

Insights into multiple enzymes produced by an indigenously isolated novel *Aeromonas veronii* strain revealing diverse applications

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Introduction

Global demand for bioenergy is rapidly increasing therefore isolation and identification the microbes having better saccharifying activity are one of the prioritized areas for the researchers working on bioenergy. Effective saccharification of lignocellulose allows to produce a cost-effective bioenergy and biomass. Such microbes have a better ability to produce some potential enzymes for industrial use. Being non-toxic and biodegradable, in nature the enzymes can replace the various industrial processes involving hazardous chemicals and consuming high energy (Illanes et al. 2012, Choi et al. 2015). Production of enzymes from bacteria is cost-effective as well as faster in comparison to other organisms. Additionally, the production of the enzyme from microbes can be controlled physicochemically and physiologically. Sincere efforts are also being made for using the enzymes in different industrial processing leading to a drift towards a more sustainable means of production (Nedwin and Oxenbøll 2005).

In several processing, multiple enzymes are needed for production rather than a single enzyme such as paper and pulp industry, animal feed, and detergent making units. Amylases, lipases, proteases, and cellulases are used in the detergent manufacturing units. *Bacillus subtilis* is well known to be the source of protease used in laundry detergents. Next lipase from *Pseudomonas* species was introduced for the removal of greasy and oily stains with the trade name Lipomax® (Cox et al. 2001). Cellulase from Alkaliphilic *Bacillus* and *Thermomonospora fusca* has been found to be useful additives in the detergent as they aid in the conditioning of the cotton fibres (Irwin et al. 1998; Jones and Quax 1999). Additionally, amylases and mannases from bacterial sources are added for better performance of the detergents. Similarly, amylases, proteases, and cellulases from bacterial sources are used in the textile industry for a better quality of fabrics. Likewise, the paper and pulp industry needs cellulase, xylanase and pectinase, and the food processing industry is also dependent on multiple enzymes.

In the current study, we have reported an indigenous bacterial isolate capable of producing multiple enzymes. Cellulase, amylase, pectinase, and protease were co-produced from this bacterial isolate. This enzyme cocktail is useful in several industrial sectors such as the paper and pulp sector and textile manufacturing units for breaking down the plant-based raw materials as well as in waste management. Optimization of temperature and pH and conditions for obtaining the maximum enzyme activity has also been attempted. The application of enzyme cocktails in retting, stain removal, and waste management have been reported. The study suggests that the integrated effect of all the enzymes in the enzyme cocktail gives better results in the application-based study as compared to the individual enzymes.

Methodology

Sample collection

Samples for the isolation of the bacteria was collected from beneath the decaying tree trunk in the Botanical Garden of DDU Gorakhpur University. The sample was collected in sterile sample bags and brought to the laboratory for isolation of the microbes.

Isolation of bacteria

For bacterial isolation, 1 gram of collected soil sample was suspended in 10 ml of water. After proper mixing, the soil suspension was serially diluted upto 10^{-5} dilutions. The diluted soil suspension was plated on LB media and incubated overnight at 37°C for the growth of bacteria. A total of 100 morphologically distinct colonies were selected and subcultured on the fresh LB Agar plate. Each discrete colony was numbered in the master plate from 1-100.

Screening

The bacterial isolates were subjected to screening for the production of extracellular enzymes. Primary screening was carried out by plate assay for investigating the extracellular production of cellulase, amylase, pectinase, and protease enzyme. A specific plate supplemented with the corresponding substrate was prepared to carry out the plate assay for above-mentioned enzymes.

Cellulase assay: CMC agar plates (0.2% NaNO₃, 0.05% MgSO₄, 0.1% K₂HPO₄, 0.05% KCl, 0.2% carboxymethylcellulose sodium salt, 1.7% agar and 0.02% peptone) were streaked from the full-grown culture plates. The plate was incubated for 24 hours at 37°C, and production of cellulase enzyme was screened by flooding the plate with Gram's iodine solution (22% w/v KI, 0.6125% w/v Iodine) for 3 to 5 minutes (Kasana et al. 2008).

Amylase assay: For detection of amylase activity, the isolates from all five sources were spotted from the master plate on the starch agarose plate (0.3% beef extract, 1% soluble starch, 2% Agar). After incubation of plates for 24 h at 37°C, the plates were flooded with Gram's iodine (2% w/v KI, 0.6125% w/v Iodine). The iodine solution coloured all the starch except a clear halo was present against the amylase-producing strain (Facchin et al. 2013).

Pectinase assay: Pectinase plate assay is carried out by inoculation of the pectin agar plate (1% Pectin, 1% Tryptone, 1% NaCl, 0.5% Yeast extract, 2% Agar) with the fungal culture. Fully grown cultures obtained by

incubating at 37°C for 24 hours was screened for the presence of pectinase activity. The zone of hydrolysis was detected by flooding the plates with gram's iodine solution (Okonji et al., 2019).

Protease assay: Protease of the bacterial isolate was screened by streaking the colonies on the skim-milk agar plate (Vermelho et al. 1996). Protease activity was detected by observing the clear zone around the bacterial colonies.

Molecular identification of potent bacteria

For molecular identification, the potential bacterial isolate with positive activity for cellulase, amylase, pectinase, and protease enzymes was used. The ZR Fungal/Bacterial DNA MiniPrep kit was used for extraction of genomic DNA from the pure bacterial culture which was used for identification (Zymo Research). The universal 27F (AGAGTTTGATCCTGGCTCAG) and 1492R primers were used for the amplification of the 16S rRNA gene (TACGGYTACCTTGTACGACTT). On a 1% Agarose gel, the PCR product was observed. QIAquick Gel Extraction Kit was used to gel elute and purify the PCR amplicon (Qiagen). Sanger DNA sequencing was used to sequence the purified PCR result in a commercial setting. The resulting sequence was compared to a non-redundant NCBI database using BLASTN. The related sequence of *Aeromonas sp.* was downloaded from the NCBI database for phylogenetic analysis. The phylogenetic tree was prepared by Neighbour Joining method through MEGA7 software (Tamura et al. 2013) with bootstrap value of 1000.

Biochemical characterization of GCEL-BG85

Pure colonies of the strain were obtained and subjected for biochemical characterization. The biochemical tests such as Gram's staining, indole, methyl red, Voges Proskauer's test, citrate utilisation, lactose, sorbitol, mannitol, glucose, adonitol, arabinose, rhamnose, sucrose, urease, and catalase utilisation tests were performed on the bacterial isolate.

Production

The production of cellulase, amylase, pectinase, and protease enzyme was carried out on LB medium. The flask was set at 120rpm at 37°C for 24 hours. The culture broth was subjected to centrifugation at 10,000rpm for 5 minutes to obtain the crude enzyme which was used for further experiments.

