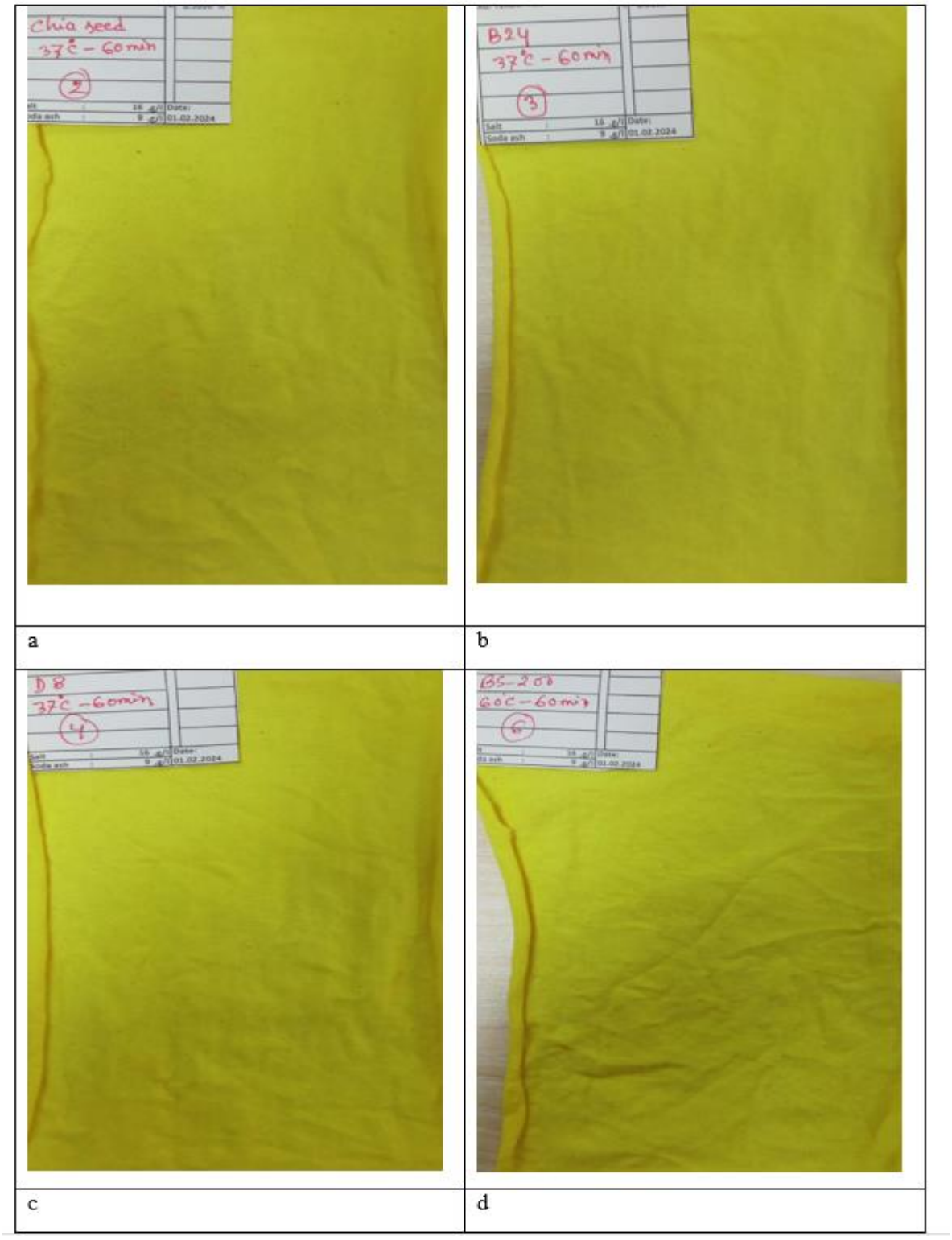
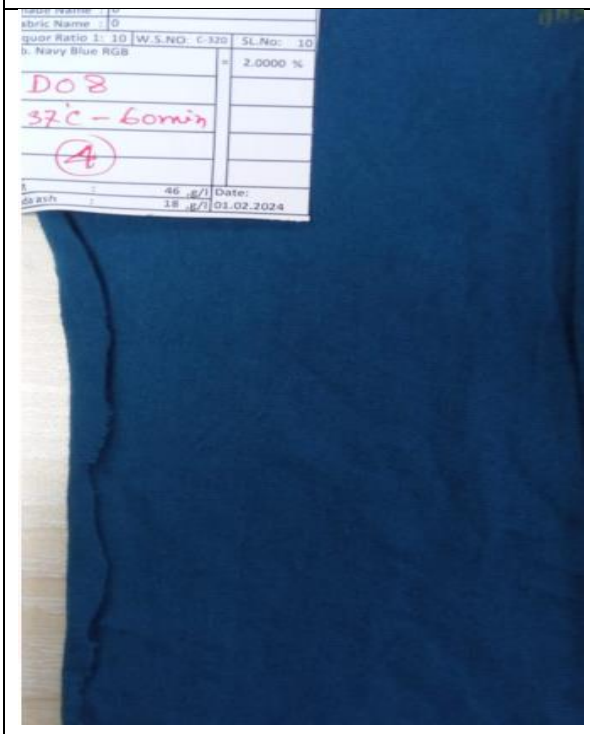


