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Isolation and characterization of cellulase producing bacteria from ant, termite and paper mill waste

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Abstract
The demand for cellulase enzymes, crucial for biofuel production, pulp and paper industry, and various biotechnological applications, has led to the exploration of sustainable and eco-friendly methods for their production. Waste materials, such as agricultural residues, food processing by-products, and lignocellulosic waste, have proven to be valuable sources for cellulase production, providing a dual benefit of waste valorisation and enzyme synthesis. This study focuses on the isolation and characterization of efficient cellulase producing bacteria found in ant and termite gut and paper mill wastes which can be considered for use in textile industry or for bio-refining. Pure isolates cultures were screened for cellulase activity and were observed under sterile conditions. Four isolates of the samples1T, 1A 1P and 3P produced halos greater in diameter, suggesting high cellulase activities. The morphological characterization of the samples collected was also done. The 16 srRNA intergenic spacer analysis through PCR technique was done and the PCR products were run in both agarose gel and polyacrylamide gel for determining the molecular weight and molecular characterization of the samples. It was found that the bacterial sample 3P showed the highest amount of cellulase production among all the rest of the samples at the optimum pH 8.5, which shows that the bacterial strain is highly basic in nature.

Keywords: Cellulose, cellulase enzymes, cellulytic bacteria, 16s rRNA, termites, waste material

Introduction
Cellulose (Cellulin) is the most abundant carbohydrate in nature. It is the main structural polysaccharide of the plants and predominant constituent of cell wall of plant cell. Its content ranges approximately 35 to 50% of plant dry weight. The crystalline structure of cellulose is relatively unusual in the polysaccharide world (Lynd *et al*., 2002) [7]. It is a homopolymer and consisting of only one type of monosaccharide that is glucose. This polysaccharide is composed of β-D-glucose units which are joined by glycosidic linkage. They are long, unbranched chain of about 6,000 glucose units with molecular weight between 0.5 to 2.5 million. Cellulose is commonly degraded by an enzyme called cellulase (Venkata *et al*., 2013) [20]. Cellulase refers to a class of enzymes generally produced by bacteria, fungi, protozoans, plants and animals that catalyzes the cellulolysis (or hydrolysis) of cellulose (Immanuel G *et al*., 2006; Kanokphorn S *et al*., 2011) [3, 5]. They hydrolyze β-1, 4 linkage in cellulose chains. The biological process of cellulolysis is basically controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β endoglucanase {(β-D-glucoside EC 3.2.1.4)}, 1, 4-β-exoglucanase {(glucohydrolase EC 3.2.1.91)}, and β-glucosidase {(cellobiase EC 3.2.1.21)}. Endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain. Exoglucanase is necessary for cleavage of non-reducing end of a cellulose chain and splitting of the elementary fibrils (protofibrils) from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Shewale, 1982; Woodward and Wiseman, 1983) [16, 24]. Cellulase is a multienzyme that can catalyse the hydrolysis of cellulose, the most abundant biopolymer on Earth and has positioned them as essential biocatalysts for sustainable processes (Ramamoorthy, Sambavi *et al*. 2019) [13]. Traditionally, cellulase production relied heavily on refined substrates, often contributing to high production costs and environmental concerns due to the need for resource-intensive raw materials. However, in recent years, there has been a notable shift towards more sustainable and eco-friendly approaches, with a particular focus on utilizing waste substrates for cellulase production. This green approach is not only aligned with the principles of a circular and bio-based economy but also addresses the dual challenges of waste management and the increasing

global demand for cellulases. A plethora of waste substrates, including agricultural residues, food processing by-products, and lignocellulosic waste, have shown promise as cost-effective and environmentally friendly sources for cellulase production (Shah, Ranawat *et al*. 2019) [14]. The cellulase was first discovered in 1983 from the anaerobic, thermophilic spore-forming *Clostridium thermocellum* (Maki *et al*., 2011) [8]. The cellulase producing organisms are the mesophilic and aerobic thermophilic bacteria. Relatively few aerobic cellulolytic bacteria have been reported to produce cellulase (Huang and Monk, 2004) [2].

Cellulase is the one of the important enzyme for lignocellulosic degradation (Mora-Pale *et al*., 2011) [9]. The lignocellulosic composition residues primarily consist of materials, which are rich in cellulose, hemicellulose, and lignin. Cellulases target cellulose and agricultural residues offer a natural source of this biopolymer. The cellulose content in these substrates can range from 30% to 50%, making them ideal for cellulase synthesis (Siqueira, Rodrigues *et al*. 2020) [27]. Carboxymethyl cellulose (CMC) has been preferentially used for the study of endo-β-1, 4-glucanase producing from gut microorganisms. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere (Tokuda *et al*., 2005; Wenzel *et al*., 2002; Lynd *et al*., 2002) [26, 23, 7]. Cellulases are used in the textile industry for cotton softening and denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications (Patagundi *et al*., 2014) [10]. Shaikh *et al.,* (2013) [15] reported that cellulase producing bacteria were isolated from various region including paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing region. Potential isolates were obtained from wood furnishing region and paper industry waste. Isolates were tentatively characterized on the basis of their cultural and morphological and biochemical characteristics, identified to be *Pseudomonas sp* and *Bacillus sp* respectively. The termite order, Isoptera, is made up of several species that are loosely classified as higher and lower termites. In their digestive tract, lower termites are home to a large number of prokaryotes and protists, which are single-celled eukaryotes. They contribute significantly to the terrestrial ecology through the recycling of lignocellulosic biomass, which is a blend of lignin, hemicellulose, and cellulose. One of the most significant soil insects, it effectively breaks down lignocelluloses linked to microbial symbionts into simpler forms of sugars and contributes significantly to the turnover and mineralisation of complex biopolymers, including wood and other materials containing cellulose and hemicelluloses (Wenzel *et al*., 2002) [23]. Identified and characterised bacterial strains that produce cellulase from the gut of higher termites *Termes propinquus* that feed on the interface between wood and soil When carboxymethyl cellulose (CMC) was added to Berg's agar, the isolates exhibited strong cellulase activity. They were able to recode their cellulase activities at a wide pH range (4–12) and at high temperatures (42–50 ℃). *Bacillus amyloliquefaciens subsp. plantarum* strain FZB42, *Bacillus sp*. strain Tp-6-2, and *Bacillus methylotrophicus* strain CBMB205 were discovered as the bacterial isolates. *Bacillus sp*. strain Tp-8-1 was also detected. The isolates produced a number of hydrolytic enzymes and demonstrated the ability to thrive in both alkaline and hot environments. Their cellulase can be utilised in the textile and detergent industries, among other industrial processes. It has been documented that the termite stomach contains a variety of species of *Enterobacteriaceae* and *Bacillaceae* families as well as cellulolytic bacteria such *Acinetobacter*, *Pseudomonas*, and *Staphylococcus* (Pourramezan *et al*., 2012) [12]. Therefore, cellulases are chiefly produced by microorganisms and they are distributed throughout the world. The interest in the search for cellulase producing novel bacterial species is increasing as bacteria can utilize wide range of cellulosic waste. Northeast India has a very rich biodiversity which generally means there are lots of microorganisms which have not been evaluated till date for their cellulolytic potential. As cellulose has tremendous economic importance, this study was aimed at the isolation and screening of indigenous bacteria with cellulolytic activity which can be used industrially. Knowledge of cellulose-degrading microbial taxa is of significant importance with respect to nutrition, biodegradation, biotechnology, and the carbon-cycle, providing insights into the metabolism, physiology, and functional enzyme systems of the cellulolytic bacteria. Cellulase enzymes play a pivotal role in a wide range of industries, from biofuel production to the paper and textile sectors. So, the present study was carried out to investigate the bacterial microflora present in inside the ants gut, termites gut and also bacterial microflora from paper mill waste industry that are associated with degradation of cellulosic material and screen these isolates for cellulase production and identify them.

