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Isolation and Characterization of *Aeromonas taiwanensis* Strain for Simultaneous Production of Cellulase, Amylase, Pectinase, and Protease Enzymes

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A study was conducted to discover a novel microorganism capable of producing multiple enzymes with industrial applications. Bacterial isolates were screened from a soil sample collected from a wood-decaying area, and their ability to produce various enzymes of industrial significance was evaluated. Among the 100 screened bacterial isolates, the strain GCEL-BGb85 was identified as Aeromonas taiwanensis through 16s RNA sequencing. Further screening revealed that this microorganism could produce cellulase, pectinase, protease, and amylase enzymes. The strain was set up for enzyme production, and the enzyme activity levels for cellulase, amylase, pectinase, and protease were 0.071, 0.201, 0.202, and 0.152 U/ml, respectively. All four enzymes demonstrated optimal activity at 40°C with a distinct pH. The zymogram analysis confirmed the presence of these enzymes in the isolated bacterial strain. As all the enzymes isolated from this strain are industrially significant, the application of the cell-free extract containing all these enzymes was evaluated at a laboratory scale for its potential use in industrial applications. The enzyme cocktail demonstrated its utility in the retting of fibers, effectively removing blood and egg yolk stains and extracting fruit juices and oil. The enzyme extract was also found to be useful in sustainable waste management.

Keywords: Aeromonas Taiwanensis; Industrial Enzymes; Hydrolytic Enzymes; Saprotroph.

The increasing annual global demand for new enzymes is expected to reach $8.7 billion by 2026, so the search for alternative sources has become imperative [1]. However, recent reports on the isolation and characterization of such enzymes for biotechnological applications remain scarce. Thorough enzymatic characterization, alongside addressing major bottlenecks such as scaling up production and scrutinizing the use of degradation byproducts, constitutes essential steps in this field [2]. The cost-effectiveness and speed of bacterial enzyme production make it a preferred method compared to other organisms. Additionally, the physicochemical and physiological control of enzyme production from microbes further enhances its appeal in industrial applications, contributing to a more sustainable production paradigm [3, 4]. In various industries, such as paper and pulp, animal

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feed, and detergent manufacturing, there is a pervasive demand for multiple enzymes. Examples include the utilization of amylases, lipases, proteases, and cellulases in detergent production [5, 6]. *Bacillus subtilis*, a notable source of protease, contributes to laundry detergent formulations. Bacterial-derived amylases and mannanases further enhance detergent performance [7]. The utilization of similar enzyme combinations from bacterial sources extends to the textile industry, augmenting fabric quality. Additionally, industries relying on cellulase, xylanase, and pectinase, such as paper and pulp, as well as the food processing industry, dependent on multiple enzymes, underscore the diverse applications of microbial enzymes [8-10].

Aeromonas spp. emerge as prolific producers of enzymes, with potential contributions to virulence potentially linked to increased bacterial adaptation to the environment [11]. Strains isolated from aquatic environments are frequently associated with the production of chitinases, which have been extensively studied over the years [12, 13]. Apart from their role in seafood waste degradation, chitinases derived from *Aeromonas spp.* show promise in controlling insect populations, including chironomids and mosquitoes [14]. Cellulase-producing strains of *Aeromonas* spp. have been isolated from various animals, suggesting potential benefits to the host through cellulose degradation [15]. Moreover, *Aeromonas spp.* isolated from sugar industry waste (molasses) indicate the potential for producing industrially relevant enzymes using inexpensive and readily available substrates [16]. Lipases, as highly valued enzymes, garner attention for their potential applications in the bio-treatment of wastewater. A strain of *Aeromonas* isolated from sludge in a dairy industry demonstrates strong potential for treating dairy wastewater with a high organic load [17]. Psychrotrophic species within the *Aeromonas* genus offer cold-adapted enzymes suitable for specific industrial processes. Cold-active lipases have already been isolated from strains of *Aeromonas*, while heat-stable enzymes, like the thermostable lipase from *Aeromonas sp.* EBB-1, are of interest for various industrial applications, including organic synthesis and detergents [18, 19]. The *Aeromonas* genus also encompasses other industrially relevant enzymes such as proteases, pullulanases, amylases, xylanases, and agarases [1, 20-23]. The primary objective of this study is the isolation of a potent bacterial strain from soil capable of concurrently producing multiple industrially significant enzymes, including cellulase, amylase, pectinase, and protease. The research also aims to characterize these enzymes and explore their applications in the paper, food, detergent industries, and waste management.