Figure: A42 Black dot marks and some patches are present in the yellow coloration with bio-scoured fabric a) CS 37 b) BL 37 c) D8 37 d) BS 60

a

B



c

D

Figure: A43 Black dot marks and patches are not visible in the dark Navy blue RGB coloration with bio-scoured fabric a) CS 37 b) BL 37 c) D8 37 d) BS 60

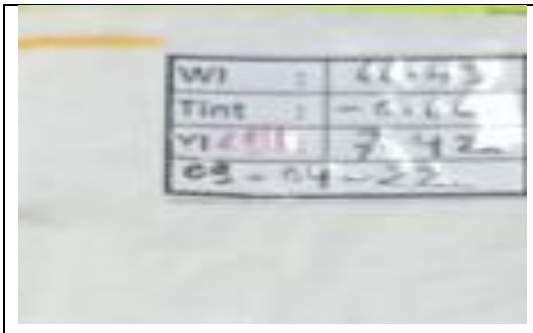
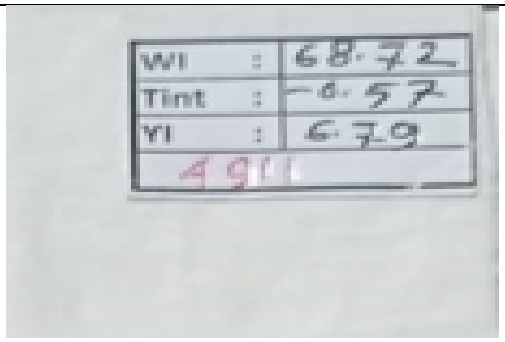
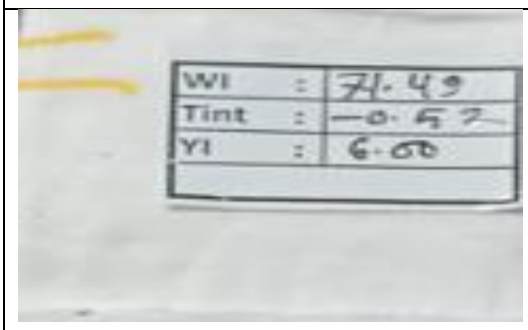
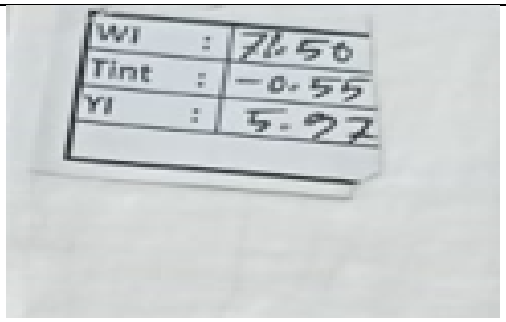
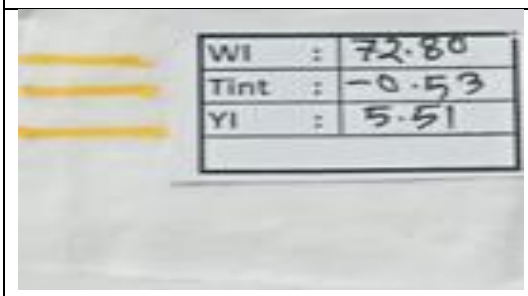
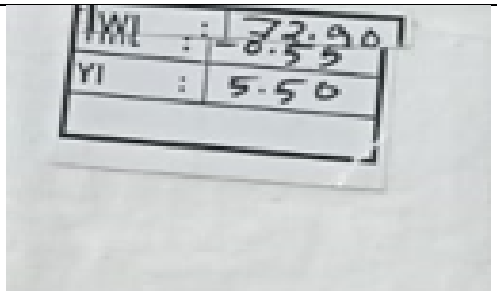
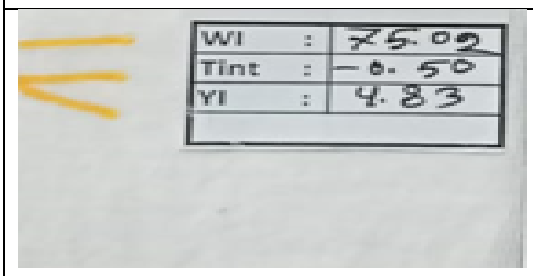
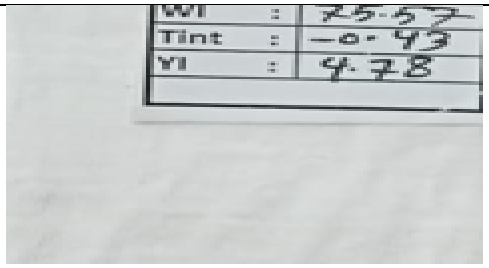
	