Materials and Methods

Sample Collection

Ants, termites and paper waste samples were collected from different locations of Guwahati. This strategy was chosen to facilitate the isolation and characterization of cellulase producing bacteria from the gut of ants, termites and paper mill effluents. Samples were aseptically collected and they were then transported to the laboratory and processed within 48 hours. Microbes inhabiting the gut of organisms feeding on cellulosic biomasses as the major feed are a good source for cellulase enzyme extraction. Termites harbor a dense and diverse population of symbiotic microbes in their guts that can degrade cellulose in wood and play an important role in turnover and mineralization of complex biopolymers like wood and other cellulose containing materials.

Termites were collected from nest. The termites were surface sterilized with 70% ethanol and then washed in sterile normal saline solution. The head of the termites were dissected aseptically and removed. The surface sterilized termites were macerated and diluted in 100ml Phosphate buffered Saline (PBS). A serial dilution was performed upto 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} volumes. The 10^{-4} dilution was used as inoculum for aseptic inoculation into preliminary growth medium.

Growth media and inoculation

The microbes were inoculated on Nutrient Agar consisting of Nutrient broth (Himedia) supplemented with agar (Himedia) in order to get plates containing pure culture which can be used for further analysis. Petri plates were taken and labelled (1A, 1T, 1P, 3P, control) and about 20 ml of nutrient agar media was poured. The experiment was done in replicates. The medium was allowed to solidify and was inoculated with bacteria using a sterile loop by quadrant streaking method. The plates were incubated at 37 ℃.

Isolation of bacteria from Ant Guts

Gut bacteria were isolated from ants and termites. Bacteria was also isolated from the paper mill waste (Guwahati). Traditional serial dilution and spread plating method was used for the isolation of cellulolytic bacteria. Four test tubes were taken. Each of the test tubes were labelled as A, B, C, and D. Then 9 ml of distilled water was added to all the test tubes. Then, 1 ml of effluent solution was transferred to the test tube a (1 ml of sample solution with 9 ml of distilled water gives 10^{-1} dilution) and mixed well. Following 1 ml of solution was aspirated from test tube A and transferred to test tube B. The contents were mixed well. The same procedure was repeated for test tube C and D. Later, 100 µl from the prepared dilutions (10^{-2}, 10^{-3}, and 10^{-4}) were used for inoculating growth medium. Using nutrient agar medium spread plate technique was performed and the plates were incubated at 37 °C for 24 hours. Then the next day dilution of a single colony was made in separate 1 mL Eppendorf tubes from the incubated plates, then again spread plate technique was used for isolation of pure bacterial culture and incubated at 37 °C for 24 hours. And the final culturing was done by quadrant streaking method and the same nutrient agar media was used. Two replica plates were made. In addition, nutrient broth was prepared using the replica plate. The broth was subculture after every 15 days to preserve the pure culture and contamination.

Morphological characterization by Gram staining

Gram stain test was carried out on the bacterial isolates from the nutrient broth. A bacterial smear from a pure culture of nutrient broth was prepared and fixed on a clean glass slide. The slide was flooded with crystal violet for 1 minute, and rinsed with running tap water. The slide was then flooded with Gram's iodine for 1 minute, and again rinsed with running tap water. This was followed by decolorization with 95% ethanol and rinsing with tap water. The slide was counter stained with Safranin for 1 minute, rinsed with running tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a microscope.

Development of Inoculum

The selected bacterial cultures were individually maintained on CMC agar slants at 4 °C till further use. The selected bacterial cultures were inoculated in broth medium at pH 7 for 24 Hrs of incubation period. After the incubation period these bacterial cells were used as inoculum.

Screening of Bacteria

Spot plate technique: The screening was performed to investigate the bacteria producing the highest cellulolytic enzyme and was done on carboxymethyl cellulose (CMC) media. Spot plate method was used to determine if the bacteria was producing cellulase. Basically, 1 mL dilution was made using the replica plate colony and was spot plated on CMC (carboxymethylcellulose) Agar (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% Carboxymethylcellulose (CMC) sodium salt, 0.02% peptone, and 1.7% agar). Plates were then incubated at 37 °C for 48 hrs. The screening of cellulase producers was done on CMC agar of four bacterial isolates and CMC Congo red agar (0.05% K_2HPO_4, 0.025% $MgSO_4$, 0.188% CMC sodium salt, 0.01% Congo red, 1.5% agar and0.2% gelatin.). The incubated CMC agar plates were flooded with 1% Congo red and allowed to stand for 15 min at room temperature. 1 m NaCl was used for counterstaining (washing) the plates. Clear zones which appeared around the growing bacterial colonies indicating cellulose hydrolysis. In addition, to further see more cellulose hydrolysis media standardization has been done.

Optimization of media to enhance growth/maximize cellulase production: It was observed that the growth of the bacterial isolates on the previous CMC medium i.e, the inorganic media, was slow so standardization of the media was done by using three different media- Media –A (CMC 0.5 g, K_2HPO_4 0.05 g, $MgSO_4.7H_2O$ 0.01 g, NH_4NO_3 0.05 g, $FeCl_3.6H_2O$ 0.025 g, $CaCl_2$ 0.01 g, Agar 1 g) in 50 ml distilled water and incubated at 37 °C for 96 hours, according to Singh *et al*., (2013) [18]. Media –B (CMC 1%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, $MgSO_4$ 0.04%, NaCl 0.005%, $FeSO_4$ 0.000125%, Agar 1.8%) in 50 ml distilled water and incubated at 37 °C for 24-48 hours, according to Khatiwada *et al*., (2016) [6] Media –C (Luria Bertani (LB) Agar 1%, CMC 1%, Peptone and yeast extract 2%) in 50 ml distilled water and incubated at 37 °C for 24 hours, according to Yang *et al*., (2014) [25]. Then 100 µl dilutions were made from the previous nutrient agar replica plate and 20 ml of each media was poured into 3 petriplates and it is divided into 4 quadrant. Later, 3 µl of bacterial samples dilution were spot plated on each quadrant and all the three plates were incubated at 37 °C for 24 hours.