Biochemical assay of enzymes

Cellulase activity: The quantity of reducing sugars produced during hydrolysis was used to determine cellulase using the DNS (3,5-dinitrosalicylic acid) technique (Miller 1959). As a substrate, a 1 percent solution of CMC was produced in phosphate citrate buffer pH 7.0. 1 ml CMC solution, 250 µl crude enzymes and 750 µl citrate

buffer were taken in reaction mixture. For 30 minutes, the mixture was incubated at 50 degrees Celsius. The reaction was then stopped by adding DNS to the solution. After adding DNS, the sample tubes were boiled for 10 minutes and then cooled in water for colour stability before being monitored at 540 nm. The quantity of enzyme required to hydrolyse CMC and release 1 mole of glucose was defined as one unit of cellulose activity.

Amylase activity: The amylase test was performed with a reaction mixture containing 1 ml substrate (1.0 percent soluble starch in citrate phosphate buffer pH 7.0), 750 µl of buffer, and 250 µl of enzyme solution (Miller et al., 1959) were used to conduct the test. Enzyme activity was measured in units, with 1 unit/ml equalling the quantity of enzyme that releases 1 mole of glucose in the test condition (Mishra et al., 2008).

Pectinase assay: The reaction mixture containing 250 µl of enzyme, 1000 µl ml citrus pectin (1 percent w/v) and 1.75 ml of 100 mM citrate phosphate buffer was used to determine pectinase activity (pH 7.0). Changes in optical density at zero time and after 30 minutes was measured at 235 nm. The pectinase activity was measured in micromoles of unsaturated product released per minute (molar extinction coefficient = 5,500 M⁻¹ cm⁻¹) (Albersheim 1966).

Protease assay: Protease activity was performed using casein as a substrate (Tsuchida et al. 1986). 1000 µl of 1% (w/v) of casein in citrate phosphate buffer, pH 7 and 250 µl of crude enzyme extract were incubated in a water bath at 50°C for 30 minutes. After 30 minutes the enzymatic reaction was stopped by adding 1 mL of 10% (w/v) trichloroacetic acid (TCA) and the reaction mixture was cooled for 15 minutes. The unreacted casein was separated by centrifuging the reaction mixture for 5 minutes at 10,000 rpm. The supernatant was transferred to new tube and combined with 2.5 ml of 0.4M Na₂CO₃ and 1 ml of three times diluted Folin -Ciocalteus phenol reagent. The blue colour generated was quantified at 660 nm against a reagent blank using a tyrosine standard after 30 minutes of incubation at room temperature in the dark (Lowry et al.,1951). Under typical supernatant solution conditions, one unit of protease is the quantity of enzyme that releases 1 g of tyrosine per ml per minute.

The total crude enzyme preparation was quantified by the Lowry method taking bovine serum albumin (BSA) as the standard.

Characterization of enzymes

The pH optima of cell free extract was determined by measuring steady-state velocity using substrate for respective enzymes at 50°C in the buffered reaction solution. The reaction was carried out in the presence of buffers in the pH range of 1.0–12.0. The different buffers used were: for pH 1.0–2.0 hydrochloric acid-potassium chloride, citrate-phosphate for pH ranging from 3.0–7.0, sodium-phosphate buffer for 8.0 pH, for pH 9.0–10.0 glycine-

NaOH buffer was used and sodium phosphate-NaOH (pH 11.0–12.0). The activities of all four enzymes were assayed by the method mentioned above and plotted in the form of pH versus activity.

The activity of enzyme was best measured at temperatures ranging from 10 - 80 °C. The temperature of the reaction solutions was plotted against enzyme activity in a graph by sigma plotter.

Applications of the enzyme

Application in retting

The enzyme extract was used to carry out retting of natural fibres of *C. juncea* (Sun hemp) mature stems using a reported method (Zhang et al. 2005) with minor modifications. For studying the retting process by the enzyme, two sets of tubes were taken, and each tube had 10 cm of the stem along with 10 ml of sodium phosphate buffer of pH 8.0. One set containing 10 mM EDTA was supplemented with 100 µl, 500 µl, and 1ml of the crude enzyme along with the control tube without any enzyme. Another set of the tubes was the same except that they did not contain EDTA. Since the pectinase enzyme is well known to for retting, one tube containing 200 IU of commercial pectinase enzyme was added to each set, with and without EDTA. For 24 hours, all of the test tubes were incubated in a water bath at 37°C. After 24 hours, the stems were violently shaken for 1 minute with 10 ml hot water. The hot water was drained out, and pictures of stems were taken.

Application in Blood and Egg Yolk Stain Removal

The efficiency of the crude enzyme in stain removal from cotton cloth was evaluated. White cloth pieces of 4X4 cm² stained with blood and egg yolk were treated with two different detergent solutions, Surf Excel and Ghadi detergent (7mg/ml) and in combination with the enzymes. The stained cotton pieces were dried in hot air oven for 5 minutes at around 95-100 °C. Each set of stained cloth, with blood and with the egg yolk were washed with 10ml of the each detergent solution alone, 500µl of the enzyme alone and in combination of enzyme with each detergent. Stained cloth incubated with water was taken as control (Nassar et al. 2015).

Waste Management: Degradation of Chicken Feather

Chicken feather was cleaned properly with water and autoclaved. The feathers were air-dried overnight and then subjected to disintegration test. Whole chicken feather was incubated in the crude enzyme solution at 70 °C for 4 hours. This reflects its potential application in both waste management as well as in the detergent industry (Bhunja et al. 2013).

Fruit Juice extraction

25 grams of freshly cut apple pieces along with 4ml of the crude enzyme were ground with the help of mortar and pestle. The paste pulp formed was transferred to 50 ml centrifuge tube along with 10 ml of the water rinsed from the mortar and pestle. The apple pastes and enzyme mixture were allowed to incubate for 1 hour at 50°C and then centrifuged at 5000 rpm for 20 min. The volume of clear supernatant formed was measured and used for analysis of the percent transmittance and percent recovery of the juice.

Application in oil extraction

For evaluating application in oil extraction, 2 grams of sesame seeds were ground with 2ml of the crude enzyme. Instead of enzyme, water was taken in the control set. The paste thus formed was transferred to 50 ml centrifuge tube along with 10 ml of distilled water. The tube was incubated at 50°C for 1 hour followed by centrifugation at 4000 rpm for 20 min. The separated emulsified oil layer was collected in a separate tube and the volume of the liquid collected was measured.

Results

Isolation and screening of bacteria

Out of the total bacteria isolated from the soil sample, only 100 bacterial isolates were selected. These selected bacterial isolates were screened according to zone of hydrolysis around the colonies for the enzymes cellulase, amylase, pectinase, xylanase and protease. The result revealed that 41 isolates were producers, of at least one enzyme, by plate assay screening. Out of those 41, isolates the numbers 29,38,60, and 79, produced two enzymes, isolates numbers 52, 56, and 58 produced three enzymes while isolating numbers 16 and 85 produced four enzymes. The isolate number 16 fail to produce pectinase, while number 85 was unable to produce xylanase. Based on the dp/dc ratio on the plate assay, the strain number 85 exhibited better activity as compared to the corresponding enzyme for strain number 16. Therefore, isolate number 85 was selected for this study and for further characterization (**Fig. 1**). This strain was named as GCEL-BGb85. The activity represented by each enzyme from this strain was expressed in terms of dp/dc ratio. Where dp is the diameter of transparent zone and dc is the diameter of the colony (**Fig. 2**).