METHODOLOGY

Sample collection

Samples for the isolation of the bacteria was collected from beneath the decaying tree trunk in the Botanical Garden of Deen Dayal Upadhyaya Gorakhpur University, India. The soil 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 deionised sterile water. After proper mixing, the soil suspension was serially diluted upto 10^{-5} dilutions. The diluted soil suspension was plated on fresh Luria-Bertani (LB) (Himedia) Agar medium and incubated overnight at 37ºC 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.

Primary 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 enzymes. A specific plate supplemented with the corresponding substrate was prepared to carry out the plate assay for above-mentioned enzymes.

Cellulase assay: Carboxymethylcellulose agar plates (0.2% $NaNO_3$, 0.05% $MgSO_4$, 0.1% K_2HPO_4, 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 [24].

Amylase assay: For detection of amylase activity, the isolates 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 37ºC, 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 [25].

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 bacterial 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 [26].

Protease assay: Protease of the bacterial isolate was screened by streaking the colonies on the skim-milk agar plate [27]. Protease activity was detected by observing the clear zone around the bacterial colonies.

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.

Molecular identification of potent bacterium

The bacterial isolate exhibiting positive activity for cellulase, amylase, pectinase, and protease enzymes was subjected to molecular identification. Bacterial genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit. (Zymo Research). The universal 27F (AGAGTTTGATCCTGGCTCAG) and 1492R primers were used for the amplification of the 16S rRNA gene (TACGGYTACCTTGTTACGACTT). QIAquick Gel Extraction Kit (Qiagen) was used for gel elution and the amplicons were sequenced by sanger DNA sequencing method. The resulting sequence was compared to a non-redundant NCBI database using BLASTN. Ten *Aeromonas* strains exhibiting maximum identity with *GCEL-BGb-85* were downloaded from the NCBI database for phylogenetic analysis. The phylogenetic tree was prepared by Neighbour Joining method through MEGA7 software [28] with bootstrap value of 1000.

Simultaneous Production of Multiple Enzymes

The production of cellulase, amylase, pectinase, and protease enzymes was carried out on LB medium. The flask was set at 120 rpm at 37ºC 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 [29]. 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 [29] 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 [30].

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 M1 cm1) [31].

Protease assay: Protease activity was performed using casein as a substrate [32]. 1000 ìl of 1% (w/v) of casein in citrate phosphate buffer, pH 7 and 250 ìl 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 Na_2CO_3 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 [33]. 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 rection 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.

Zymogram analysis

The presence of all the screened enzymes in the bacterial strain was confirmed by zymogram analysis. Zymogram of pectinase was performed according to the method mentioned by Balaji et al. [34] and the gel was stained by KI/I_2 solution. The method used by Champasri et al [35] with slight modifications was used to confirm the presence of cellulase enzyme. A zymogram of amylase was performed according to A Andrades, LM Contreras [36] while protease was confirmed according to Heussen and Dowdle [37] (with slight modifications).

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 [38] 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 ìl, 500 ìl, 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 cm^2 stained with blood and egg yolk were treated with two different detergent solutions, Surf Excel (Hindustan Lever Ltd, India) and Ghadi detergent (RSPL Limited) (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 [39].

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 [40].

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 (Figure 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 (Figure 2).

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

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 bacterium. The 16S sequence was used to perform BLAST with database of NCBI Genebank. The sequence exhibited closest similarity with *Aeromonas taiwanensis*. The strain has been submitted to MTCC with Accession no. 13240. The 16S rDNA gene sequence was used to carry out BLAST with the GenBank database. The first ten sequences exhibiting maximum sequence identity were selected and aligned using multiple alignment software program, Clustal W. Next a phylogenetic tree was constructed using MEGA 7. On performing molecular phylogenetic analysis and the tree prepared by neighbour-joining method, the isolate showed close association with *Aeromonas taiwanensis*. (Figure 3). The biochemical characterization tests results are listed in 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