Estimated growth at variable pH and temperature (organic media): It was done in order to determine the growth ability of bacteria under various conditions by growing them at variable ranges of pH and temperature. Preparation of the broth cultures was done using the organic media, among them 3sets were made at 3 different pH (acidic, basic, neutral) and were kept at 2 different temperatures (45 °C and 37 °C) for the samples: 1T, 3P, 1A, 1P (These were bacterial isolates which had maximum cellulase activity – 'T' stands for termite, 'P' stands for paper mill waste and 'A' stands for ants).

Screening for cellulase enzyme

Molecular identification of cellulolytic bacteria: The strain which show maximum cellulase activity was further subjected to molecular identification by analysing 16S r RNA sequence.

Isolation of genomic DNA: The genomic DNA of the bacterial cultures was extracted following the protocol of Pospiech and Neumann (1995) [11]. Cells from an overnight grown culture (1.15 ml) were harvested at 6000 rpm for 15 minutes in a 2 ml microcentrifuge tube. After being rinsed in 1 ml of 1X phosphate buffer saline (1X PBS), the cell pellet was re-suspended in 0.5ml of freshly made SET buffer containing 1 mg/ml lysozyme and incubated at 37 °C for 1hr with repeated inversion. After the initial incubation, 1/10th of a volume of sodium dodecyl sulphate (50 µl) was added, and the mixture was then incubated again for a further 30 minutes at room temperature. After that, 800 µl of Phenol: Chloroform: Iso-amyl alcohol (25:24:1) and 1/3 of a volume of 5 M NaCl were added. The mixture was then incubated at room temperature for 30 min while being repeatedly inverted. After incubation, the mixture was centrifuged at 4500 rpm for 15 minutes, collecting the upper aqueous phase in a new microcentrifuge tube. The DNA in the supernatant was precipitated by adding one volume of chilled

isopropanol (700 µl), which was then centrifuged at 12000 rpm for 15 minutes after being incubated at 37 °C for 30 minutes. The pellet thus obtained was cleaned with 1 ml of 70% ethanol before being allowed to air dry at room temperature. Finally, 50 µl of autoclaved TE (Tris EDTA) buffer (pH 8.0) wer added to the DNA pellet, which was then dissolved.
The DNA pellet was then stored at -80 °C pending further use.

Qualitative analysis of the DNA

Agarose gel electrophoresis: Agarose at the rate of 1.8% was dissolved in 1 X Tris Acetate EDTA (TAE) buffer (40 mM Tris-Cl, 20 mM acetic acid and 1 mM EDTA) (pH 8.0) by heating in a microwave oven. Prior to the agarose gel casting, 2-5 µl ethidium bromide was added at the rate of 0.5 µg/ml to the gel. Molten agarose was cooled to 50 °C and poured into gel casting tray using appropriate comb. After complete solidification of the agarose gel, the comb was carefully removed and the gel plate was mounted on electrophoresis unit (Cleaver Scientific Ltd.). The DNA samples were mixed with 2 µl of tracking dye and loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 80 V for 20-30 min. After completion of electrophoresis, imaging was carried out under UV using Alpha imager EC gel documentation system and results were noted.

Electrophoretic analysis of amplification profiles of cellulose positive bacterial isolates by SDS-PAGE (Polyacrylamide Gel Electrophoresis)

PCR (Polymerase Chain Reaction): The PCR was performed using the reaction mixture contained sterile water, 1X PCR buffer with MgCl2, 4nmol deoxynucleotide triphosphates, 25pmol forward and reverse primer, 1.25 units of Taq DNA polymerase, 1ng of template DNA and with the following amplification for 16s rRNA initial denaturation at 95 °C for 5 min of initial denaturation, followed by 35 cycles annealing and extension (94 ºC for 1 min, 55 °C for 1 min and 72 °C for 2 min) and final extension at 72ºC for 10 min followed by hold for infinity at 4 °C. The presence of PCR products were detected on 1.5% Agarose gel and 12% PAGE gel to confirm size.
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was performed according to Singh *et al.* (2012) [17] methodology with slight modification. The electrophoresis was performed at 120V and Coomassie Brilliant Blue R-250 staining was used to visualise the protein bands. By comparing the protease's molecular weight with reference molecular weight markers, the molecular weight of the enzyme was found.

Result and Discussion: Cellulose is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere. Cellulose biodegradation by cellulases, produced by numerous microorganisms like bacteria represents a major carbon flow from fixed carbon sinks to atmospheric CO_2, is very important in several agricultural and waste treatment processes and could be widely used to produce sustainable bio-based products and bioenergy to replace depleting fossil fuels. Effective conversion of lignocellulosic biomass is one of the most important and difficult technological challenges faced as enzymatic hydrolysis of these materials are required to produce the fermentable sugars. Currently most commercial cellulases are produced by fungi. But bacterial cellulases are starting to get attention as bacteria have faster growth compared to fungi**.** Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Habitats that contain these substrates are the best sources in which to find these microorganisms (Huang and Monk, 2004) [2]. So the sites for sample collections were selected as those were rich in cellulosic biomass such as paper mill effluents, termite and ant guts, hence there were maximum possibilities to get potential cellulase producing bacterial strain.

Isolation of bacteria

In this investigation, the guts of termites, ants, and paper mill waste were chosen as sources of desired cellulase-producing bacteria: A total 4 distinct bacterial colonies was selected 1T, 1A, 1P, 3P (laboratory coding) which showed distinct colonies on nutrient agar plates and the edge, opacity and morphology observation were recorded. They showed distinct colonies on nutrient agar plates and the edge, opacity and morphology observation were recorded (Table 1).

Table 1: Morphological characteristic of the four bacterial isolates

S. No.	Sample Code	Opacity	Edge	Morphology
1.	1P	Opaque	Undulate	Circular
2.	1T	Opaque	Entire	Circular
3.	1A	Opaque	Filamentous	Rhizoid
4.	3P	Opaque	Entire	Circular

Congo red Assay

The four bacterial colonies were spotted on CMC plates to screen for cellulase activity and the following result was observed (Table 2): Following Congo-red staining, these strains displayed hydrolyzing zones on agar plates. Each colony showed variable decolonization after flooding the plates with Congo red as shown Figure 1. The size of depolarization and the size of the colonies were measured. The sample 1T showed the largest area of decolourization among all the four sample. After 48 hours of incubation, all 4 strains of bacteria (1P, 1A, 1T, 3P) showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. The sample1T gave the highest ratio of clear zone diameter to colony diameter i.e., 2.9 cm. This indicated more cellulose degradation in CMC agar plate cultured with 1T as compared to plates cultured with the other strains.