a. P1+P2a	b. P1+P2a+P3
	
c.P1+P2b	d. P1+P2b+P3
	
e. P1+P2c	f. P1+P2c+P3
	
g. P1+P2d	h. P1+P2d+P3

Figure: A44 W.I. for the bleached samples of 4,6,8 and 10 g/L of hydrogen peroxide and bleaching with thiourea at the same hydrogen peroxide bath

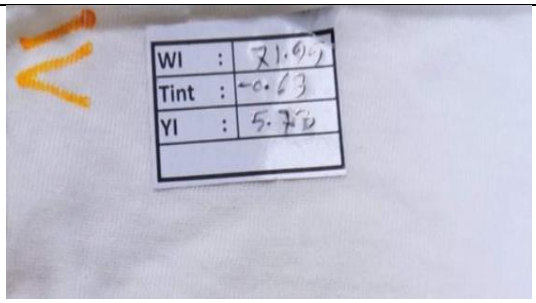
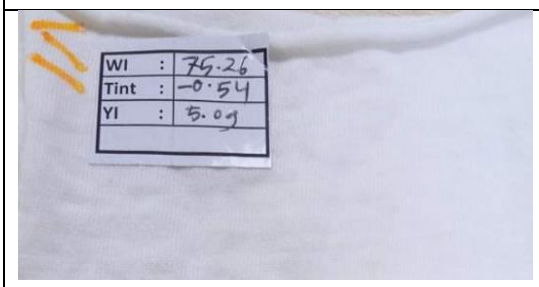
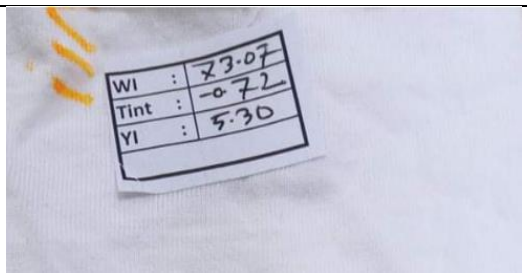
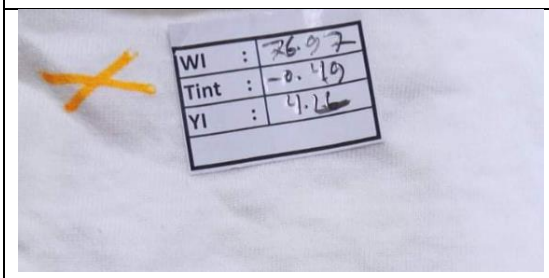
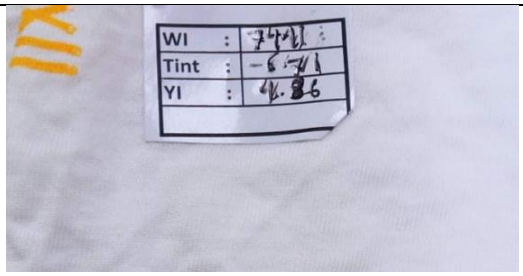
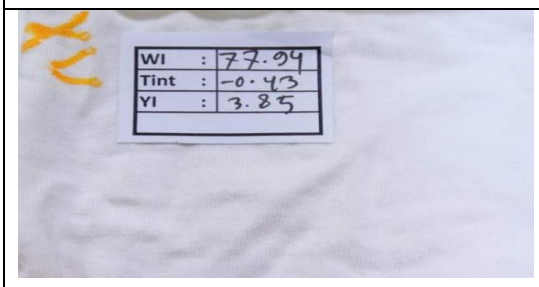
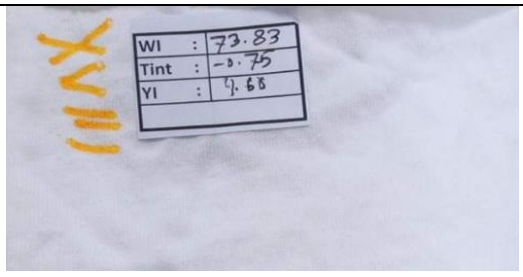
	