Efficacy of this enzyme in the production of extracellular enzyme by liquid culture was also assessed.

Fig. 1

Fig. 2

Identification, phylogenetic analysis and characterization of the isolate

A molecular approach based on the 16S rRNA sequencing was used for the identification of the isolated bacteria. The 16S sequence was used to carry out the BLAST with database of NCBI Genebank. The sequence exhibited closest similarity with *Aeromonas veronii*. The sequence has been submitted in the NCBI Genebank and have been assigned Accession number OM190479. Sequences of all related *Aeromonas* species were subjected to phylogenetic analysis along with *Aeromonas veronii* to evaluate its phylogenetic ancestry. This strain grouped closely with *Aeromonas rivipollensis* and *Aeromonas hydrophyla*. However, it exhibits distant relationship with *Aeromonas dhakensis* and *Aeromonas enteropelogenes* (**Fig. 3**). The biochemical characterization tests results are listed in **table 1**.

Fig. 3

Table 1

Enzyme activity and protein concentration

The crude enzyme produced by liquid fermentation has concentration of 12.53 mg/ml. The activity of cellulase, amylase, pectinase and protease were 0.071, 0.201, 0.202, 0.152 U/ml respectively.

Effect of pH and temperature on enzyme activity

The temperature and pH optima of all the four enzymes produced by bacterial strain were estimated. All the four enzymes, cellulase, amylase, pectinase and protease exhibited maximum activity at 40°C (**Figure 4**) while the pH optima were different for each of the targeted enzyme. Amylase had best activity at pH 9 but significant activity was also observed at the acidic pH of 5. Cellulase also exhibited activity at both acidic and alkaline pH i.e. at pH6 and pH 12. Maximal activity of pectinase enzyme was observed at pH 12 but activity was also observed at acidic pH (pH 6.0). Protease had optimal activity only at alkaline pH 11 (**Figure 5**).

Fig. 4

Fig. 5

Applications of the enzyme

Retting efficiency of the enzyme

The crude enzyme preparation was tested for its ability for retting *Crotolaria juncea* natural fibre. Better retting was observed in the absence of EDTA as compared to the conditions where 10mM EDTA was added in the reaction condition. Retting efficiency was very low when the stem was incubated with 200 Units of commercial pectinase enzyme (Himedia) (**Figure 6**).

Fig. 6

Wash performance analysis

The enzyme in combination with both the detergents, Surf excel and Ghadi detergent completely removed the blood stain while the egg stain was reduced to great extent (**Figure 7**).

Fig. 7

Waste management- Chicken feather degradation

Analysis of feather disintegration by the crude enzyme containing protease suggested that a considerable amount of feather enzyme was degraded. Thus, this enzyme may play role in the waste management (**Figure 8**).

Fig. 8

Application of the enzyme cocktail in fruit juice extraction and oil extraction

The apple juice recovery was better with the crude enzyme as compared to the control where water was taken instead of the enzyme. Moreover, there was also increase in the % Transmittance of the extracted fruit juice with better clarity than the control sample **Table 2**.

Table 2

Discussion

A large number of studies have reported isolation of novel microbes from a diverse soil sample and diverse bacteria are predominant in the soil microflora (Jacoby et al. 2017). These bacteria maintain the ecological balance, maintain the natural properties of the soil and convert toxic compounds into molecules that can be easily used by the plant in metabolic pathways (Arasu et al. 2019; Arasu et al. 2017; Lladó et al. 2017). Apart from carrying out these natural processes, the soil bacteria are extensively being used in diverse industrial processes (Khusro et al. 2014; Sankari and Khusro 2014). To cater the increasing demand of the enzyme in industrial applications, researchers are constantly looking for enzymes with better catalytic application. Moreover, many industrial processes need multiple enzymes at different steps of the manufacturing process. In this context, the bacterial isolate mentioned in this report to coproduce multiple enzymes that can be used in several industrial applications.

A study by Singh et al., 2015 has also reported optimization of xylano-pectino-cellulolytic bacteria after extensive screening on the agriculture residues and commercial substrates (Singh et al. 2015). Protease, amylase and cellulase production has been carried out by lactic acid bacteria (Ho and Yin Sze 2018). The bacteria have been isolated from traditional fermented food. They have proposed the use of this strain for silage production. Halophylic bacterial strains have been screened to produce extracellular hydrolytic enzymes (Rathakrishnan and

Gopalan 2022). Out of the 28 cultures screened, nine isolated exhibited positive activity for amylase, lipase, protease and cellulase enzymes.

Conventionally, the enzymes used in the processing are individually produced by the microbe are applied for processing. But it would be more feasible if a single organism producing multiple enzymes can be used. There are several reports of multiple enzymes producing microorganisms. Fungi are the preferably used for multiple enzymes production as there are very few reports for bacterial strains (Chugh et al. 2016). Since enzymes are powerful biocatalysts, industrial biotechnology based on enzyme production and application has become a priority. Applications of enzymes range from the synthesis of new products to product development, environmental management, and service production. (Ibrahim 2007). A multienzyme complex comprising of amylolytic/xylanolytic/cellulolytic enzyme has been discovered from mesophilic anaerobic bacterium *Clostridium manihotivorum* CT4 (Cheawchanlertfa et al. 2021).

The multiple enzymes thus produced can be applied in industrial applications such as retting. It is an important step for releasing the fibres from the stems to make them suitable for use in textile and paper and pulp industries. Pectinolytic enzymes polygalacturonases and pectin lyases have been reported to be produced by microbes during the water retting process (Zhao et al. 2016). Pectinolytic enzymes are widely used in fruit juice clarification. However, cellulases and xylanases have also been reported in the clarification of several fruit juices (Li et al. 2020; Santana et al. 2021). Since the enzymes-based process is eco-friendly and provides a sustainable alternative to chemical-based processing. But apart from industrial processing, it can be used in waste management. Proteases can be exploited in the degradation of chicken feathers which is an environmental concern to be addressed. Most of the study has reported keratinase for this process (Parinayawanich et al. 2021; Tamreihao et al. 2019).

Several bacterial isolates from the soil sample have been shown to generate these enzymes individually. Cellulase isolated from soil bacteria are significantly used several industries. *Pseudomonas fluorescens*, *Bacillus subtilis*, *E. coli*, and *Serratia marcescens* that produce cellulase were isolated from soil sample by Sethi et al (Sethi et al. 2013). *Pseudomonas* sp., obtained from a trash dumping site, was purified to produce a 60 kDa cellulase enzyme (Castaeda-Cisneros et al., 2020). It was reported to have optimum activity 50°C and pH 7.0, the enzyme stability was assessed in the presence of metal ions, inhibitors, surfactants and commercial detergents.