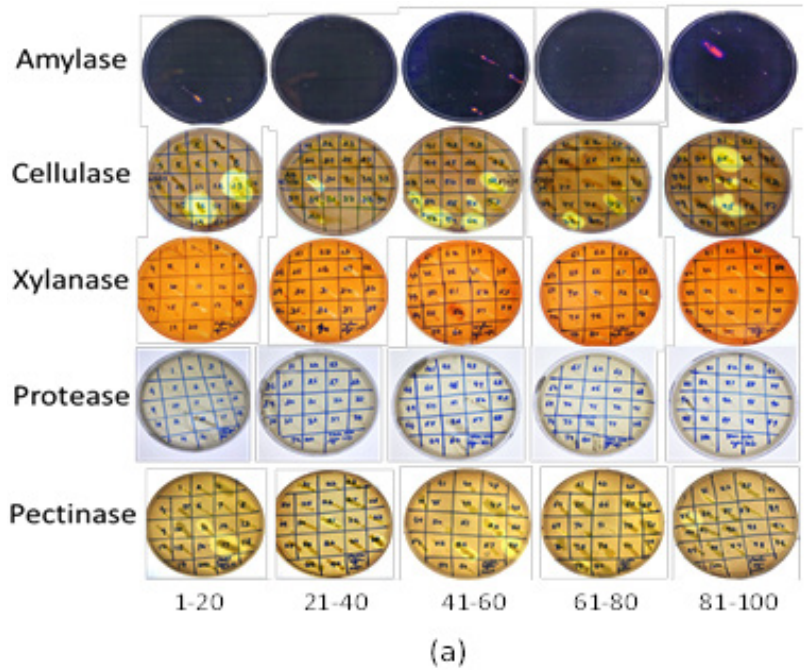
The temperature and pH optima of all the four enzymes produced by the bacterial strain were estimated. All the four enzymes, cellulase, amylase, pectinase and protease exhibited maximum activity at 40ºC. while the pH optima were different for each of the targeted enzyme. Amylase had best activity at 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 4).

Zymogram analysis

- Amylase- ~54kDa
- Cellulase- ~ 32 kDa

• Proteases- ~ 33 kDa
• Pectinases- ~ 91kDa
• SDS PAGE of crude enzyme.

The zymogram analysis confirmed the presence of these enzymes in the isolated bacterial strain. The presence of pectinase was detected at ~ 91kDa and amylase showed presence at ~54 kDa, cellulase showed a prominent band at ~32 kDa. The presence of protease was also confirmed by the clear zone at ~33kDa (Figure 5)



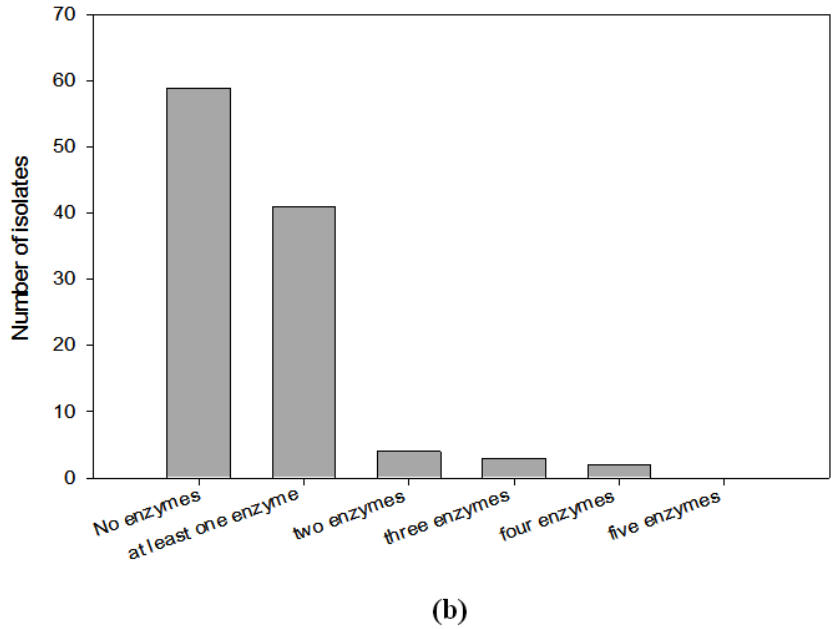


Fig. 1. (a) Primary screening by plate assay of the 100 bacterial isolates isolated from the soil sample collected from the Botanical Garden of DDU Gorakhpur University, Gorakhpur. (b) Graphical representation of the number of strains exhibiting multiple enzymatic activities

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).

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).