a.P1+P2d	b. P2d
	
c. P1+P2d+P3	d. P2d+P3
	
e. P1+P2d+P3+P4c	f. P2d+P3+P4c
	
g. P1+P2d+P3+P4g	h. P2d+P3+P4g

Figure: A 45 W.I. for the bleaching demineralized and grey fabric with 10 g/L hydrogens peroxide oxidative bath and sequential reductive bath

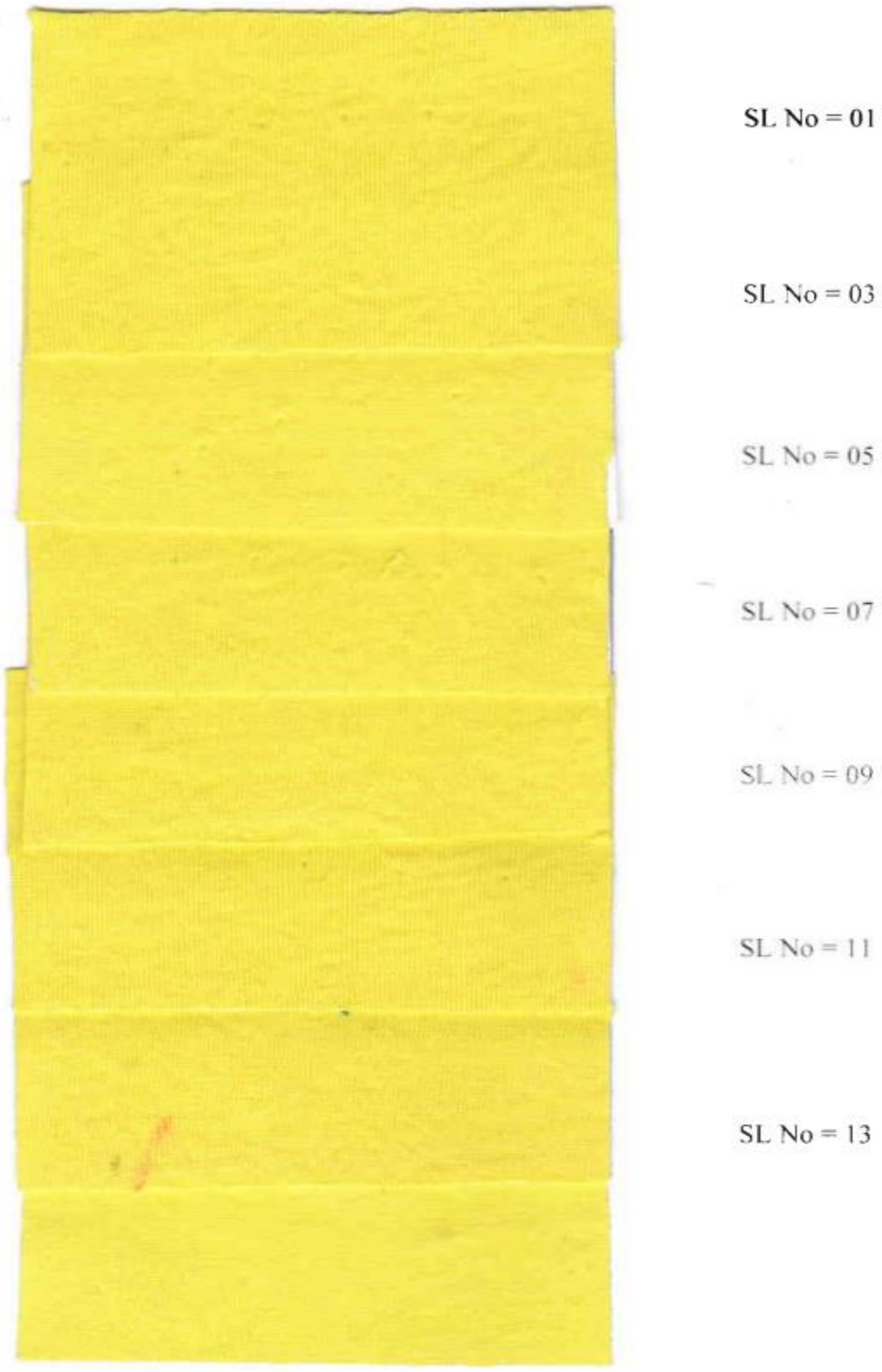


Figure: A 46 The dyed cotton knit fabric with 2% Yelow 4GL with 1) HP50 3) HP50+SB 5) BL HP50 7) BL HP50+SB 9) D8 HP50+SB 11 D8 HP50+SB 13)CSBP 10 treated fabric