A thermostable amylase producing bacteria, *Pseudomonas aeruginosa* has been isolated from brick kiln soil (Ullah et al. 2021). This isolate exhibited maximum amylase activity of 5.9 U/ml. Thermophilic and halophilic

amylase producing bacteria have been isolated from hot and arid region (Yassin et al. 2021). These isolates were identified as genus *Bacillus*. These strains exhibit activity in the range of 55 to 80°C and pH varying from 5-9.

Pectinase enzyme producing *Enterobacter sp.* MF84, isolated from soil have been used for pectinase enzyme production (Abdollahzadeh et al. 2020). This enzyme was maximally active at 30 °C and at pH 9. Moreover, its optimal production has optimized using response surface methodology (RSM). Pectinase producing bacterial have been isolated from forest soil. They have been identified as *Streptomyces sp.*, *Cellulomonas sp.*, and *Bacillus sp* (Shrestha et al. 2021). The crude extract from these bacteria have been found useful in oil and juice extraction. Another pectin producing *Bacillus* strain has been isolated from soil sample of fruit waste (Murugan et al. 2021). It exhibited specific activity of 484.70 U/mg and worked best at pH 6.0 and at 40 °C.

Protease-producing bacteria have also been recovered from soil samples, according to multiple publications. Serratiopeptidase enzyme-producing bacteria have been identified from mulberry plant tissues and rhizospheric soil, according to a recent publication (Koul et al. 2021). *Serratia marcescens* MES-4 and *Serratia marcescens* MRS-11 were found as the bacteria. Both strains had activity levels of 95 and 156 units per millilitre, respectively. *Bacillus amyloliquefaciens* strain HM48, another protease producer, has been discovered in the Dachigam National Park soils (Mushtaq et al. 2021). The maximum activity of this enzyme was observed to be about 25 kDa at pH 8.0 and 70 °C. Its effectiveness in terms of washing and garbage disposal has been documented by the authors. Another *Bacillus* strain, *B. pumilus*, has been discovered to generate cold-active protease, which was isolated from the Himalayan Thajwas Glacier (Farooq et al. 2021). This enzyme has a specific activity of 37.02 U/mg and peaks at pH 9.0 at 20°C.

The most frequent molecular approach for identifying and classifying bacteria isolates is analysis the of 16S rRNA gene sequence. Because the 16S rRNA gene is conserved for all bacteria, it may be utilised to differentiate taxa (Ntushelo et al. 2012). *A. veronii* was discovered to be the bacterial strain. Hickman-Brenner et al. characterised it for the first time as a Gram-negative, rod-shaped bacteria (Hickman-Brenner et al. 1987). It has been reported from environmental, clinical, and dietary samples, among other places (Ghenghesh et al. 2008). *A. veronii* NS07 strain generating -amylase was isolated from agricultural soils, according to research (Samie et al. 2012). The enzyme was psychrophilic as well as unique, as its N-terminal amino acid sequence did not match any other known -amylases. *Aeromonas* has 38 species, according to the NCBI taxonomy bank, with the majority of them being pathogenic (Rodriguez-Morales and Castaeda-Hernández 2019). *Aeromonas hydrophila*, *A. caviae*, *A. veronii*, *A. biovar* and *A. sobria* are pathogenic strains of the genus *Aeromonas* that may be found in both fresh and brackish water.

The application of the enzyme cocktail containing cellulase, amylase, protease and pectinase enzyme was investigated in retting, fruit juice and oil extraction, and in waste management in degradation of chicken feathers.

Retting is the separation and extraction of fibrous components from non-fibrous components in bast fibres such flax, hemp, jute, sunn hemp, ramie, and kenaf (Kumar et al., 2020). Bast fibres are a great source of fibre that may be utilised in a variety of goods, including textiles, high-quality papers, reinforcing materials, plastic replacement, composites, and so on (Fernando et al., 2019). A high percentage of cellulose and a low amount of hemicellulose, lignin, and pectin are required in a high-quality fibre. Dew or field, pool, stream, and water retting are all traditional procedures. The water retting method is the most popular, although it is a costly, time-consuming, and energy-intensive procedure. It also emits undesirable odours and releases toxic fermentation waste such as galacturonic acid, butyric acid, acetic acid, lactic acid, Ca^{2+} , Mg^{2+} , and bicarbonate content (Majumdar et al., 2019). Hence, alternative retting processes, such as enzymatic and microbiological retting, are necessarily needed (Kumar et al., 2020). Enzymatic and microbial retting has proved to be a viable and cost-effective alternative to chemical degumming and traditional retting. Moreover, the enzymatic fibre processing is easier to control, less time, and produces less effluents. All microbial bast fiber retting methods are entirely functions of microbial enzymatic activities (Yadav et al., 2016, Hasan et al., 2020). In microbial retting process, fibres are separated by using solvent maceration and bacteria enter through the stomata in cell wall tissues. Bacterial retting depends on their optimal growth depending upon temperature, pH, and oxygen availability. (Kapoor et al. 2001) investigated chemical and enzymatic degumming of ramie and sunnhemp fibers, concluding that individually none of above treatment is adequate to degum these bast fibres. The outcomes were improved by using a modest chemical treatment prior to the enzymatic treatment. (Sharma and Satyanarayana 2012) reported enzymatic retting of ramie fibres with thermoalkalstable pectinases from *Bacillus pumilus* DCSR1. Improvement in Young's Modulus and tensile strength of the fibres was observed after treatment of fibers with NaOH (0.04%) followed by pectinase treatment (300 U/g dry fibre). Enzymatic retting process is carried out by alkaline pectinases, hemicellulase, and laccases. Alkaline pectinases are mainly used in retting and the main enzymes are polygalacturonase, pectate lyase, and esterase. Pectinolytic microbes with xylanolytic activity but no cellulase activity can help enhance fibre quality. Xylanase enzymes for the retting process that are cellulase-free and alkaline resistant. Bacterial xylanase is alkali tolerant, unlike fungal xylanase, which makes it ideal for bast fibre retting. As a result, during the retting process, cellulase-free xylanase and pectinase enzymes are preferred. *Escherichia coli*, *Rhodobacter sp.*, *Pseudomonas fulva*, *Rhizobium huautlense*, *Massilia timonae*, *Pantoea*

agglomerans, *Pseudomonas rhizosphaerae*, are among the bacteria employed in retting, whereas fungi such *Epicoccum nigrum* *Cladosporium herbarum*, and *Alternaria alternata* (Fernando et al., 2019).