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	–

Waste management- Chicken feather degradation

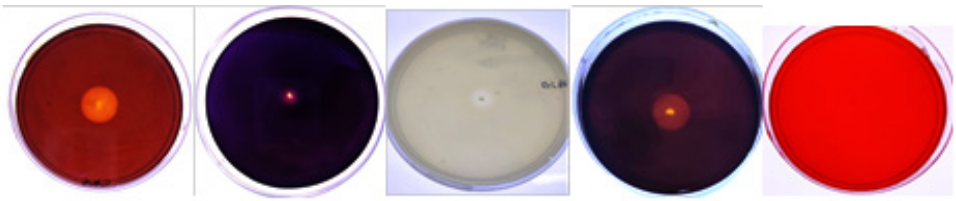
Analysis of feather disintegration by the crude enzyme containing protease suggested that a considerable amount of feather enzyme was degraded. Thus, this enzyme may play role in the waste management (Figure 8

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.

DISCUSSION

A large number of studies have reported isolation of novel microbes from a diverse soil sample. The soil microflora comprises of diverse bacterial population. These bacteria maintain the ecological balance and natural properties of the soil and convert toxic compounds into molecules that can be easily used by the plant in metabolic pathways. Apart from carrying out these natural processes, the soil bacteria are extensively being used in diverse industrial processes. 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, we have reported a bacterial isolate that can coproduce multiple



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

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

enzymes namely cellulase, pectinase, amylase and protease that can be used in several industrial applications.

A study by Singh et al. has also reported optimization of xylano-pectino-cellulolytic bacteria after extensive screening on the agriculture residues and commercial substrates [41]. Multiple enzymes protease, amylase and cellulase production have been reported to be produced by lactic acid bacteria isolated from traditional fermented food [42]. They have proposed the use of this strain for silage production. Halophylic bacterial isolates have also been screened to produce extracellular hydrolytic enzymes namely amylase, lipase, protease and cellulase enzymes [43]. These reports suggest that the multiple enzymes produced by individual bacteria can assist in various industrial applications as compared to the conventional method where individual microbes are used for the production of only single enzyme. There are large number of reports where fungi have been used for the simultaneous production of multiple enzymes however there are very few such reports for bacterial isolates [44].

A bacterial strain might produce multifunctional proteins with different molecular weights. A single enzyme may have different molecular weights in different species. The *Aeromonas* strains evaluated in this study produced enzyme extracellularly which was also confirmed by zymography which is a sensitive and reliable method. Many Aeromonas species produced proteases between 22kDa to 70kda. Zacaria et al study protease diversity among *Aeromonas species* and corresponding *A. hydrophila* proteins have molecular weight of 22 and 56 kDa [45]. Van Dyk et al. examined the cellulolytic and hemicellulolytic systems of *Bacillus licheniformis* SVD1 and discovered that SDS-PAGE revealed up to eight visible bands, while in zymogram only two active bands of CMCase ranging from 25 to 30 kDa were present [46]. According to a study the SDS-PAGE and zymogram activity staining showed that the molecular mass of strain 7193 and 7197 amylases were about 60 and 50 kDa respectively [47]. Shrestha et al., have discussed how the molecular weight of pectinases varies with different microorganisms; for example, PG from *Bacillus licheniformis* was observed to be of 153 kDa [48], PNL of 25 kDa in *Bacillus pumilus* [49], and polygalacturonase of 153 kDa in *Bacillus licheniformis* [48]. The polygalacturonase from *Rhizopus pusillus* was purified by two chromatographic steps (Sephadex G-200 and Sephacryl S-100) gave a band around 32 kDa in SDS-PAGE [50].