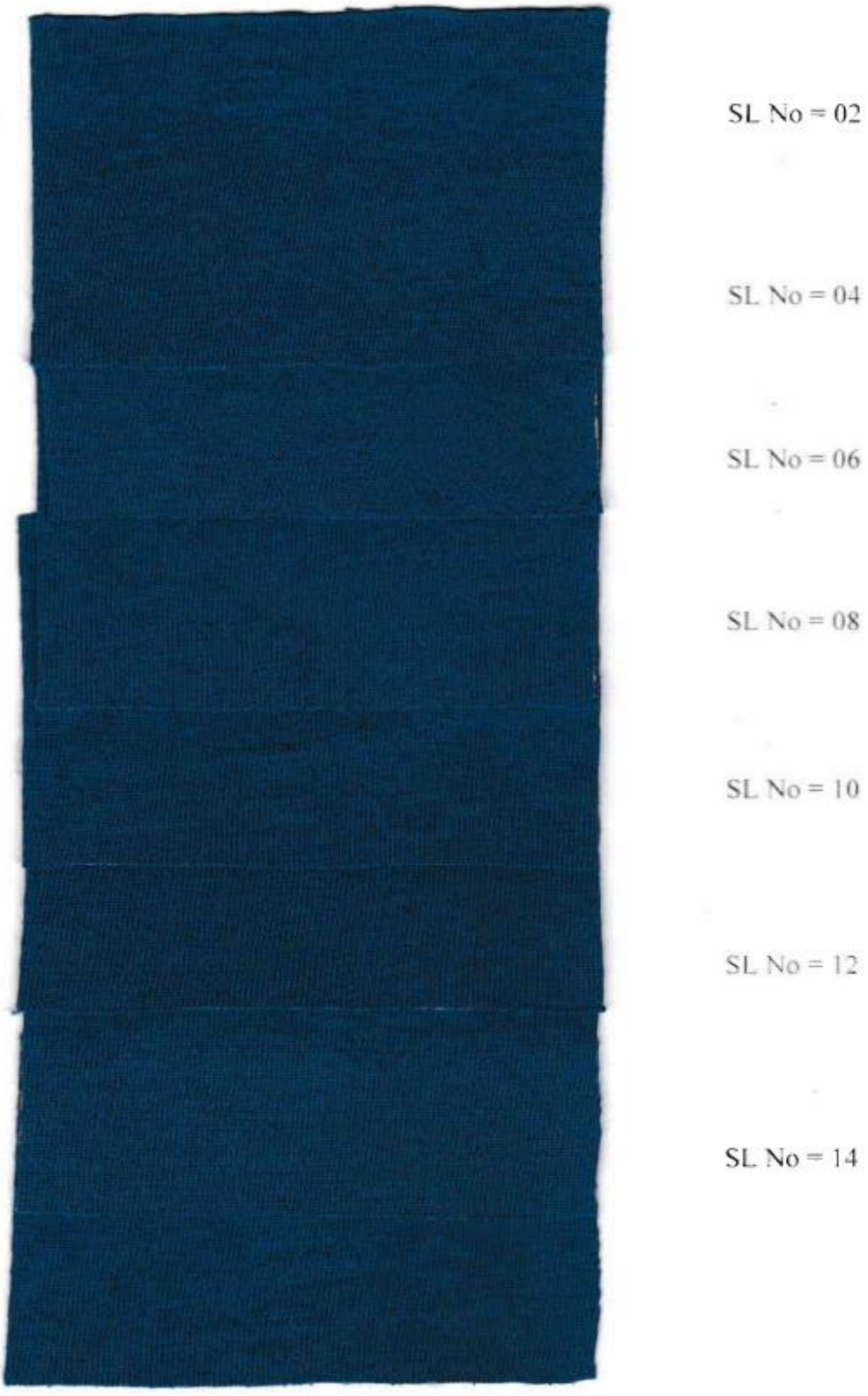


Figure: A 47 The dyed cotton knit fabric with 2% Navy Blue RGB with 2) HP50 4) HP50+SB 6) BL HP50 8) BL HP50+SB 10) D8 