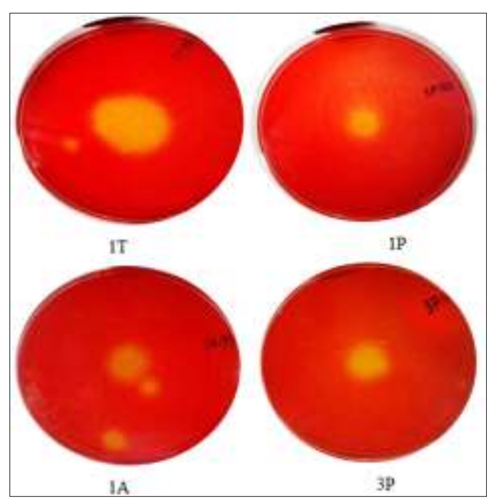


Fig 1: Congo red assay of isolated bacterial colonies

Morphological characterization by gram staining and optimization of culture media and conditions

Optimisation of media was done which showed that the growth in organic media was better as compared to inorganic media for the growth of the cellulose degrading bacteria (Table 2). Morphological study of the colonies suggested that they had both positive (1A) and negative (1T, 1P, 3P) gram stain reaction (Figure 2)

Table 2: Congo red screening of bacterial colonies using three different media composition

S. No.	Different Media	Observation	Size of Decolourization
1.	Media A (Inorganic)		1A:1.5 cm 1T:1.8 cm 3P:1.7 cm 1P:1.4 cm
2.	Media B (Inorganic)		1A:1.4 cm 1T: 1.9 cm 3P: 1.8 cm 1P:1.6 cm
3.	Media C (Organic)		1A:1.6 cm 1T:2.3 cm 3P:2.1 cm 1P:

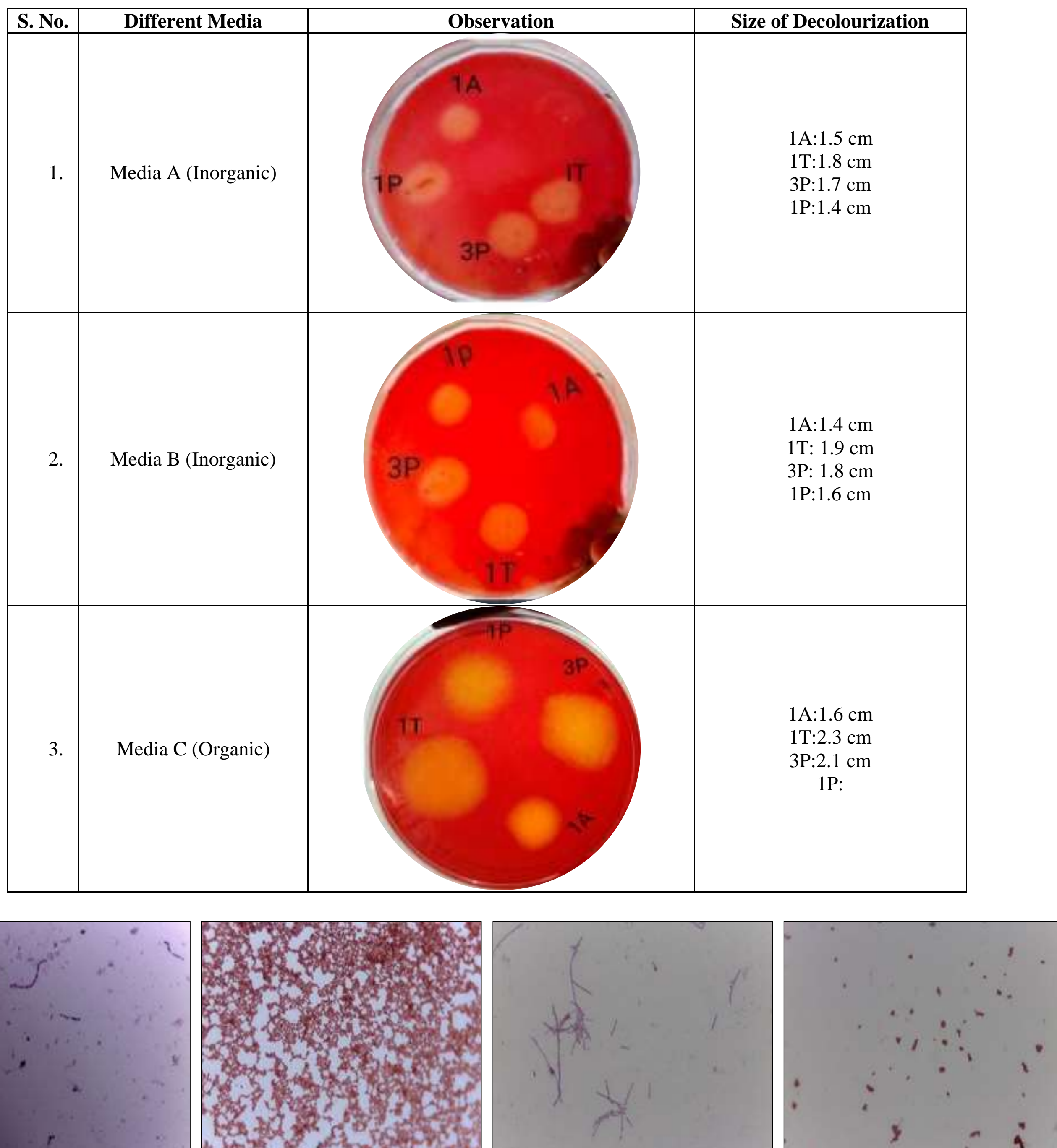
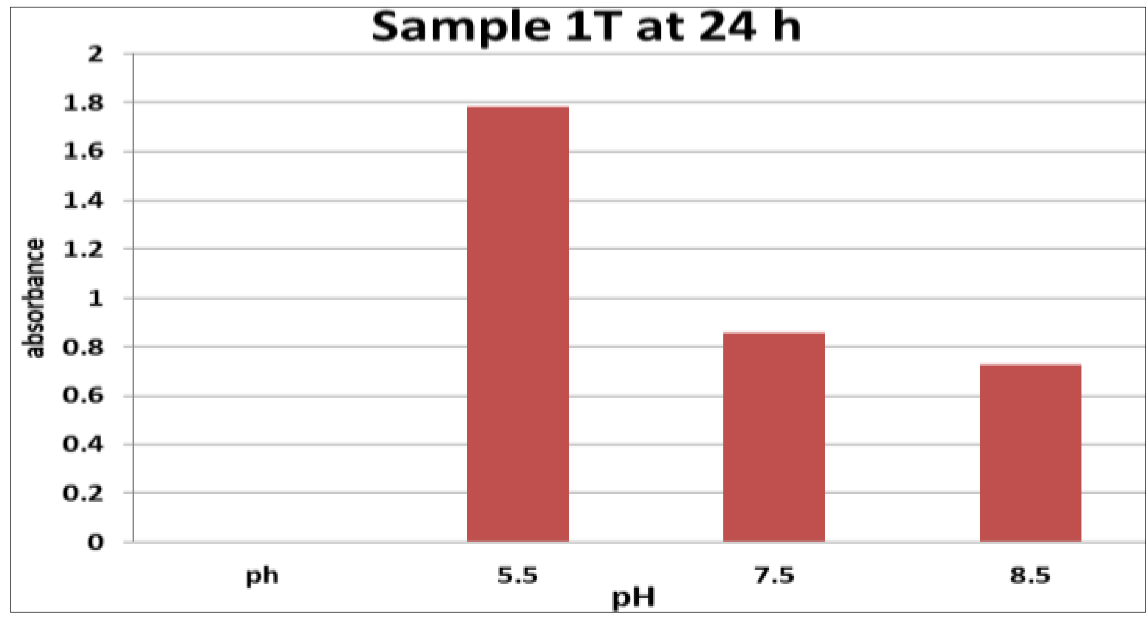