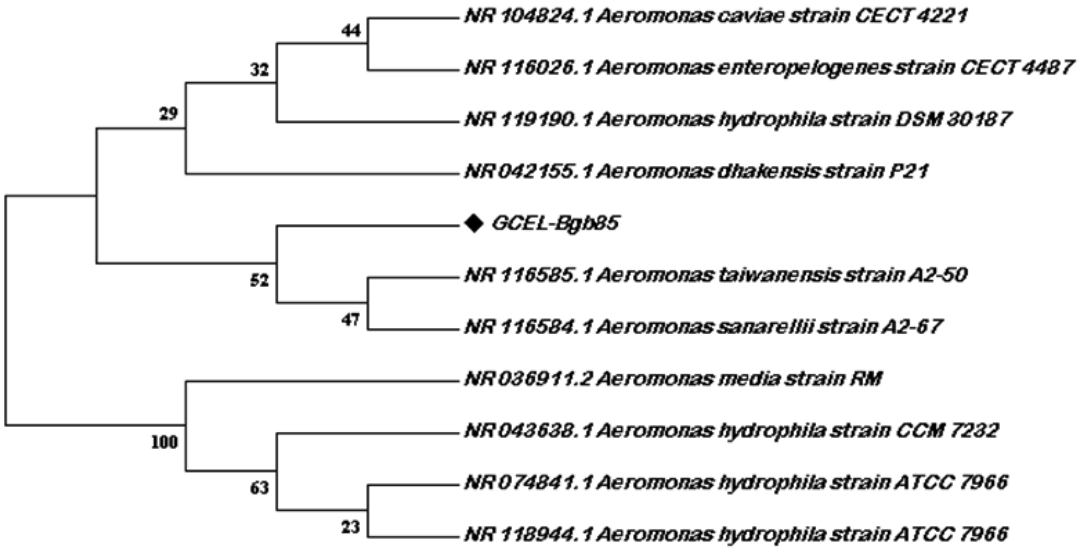
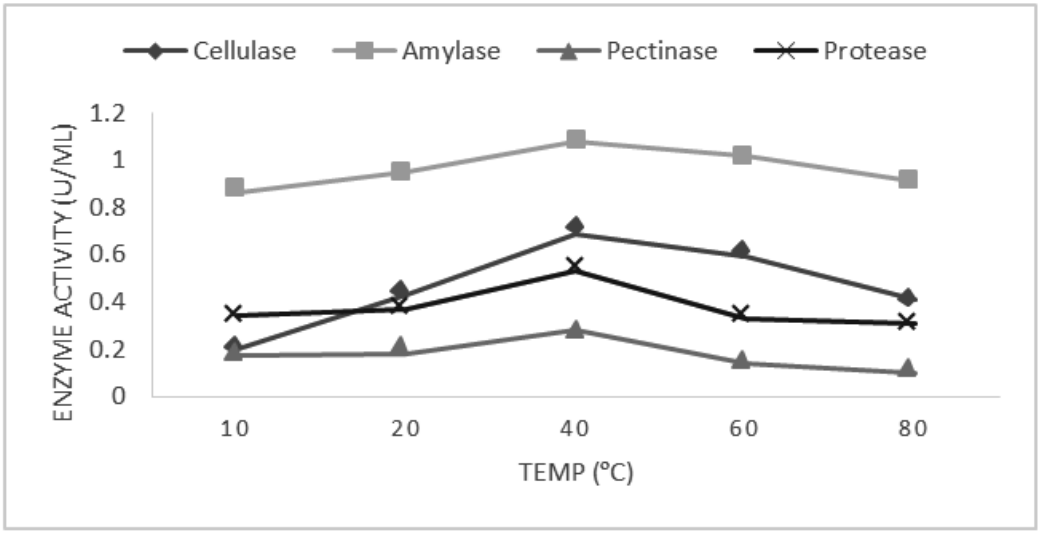
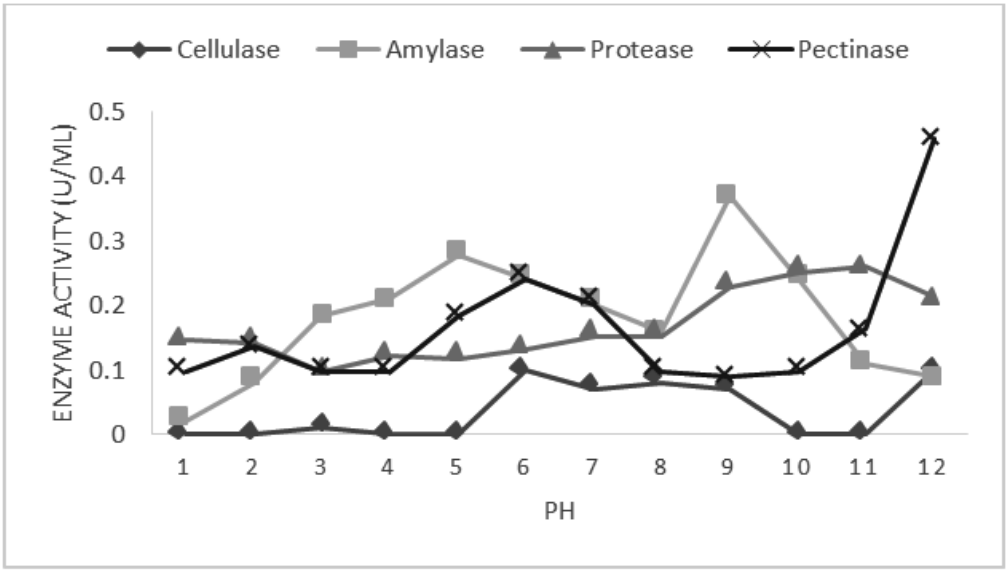