Pectinase has been solely and predominantly being used in the fruit juice industrial sector since decades. In this study we have tested the ability of cell free extract in the extraction of apple juice and oil extraction from sesame seeds. As evident from **the table 2** better extraction was obtained in the presence if the enzyme as compared to the control. Pectinase aids in the process by breaking down the pectin present in the apple and in the seeds (Demir et al. 2014). The degradation of pectin leads to reduced water holding capacity of pectin which causes release of water from the system hence the extraction yield is increased (Kashyap et al. 2001). The synergistic action of other enzymes, cellulase, amylase and protease produced by the isolate facilitates the process (Sharma et al. 2014). Thermostable protease, lipase and amylase produced by *Bacillus amyloliquefaciens* KUB29 have been effectively used in bio-oil extraction process (Devaraj et al. 2019). Cellulase and pectinase pre-treatment together is effective in increasing the yield of oil extraction along with reducing the viscosity of the extracted oil (Huang et al. 2022). These reports suggest that the combined synergistic effect of multiple enzymes is more effective in oil extraction than the conventionally used pectinase alone. Similarly in the fruit juice extraction, endo-xylanase from *Aspergillus japonicus* (UFMS 48.136) has been used in the clarification of mango, banana and tangerine juice (da Silva et al. 2019). Extraction of banana juice has been optimized with the presence of cellulase and pectinase enzymes (Handique et al. 2019). Cellulase-free xylanases from *Fusarium* sp. 21 has also been studied to be useful in fruit juice processing (Li et al. 2020).

Cellulase, lipase, protease and amylases are the most commonly used enzymes in the detergent industry (Singhania et al. 2015). Since the cell free lysate in the current study contained cellulase, amylase and protease enzymes, it was evaluated for its application in the detergent industry by subjecting the enzyme to cleaning the blood and egg yolk stains on cotton cloth cuttings. Currently detergent compatible enzymes are being added by most of the detergent industries as it acts as an eco-friendly substitute to the toxic chemicals which are otherwise added to the detergents. Cellulases have been found to be compatible with a wide range of commercial detergents as evident from large number of reports (Niyonzima 2019). In some studies, cellulases have been coproduced with lipases, proteases and amylases. A study has reported extracellular coproduction of cellulase along with amylase and protease by *Bacillus* sp. SMIA-2 and these enzymes were protected from proteolysis by protease enzyme (Ladeira et al. 2015). *Bacillus flexus* XJU-1 has also been subjected to co-production of lipase, amylase and protease by submerged fermentation and in this case too it was not degraded by the protease (Niyonzima and More 2014). So, the enzymes used in the detergents should be compatible with other enzymes used in the

detergents (Caparrós et al. 2012). This is in agreement of our study where the cellulase, amylase, pectinase and protease were coproduced and the crude enzyme was effective in stain removal. Enzyme was compatible with both the detergents used and was effective in stain removal. Mushtaq et al. have reported that extracellular protease from bacterial strain *Bacillus amyloliquefaciens* is compatible with commercial detergent as exhibits effective wash performance on blood and egg-yolk stains (Mushtaq et al. 2021).

Chicken feather is a by-product of the poultry industry that accounts for 5-7 percent of the chicken's body weight. The refractory pollutant material in chicken feathers is keratin protein (over 90 percent). Because of their resistance to protease breakdown, untreated chicken feathers may pose an environmental threat as a result of rising waste feather output from poultry operations (Li 2019). Untreated feather waste may harbour a variety of dangerous microorganisms and create pollutants including N₂O, NH₃, and H₂S, all of which are damaging to the environment, plants, animals, and people's health (Tamreihao et al. 2019). As a result, many academics are keen in using economic ways to transform chicken feathers into value-added items (Kang et al. 2018).

Tesfaye et al. described in their review, chicken feathers can be changed into different valuable products; in decorative applications, fertilizer, dusters, medical devices, bedding materials, and feedstock (Tesfaye et al. 2017). The conventional feather processing approaches such stem pressure cooking method and chemical treatment can convert it into animal feeds such as feather meals, but the processes require a huge amount of energy and some important amino acids are destroyed during treatment. The third most abundant material keratin after cellulose and chitin in environment can be decomposed by many microbes such as fungus, actinomycetes and bacteria.

The breakdown of chicken feather keratin protein by fungus proteases has been documented. Masood et al. found that *Aspergillus flavus* produces keratinases, which might help decompose chicken feather waste (Masood et al. 2021). Kim describes the purification and characterization of *A. flavus* keratinases protein (Kim 2007). *Bacillus* sp., *Brevibacillus*, *Geobacillus*, *Chryseobacterium*, *Xanthomonas*, *Nesterenkonia*, *Stenotrophomonas*, *Pseudomonas*, and *Serratia* are just a few of the bacteria that may produce feather-degrading enzymes. The ideal temperature (28–90°C) and pH (5.8–11) for bacteria-produced keratinases were found to act in a wide range (Tamreihao et al. 2019).

This study reports the isolation of indigenous bacteria from soil samples, its characterization, screening, identification, and application study. The cell-free extract of the bacterial isolate was effective in several applications. Hence the enzyme cocktail acts by the synergistic action of the enzymes and give effective results as compared to the activity of individual enzymes alone.

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Tables

Table 1. Biochemical characterization of the isolate GCEL-BG85

TEST	ACTIVITY
Indole	-
Methyl Red	+
Voges Proskauer's	+
Citrate Utilization	-
Glucose	+
Adonitol	+
Arabinose	+
Lactose	+
Sorbitol	+
Mannitol	+
Rhamnose	+
Sucrose	+
Urease	-
Catalase	-

Table 2. Application of the enzyme isolated from GCEL-BG85 in oil and juice extraction.

Sample	Oil Extraction (ml)	Juice Extraction (ml)	% Juice recovery	% Transmittance
Control	4.5	7	39.2	10
Enzyme	5.5	10	41.2	11.48

Figure legends

Fig. 1. (a) Primary screening by plate assay of the 100 bacterial isolates isolated from the soil sample collected from the Botanical Garden of DDU Gorakhpur University, Gorakhpur. (b) Graphical representation of the number of strains exhibiting multiple enzymatic activity.

Fig 2. Primary screening of GCEL BGb85 by plate assay and dp/dc ratio estimation for all the enzymatic activity.

Fig 3. Neighbour Joining (NJ) tree based on DNA sequences of the 16S rRNA gene showing the phylogenetic relationships. The values present on the branches represent the bootstrap support values.

Fig 4. Temperature is optimal for cellulase, Amylase, Pectinase, and protease enzymes from the Bacterial isolate GCEL BG85. All of these enzymes exhibited maximal activity 40°C.

Fig 5. pH optimal of cellulase, Amylase, Pectinase, and protease enzymes from the Bacterial isolate GCEL BG85. Except for protease, all the enzymes exhibited activity at both acidic and alkaline pH.