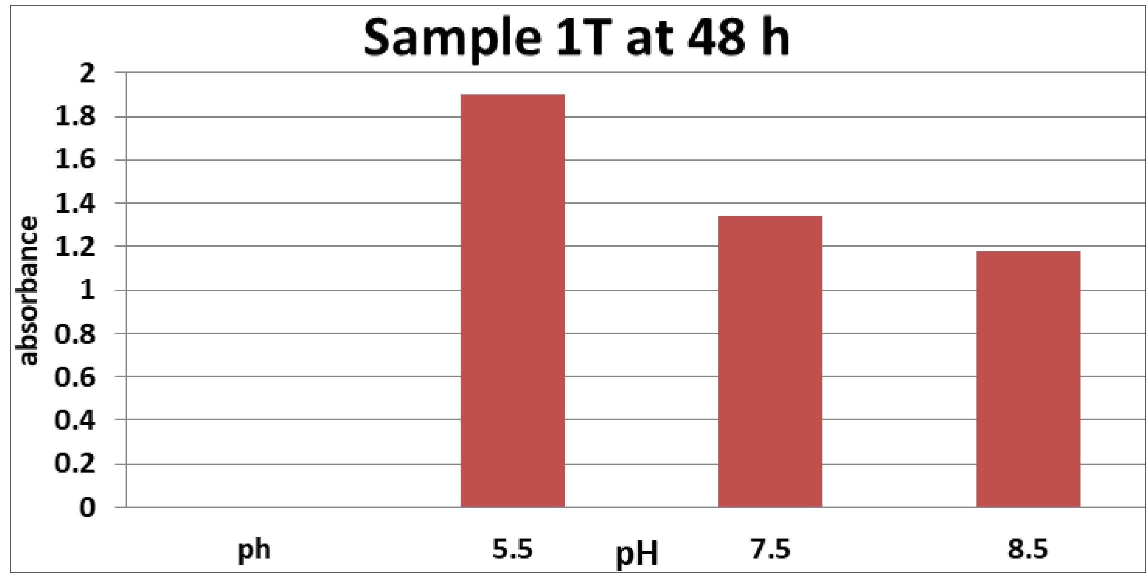
Fig 2: Morphological characterization by gram staining observed under oil immersion microscope

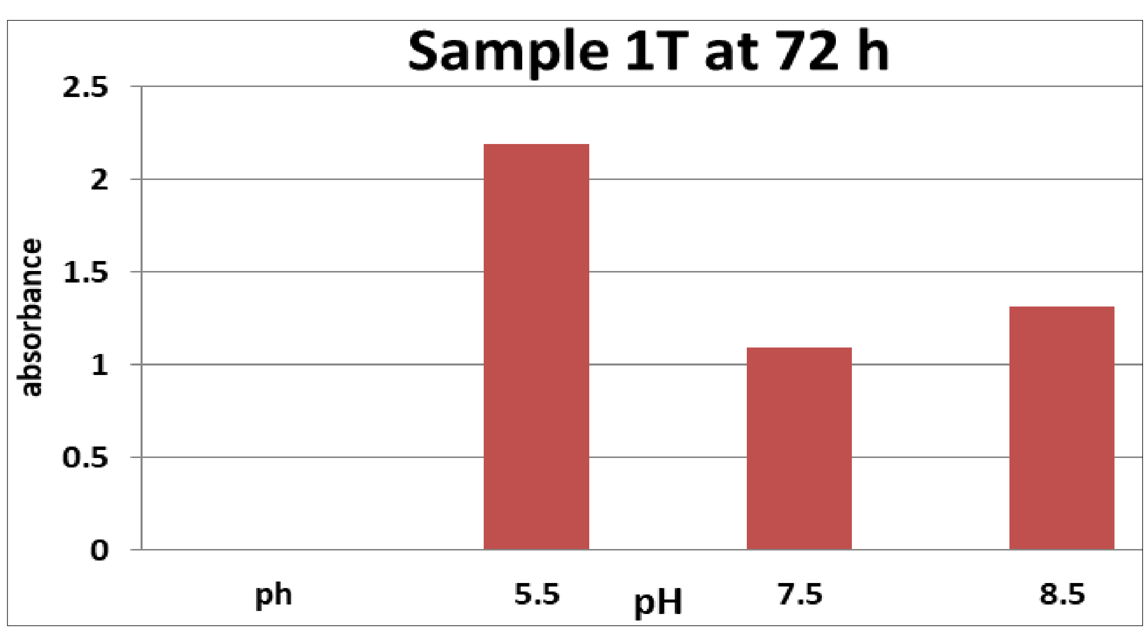
To find the optimum temperature and pH for growth of bacteria, the CMC broth containing the four inocula were incubated at a temperature range of 37 and 45 °C and various pH (pH 5.5, 7.5 and 8.5). This was done as temperature and pH also affects cellulase activity. The highest bacterial growth was found at pH 8.5 (1A), 7.5 (1P), 7.5 (3P), 1T (8.5). The optimum temperature at which highest bacterial growth was produced was 37 °C (1A, 3P, 1T) and 45 °C (IP). The growth of bacteria also occurred at other temperature values which showed that cellulase degrading bacteria strains aretolerant to both higher and lower temperature.