Fig. 3. Neighbour Joining (NJ) tree based on DNA sequences of the 16S rRNA gene showing the phylogenetic relationships. The values present on the branches represent the bootstrap support values

The bacterial strain was identified as *Aeromonas taiwanensis*. *It* is a comparatively new species, reported as a wound contaminant from two Taiwanese individuals [51]. These bacterial species live in a variety of aquatic and terrestrial ecosystems, are commonly found in water, but are also found in soil and food sources [52]. They have also been linked to gastrointestinal infections, particularly in immunocompromised people. *Aeromonas* has 38 species, according to the NCBI taxonomy bank, with the majority of them being pathogenic. *A. taiwanensis* was isolated a couple of



a)



b)

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

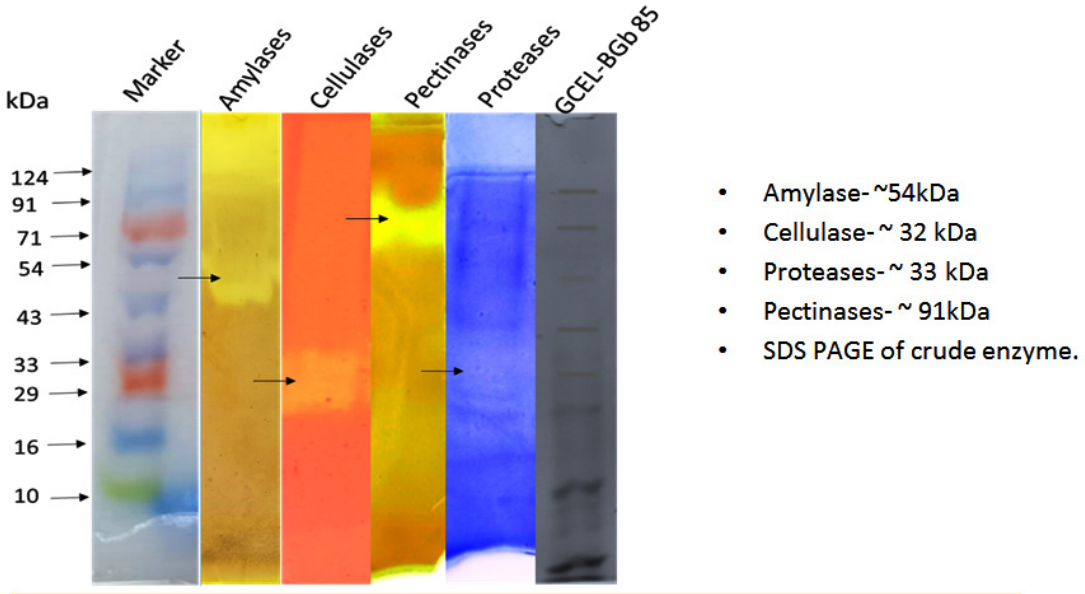


Fig. 5. Zymogram of cellulase, amylase, pectinase, and protease present in the crude fungal extract along with the marker in lane 1.