Fig 6. Effect of enzyme produced from GCEL-BG85 on retting of natural fiber: *C. juncea* (Sunn hemp); Stems were treated with the enzyme in the presence or absence of 10 mM EDTA. The control stem was treated with water instead of an enzyme. Since pectinase plays the main role in retting, the retting efficiency of the enzyme produced was compared with that of 200U of commercial pectinase (Himedia)

Fig 7. Wash performance analysis of the enzyme from GCEL BG85. Enzyme alone and in combination with the detergents were used for removal of egg stain and bloodstain from the cloth.

Fig 8. Application of the enzyme in waste management. Significant feather disintegration was observed on incubation of the chicken feather with the enzyme solution.

Figures

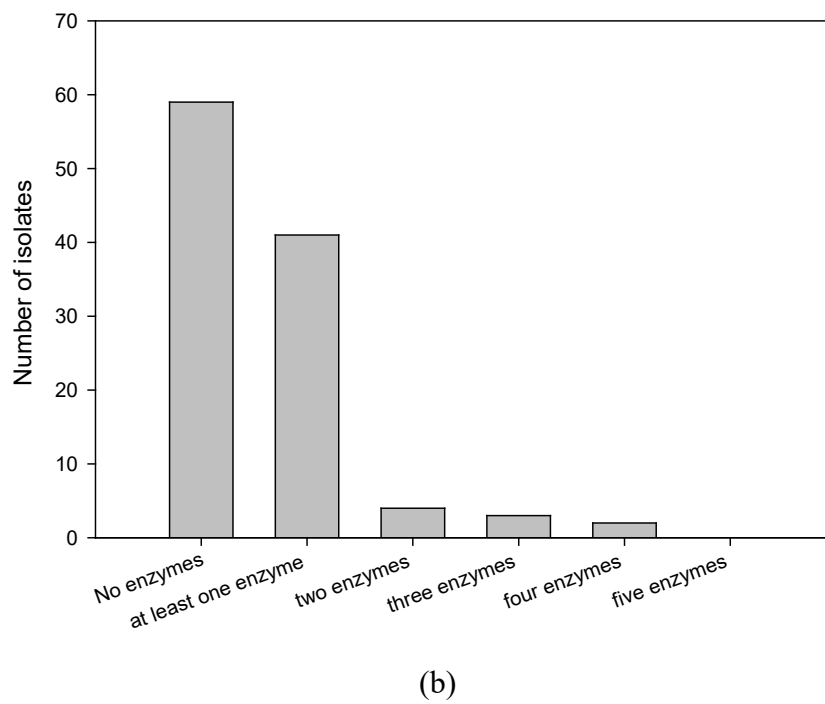
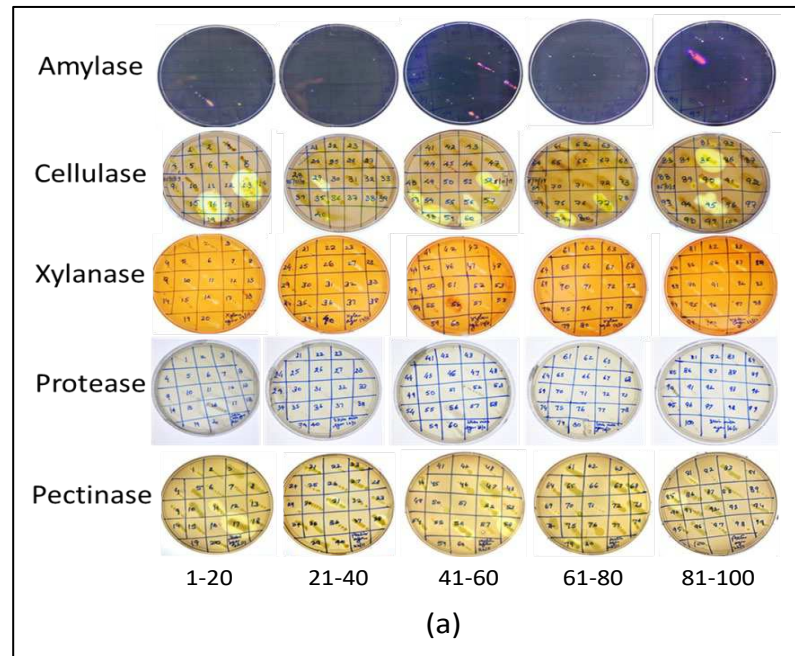
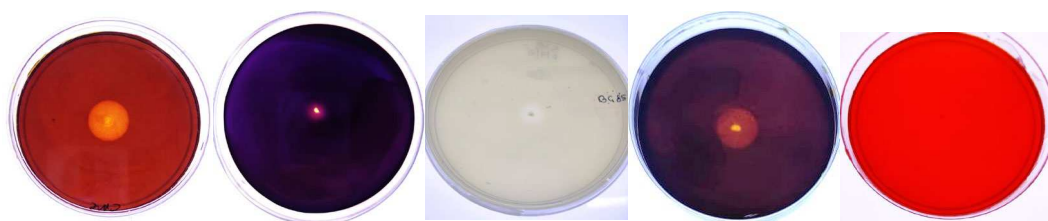


Fig 1



Enzymes	Cellulase	Amylase	Protease	Pectinase	Xylanase
Activity	+ve	+ve	+ve	+ve	-ve
Dp/Dc ratio	3.04	1	4	4.6	--