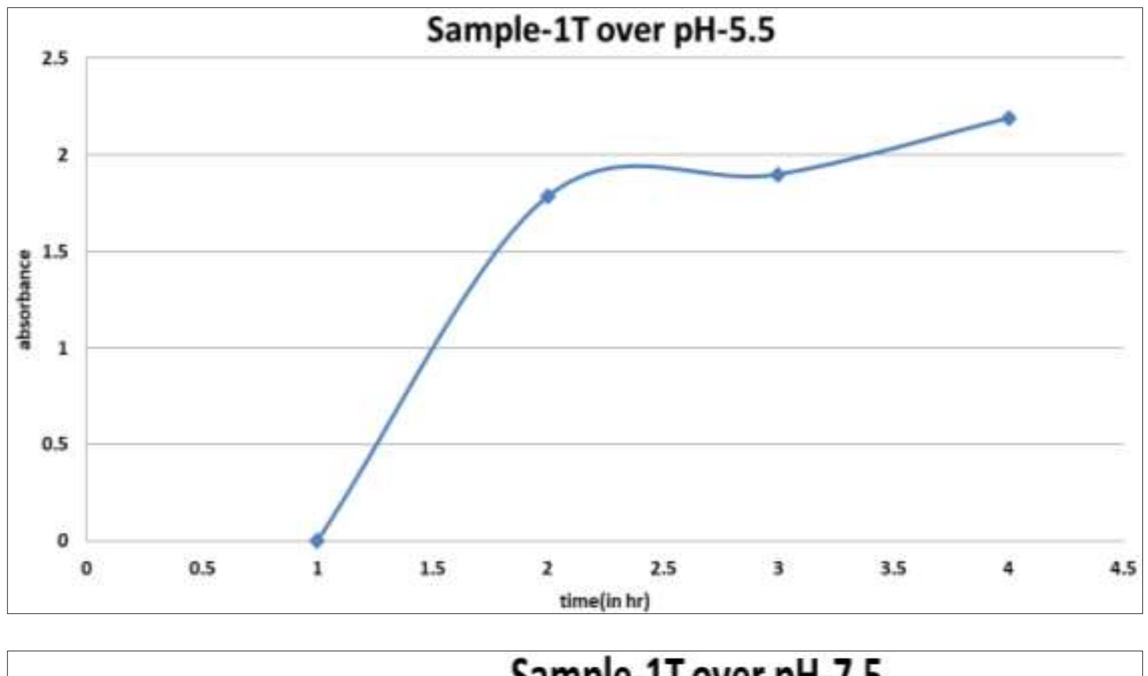
Different cultural parameters, such as pH, temperature, substrate concentration, and incubation length, were examined for the individual strains' ability to produce cellulase. Carbon and sources of nitrogen (Arusha *et al.*, 2016) [1].

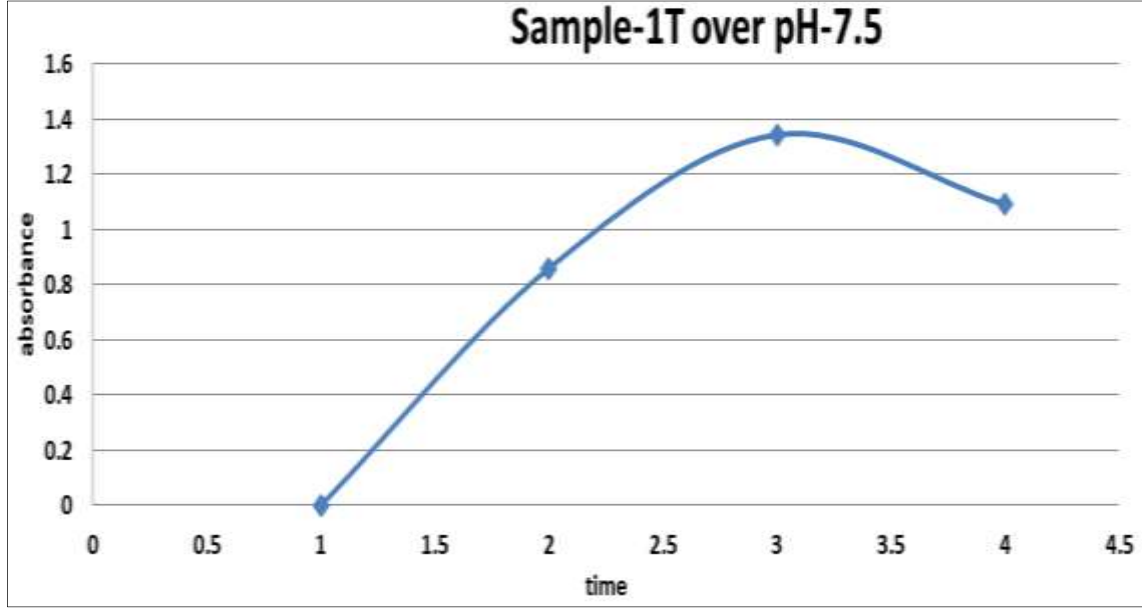
Optimization of bacteria isolates over different pH and time interval
a) Growth Pattern of 1T