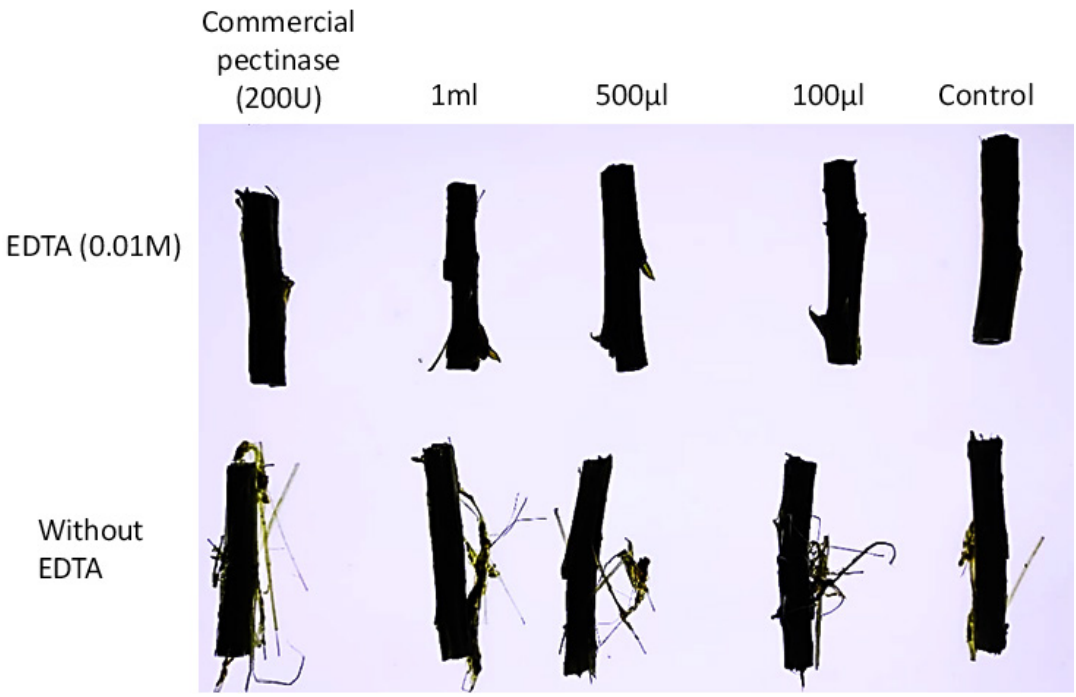


Fig. 6. Effect of enzyme produced from *GCEL-BG85* on retting of natural fiber: *C. juncea* (Sunn hemp); Stems were treated with the enzyme in the presence or absence of 10 mM EDTA. The control stem was treated with water instead of an enzyme. Since pectinase plays the main role in retting, the retting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

years back for the first time in India from lacustrine wetland [53]. Although considered as a pathogenic strain, *A. taiwanensis* have been reported to useful in several applications. A study has suggested its role in bioremediation as it can efficiently act on the antiparasitic agent and acaricide, Ivermectin (IVM) which is widely in use [54]. In another report, *A taiwanensis* isolated from the rhizospheric soil of mangrove plants collected from, India have been stated to act efficiently in decolorization of azo dye [55]. In the current study, *A. taiwanensis* has been reported to play role in several industrial applications.

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. The water retting method is most popular but emits undesirable odours and releases toxic fermentation waste such as galacturonic acid, butyric acid, acetic acid, lactic acid, Ca2+, Mg2+,

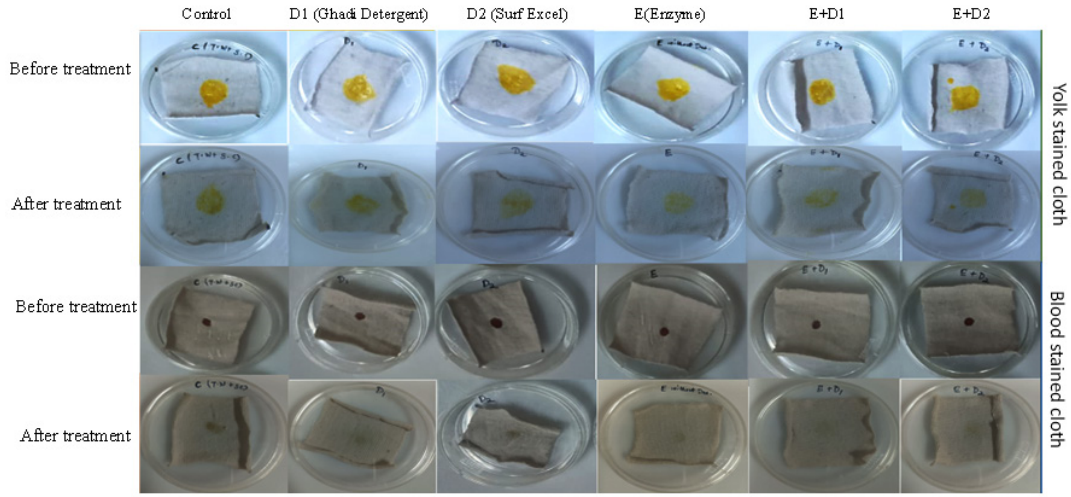


Fig. 7. Wash performance analysis of the enzyme from *GCEL BG85*. Enzyme alone and in combination with the detergents were used for removal of egg stain and bloodstain from the cloth