Fig 2

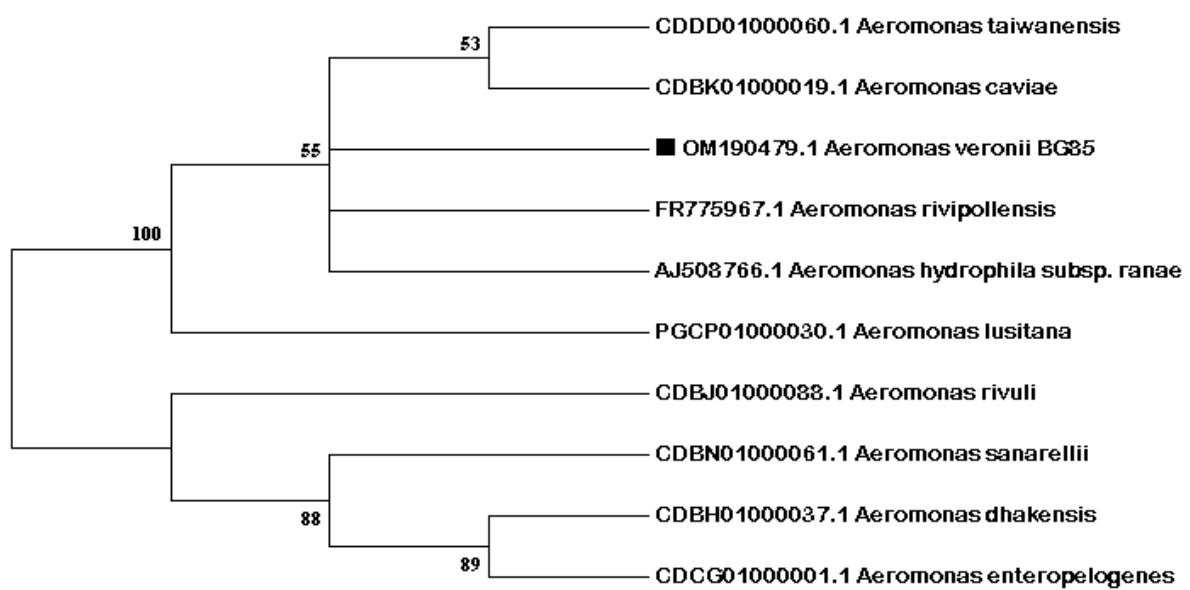


Fig 3

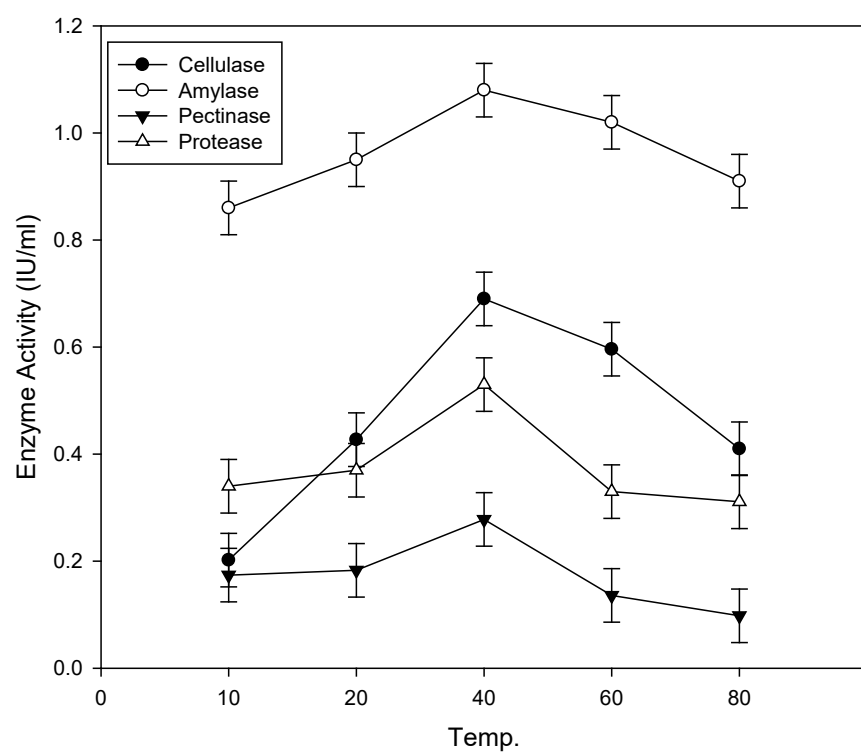


Fig 4

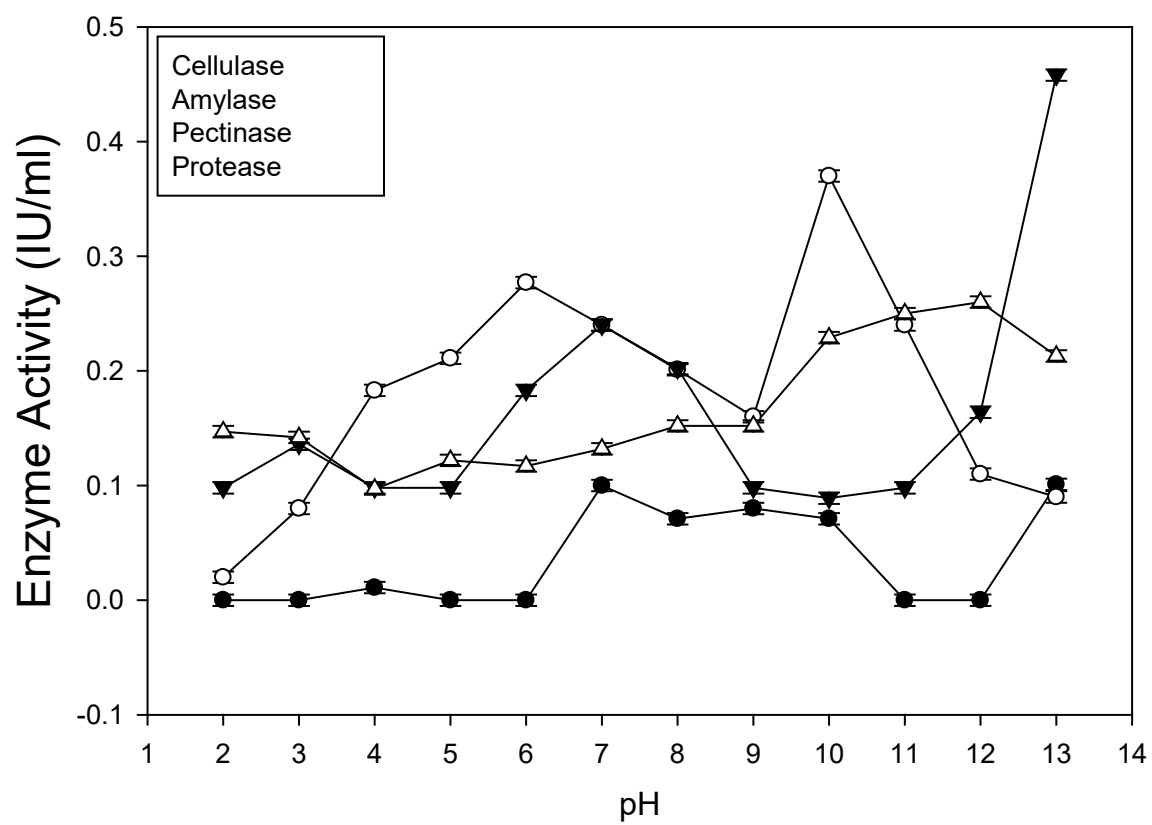