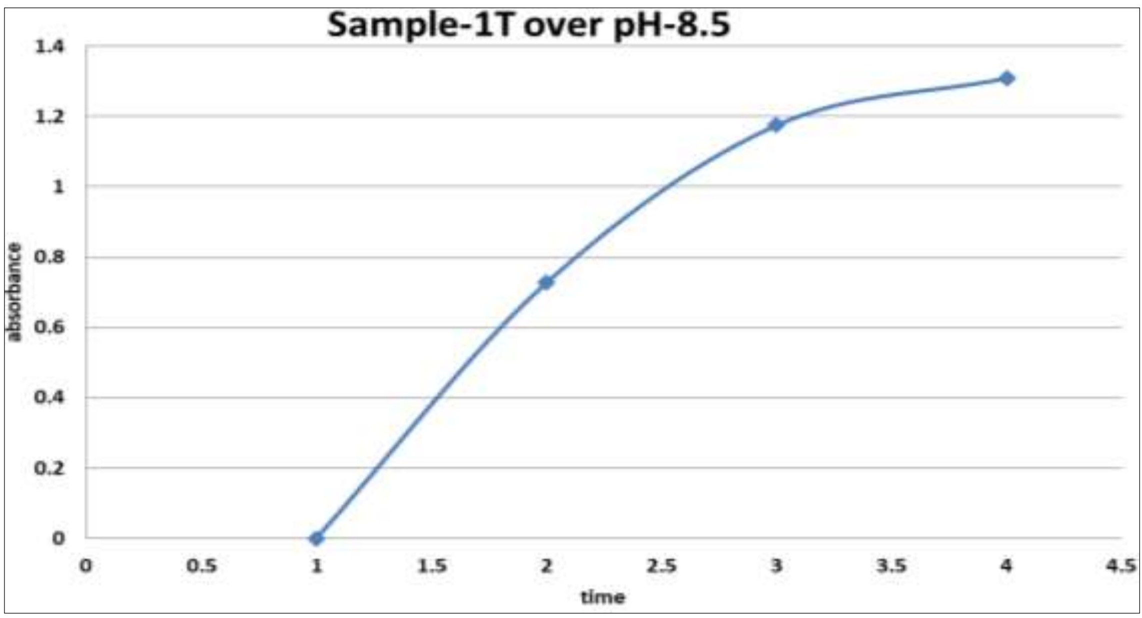
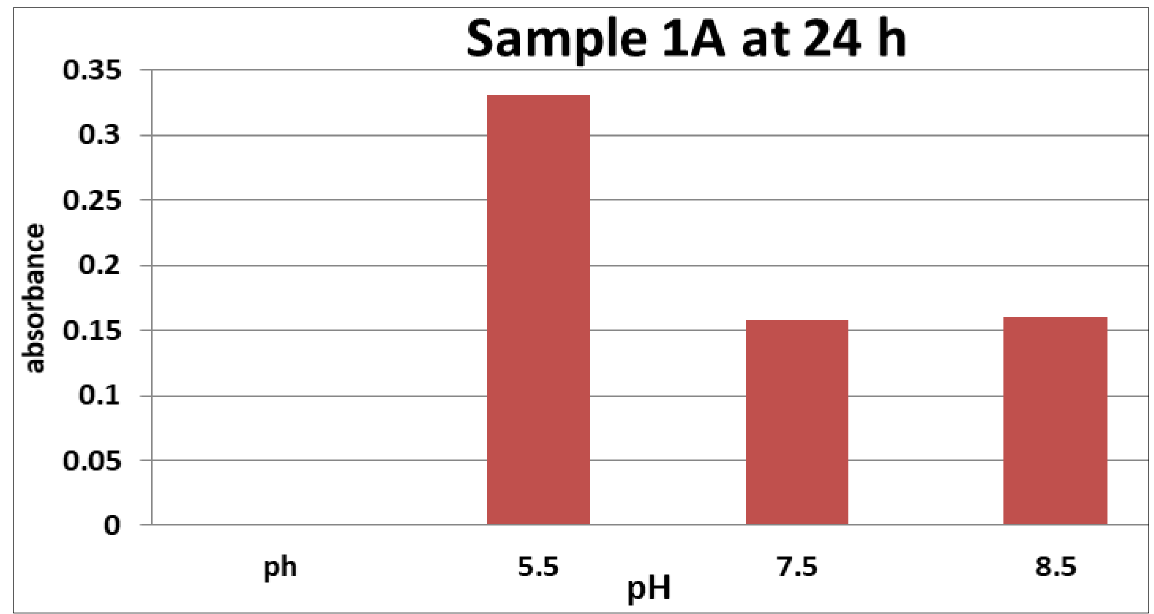
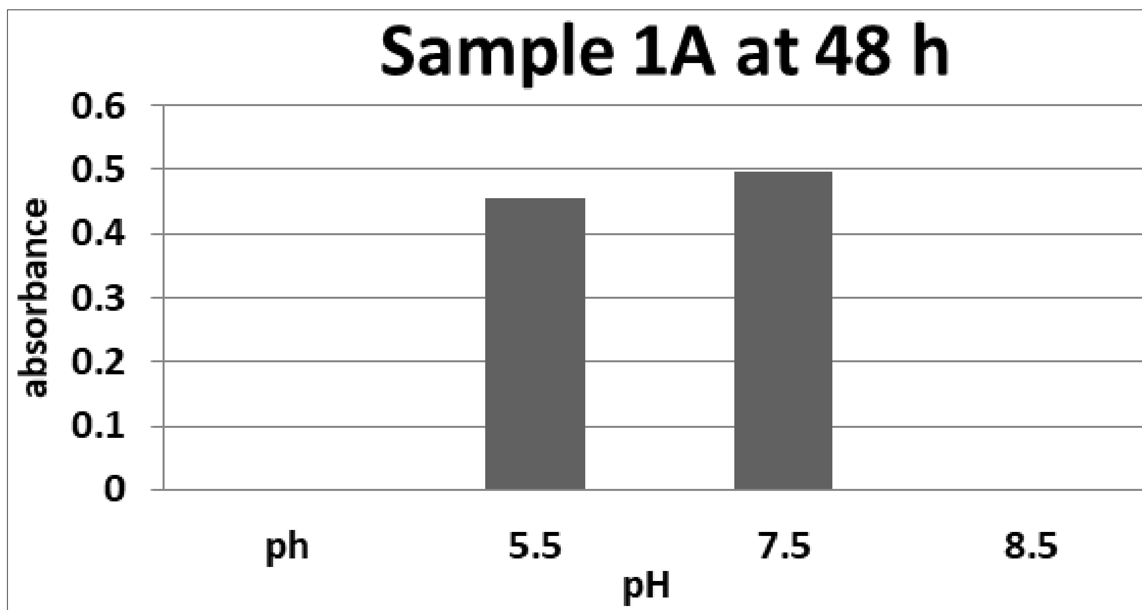
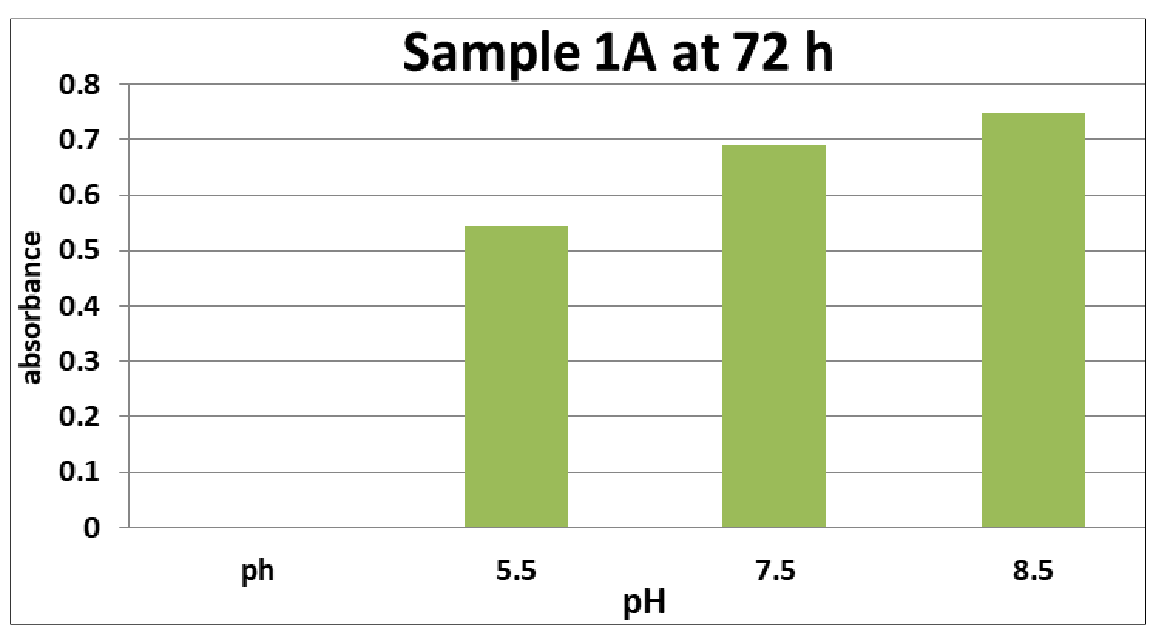