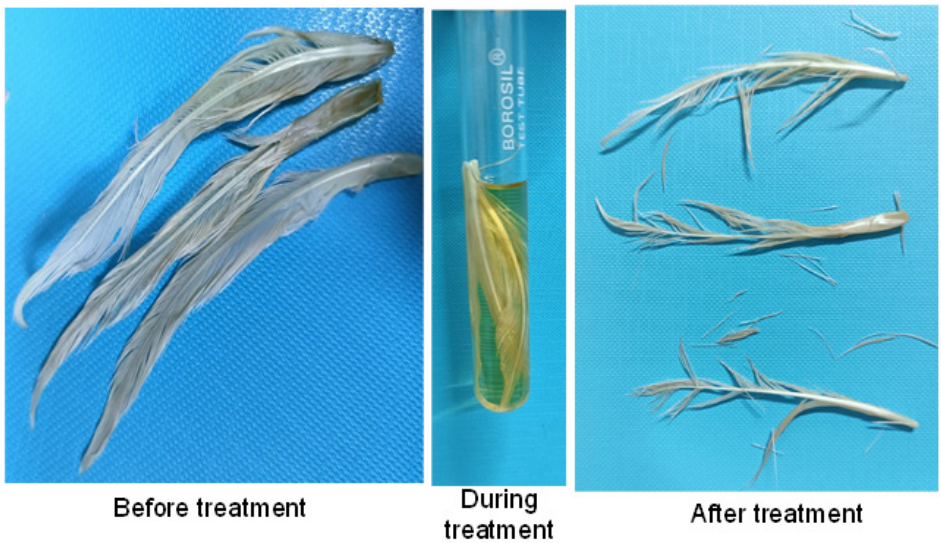


Fig. 8. Application of the enzyme in waste management. Significant feather disintegration was observed on incubation of the chicken feather with the enzyme solution

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

and bicarbonate content. Hence, alternative retting processes, such as enzymatic and microbiological retting, are necessarily needed. Since the crude enzyme extract was able to produce pectinase enzyme, its application was investigated in the retting of jute fibres. Sharma and Satyanarayana [56] have reported enzymatic retting of ramie fibres with thermoalkalistable pectinases from *Bacillus pumilus* DCSR1. Improvement in Young's Modulus and tensile strength of the fibres was observed after treatment of fibres 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. Pectinolytic microbes with xylanolytic activity but no cellulase activity can help to enhance the fibre quality. *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* are used for retting [57].

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. 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 [58]. The synergistic action of other enzymes, cellulase, amylase and protease produced by the isolate facilitates the process [59]. Thermostable protease, lipase and amylase produced by *Bacillus amyloliquefaciens* KUB29 have been effectively used in bio-oil extraction process [60]. Cellulase and pectinase pre-treatment together is effective in increasing the yield of oil extraction along with reducing the viscosity of the extracted oil [61]. 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 [62]. Extraction of banana juice has been optimized with the presence of cellulase and pectinase enzymes [63]. Cellulase-free xylanases from *Fusarium* sp. has also been studied to be useful in fruit juice processing [64].

Cellulase, lipase, protease and amylases are the most commonly used enzymes in the detergent industry. 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. 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 [65]. *Bacillus flexus* XJU-1has 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 [66]. So, the enzymes used in the detergents should be compatible with other enzymes used in the detergents [67]. 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 [68].

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 . Untreated feather waste may harbour a variety of dangerous microorganisms and create pollutants including N_2O, NH_3, and H_2S, all of which are damaging to the environment, plants, animals, and people's health [69]. As a result, many academics are keen in using economic ways to transform chicken feathers into value-added items [70].

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 gives effective results as compared to the activity of individual enzymes alone.

CONCLUSION

The search for novel microbial sources, using conventional and metagenomics-based approaches, from diverse environmental samples, with potential for diverse applications, is an important area of microbiological research. In the present study, a bacterial strain *Aeromonas taiwanensis* indigenously isolated from a soil sample, revealed the production of multiple enzymes namely cellulase, amylase, pectinase, and protease. The crude enzymes produced by this strain were characterized for several enzymatic attributes, mainly pH and temperature optima, to elucidate relevant applications. The enzyme cocktail was assessed for several applications like fruit juice and oil extraction, blood and egg yolk stain removal, retting of natural fibers, and sustainable waste management. This enzyme cocktail could serve as an alternative to the chemical components, conventionally applied in relevant industrial processing, as an eco-friendly approach.

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Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

DY and AT have conceptualized, supervised, investigated, and edited the manuscript. SG has performed, analyzed, and written the manuscript. VKM, SD, and KY reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Availability of data and materials

The strain has been submitted to MTCC with Accession no. 13240. The 16S rRNA gene sequence showing close association with Aeromonas taiwanensis has been deposited in GenBank with Accession number OP289101.1

Ethics Approval Statement

The study does not involve an experiment on humans and animals. No Ethical approval was conducted.

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