Fig 5

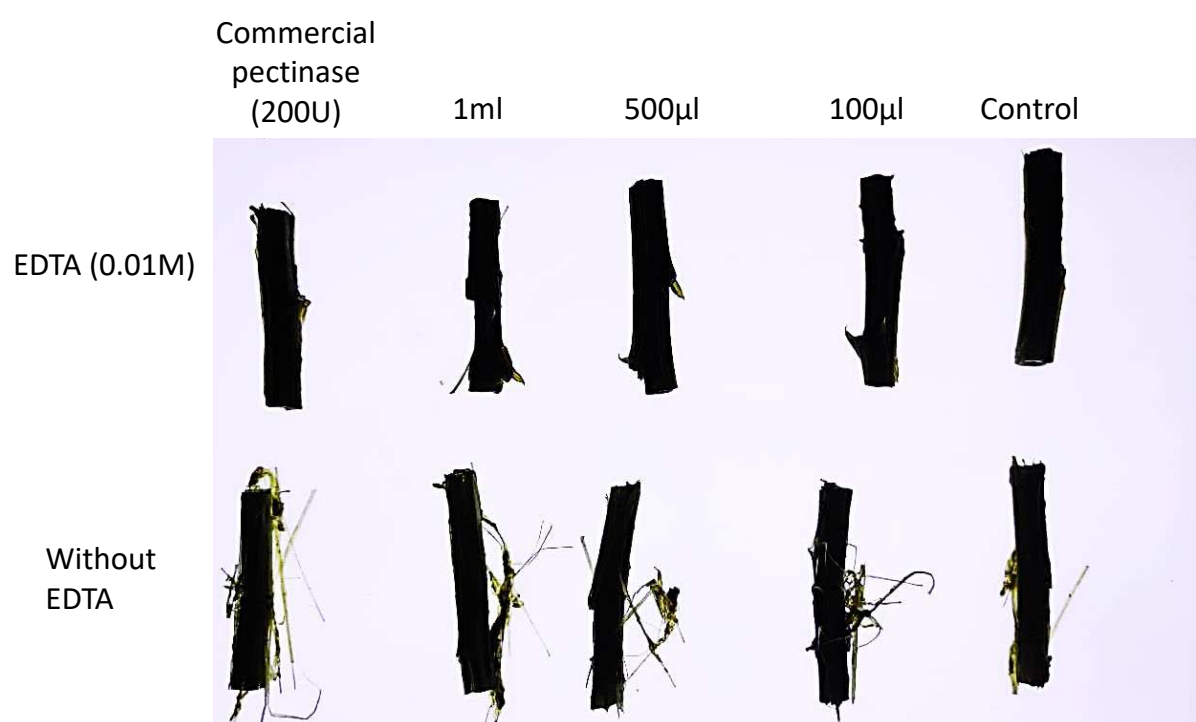


Fig 6

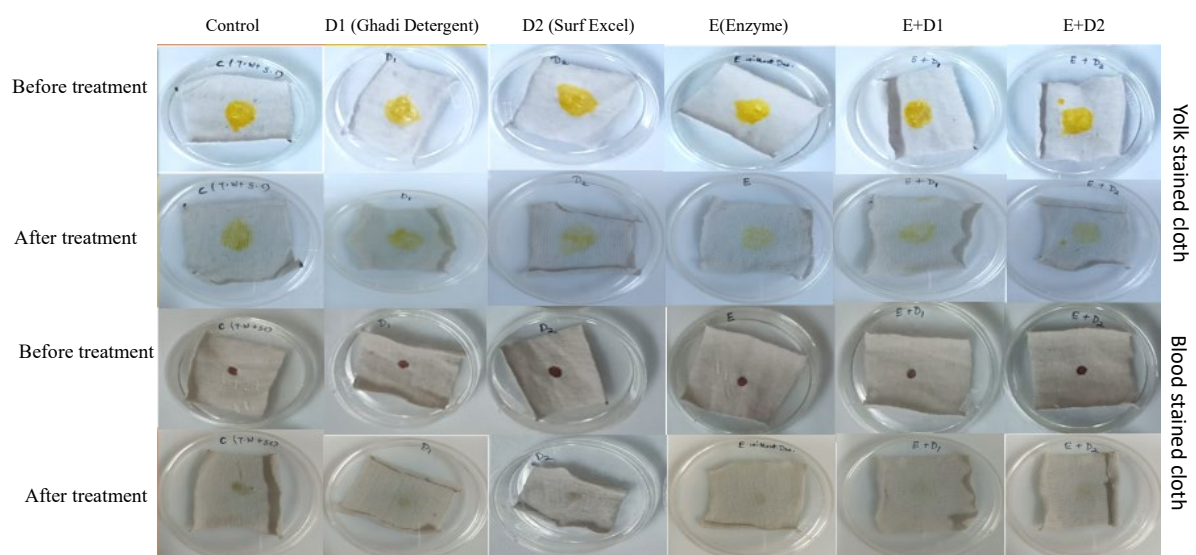


Fig 7

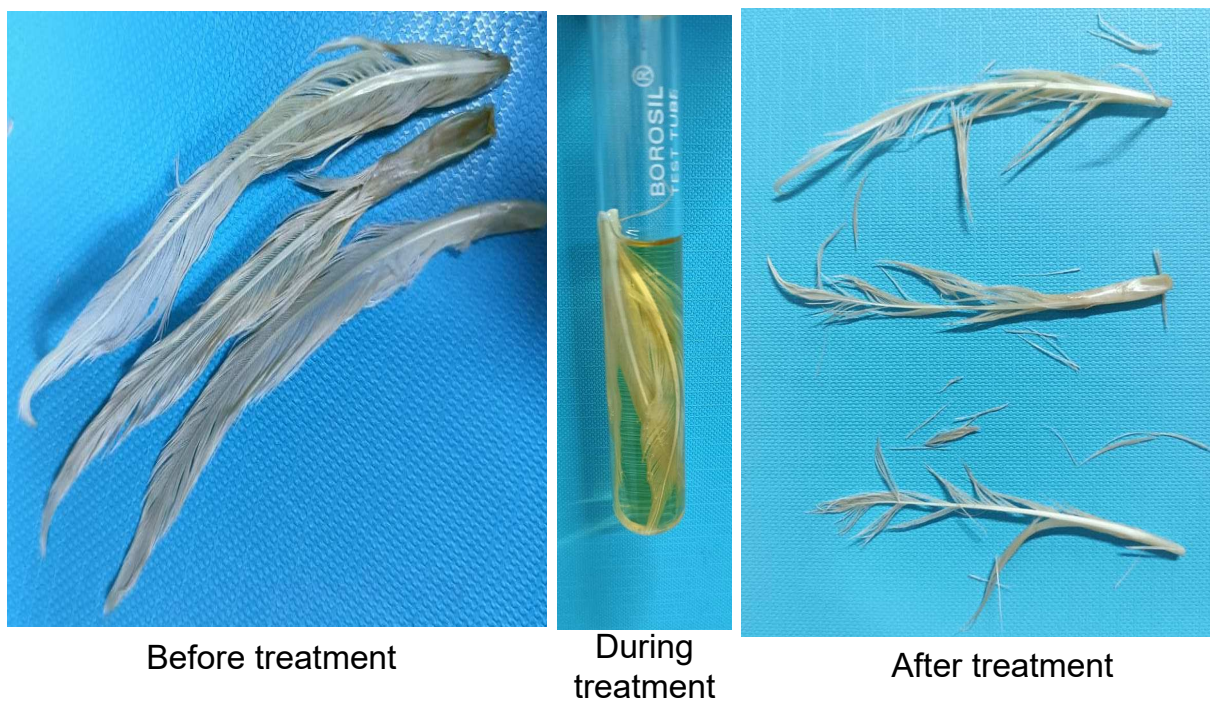


Fig 8