HP50+SB 12) D8 HP50+SB 14)CSBP 10 treated fabric

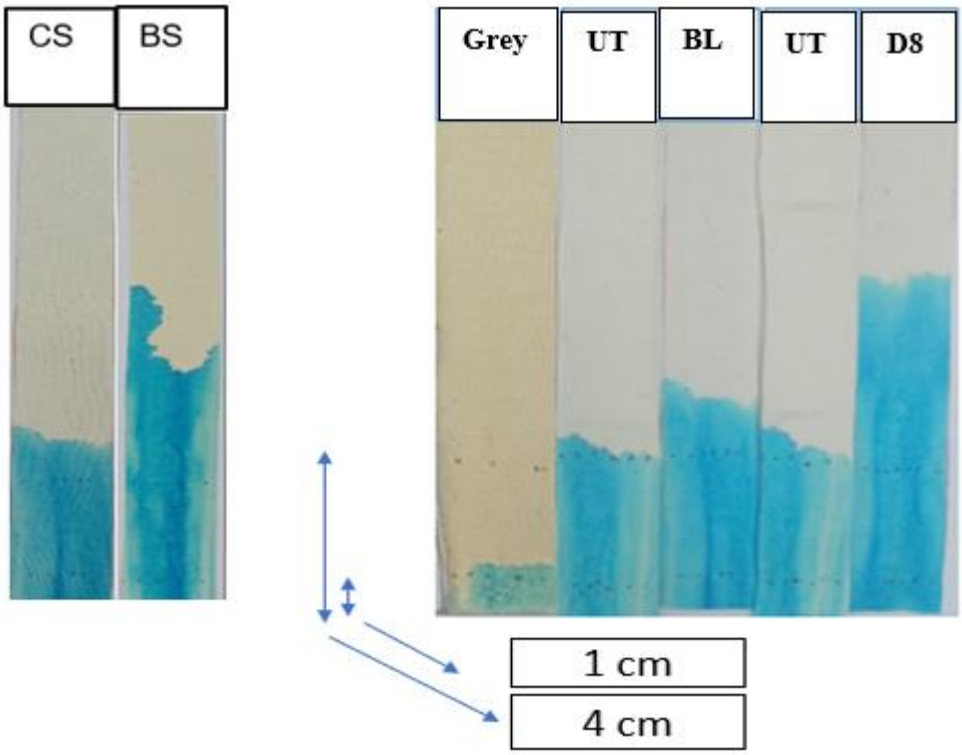


Figure: A 48 Wicking height in dry condition with the fabric treated at 37 °C