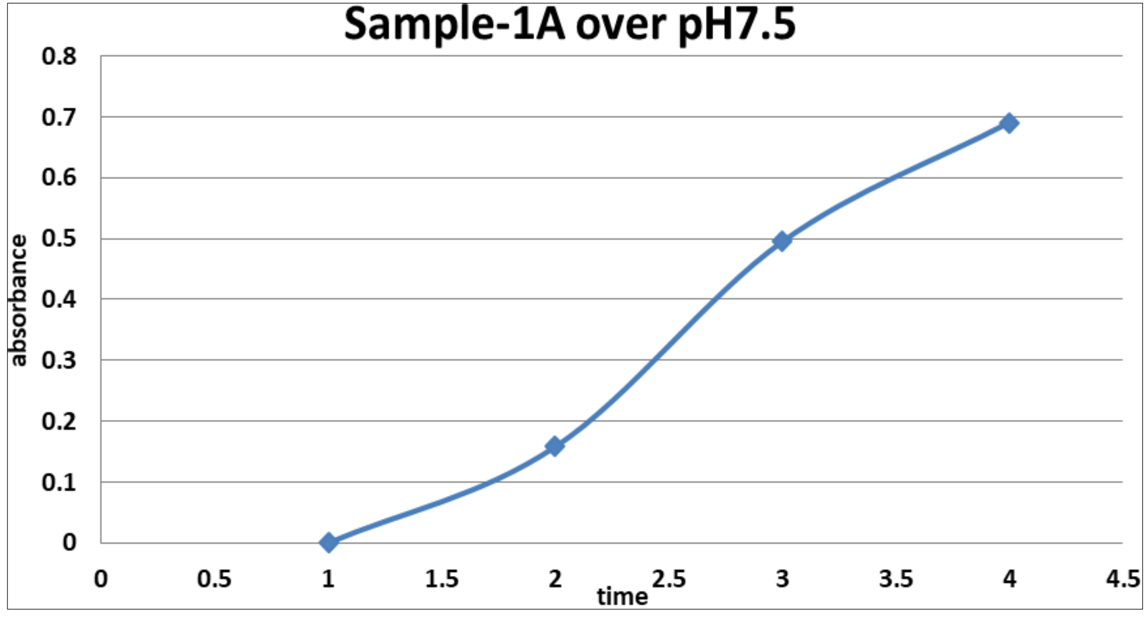
Fig 3: At the pH 5.5 the bacteria of sample 1T shows maximum growth but on other pH growth has increased linearly i.e, acidic

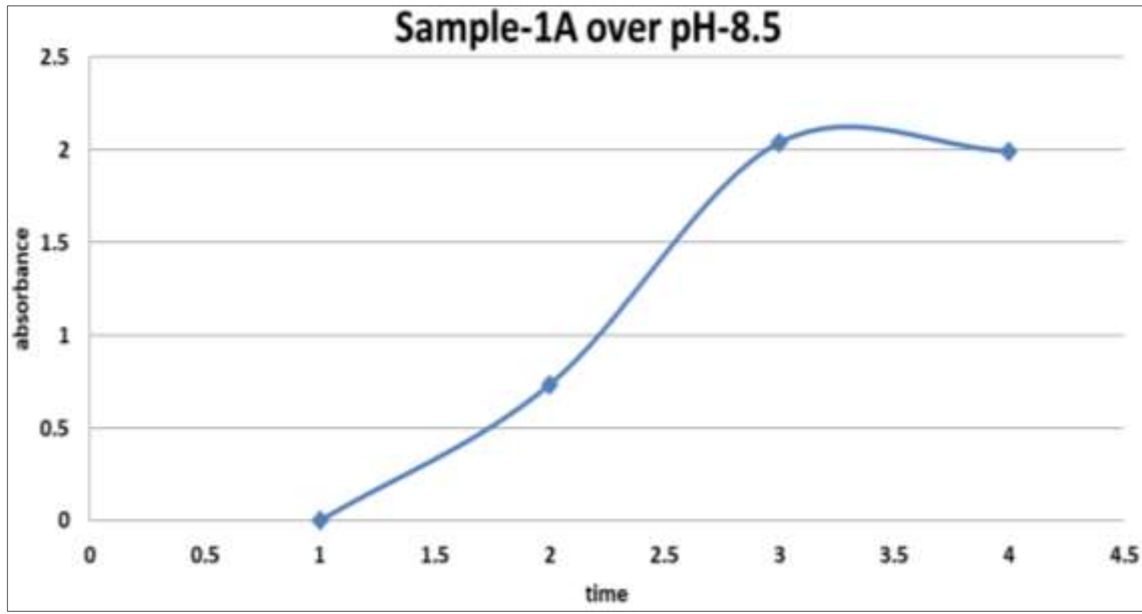
b) Growth Pattern of 1A











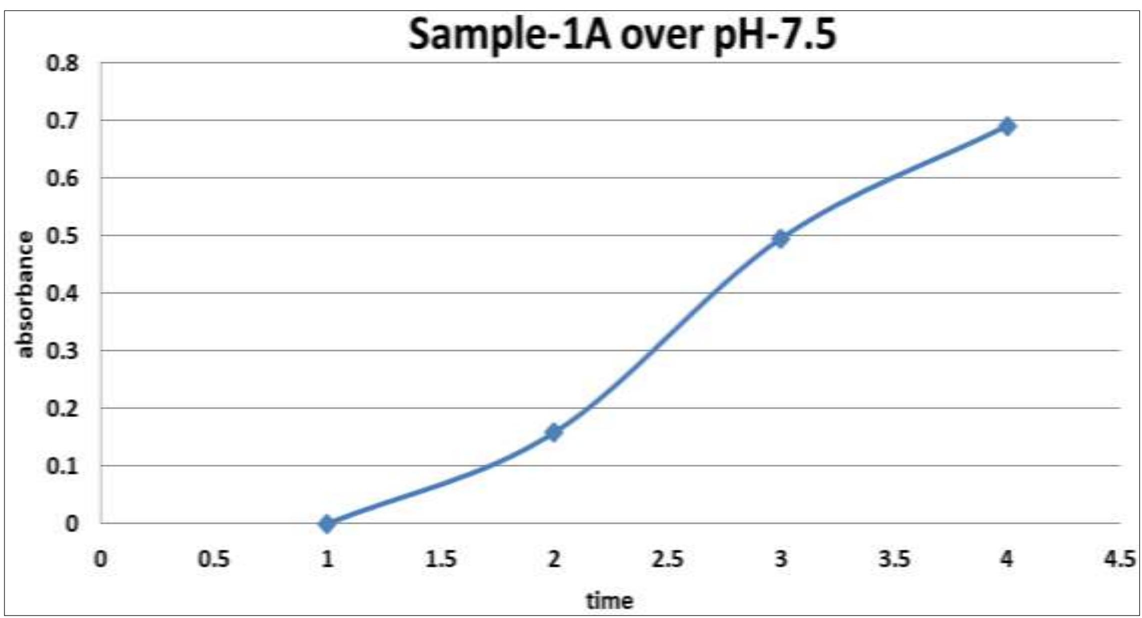
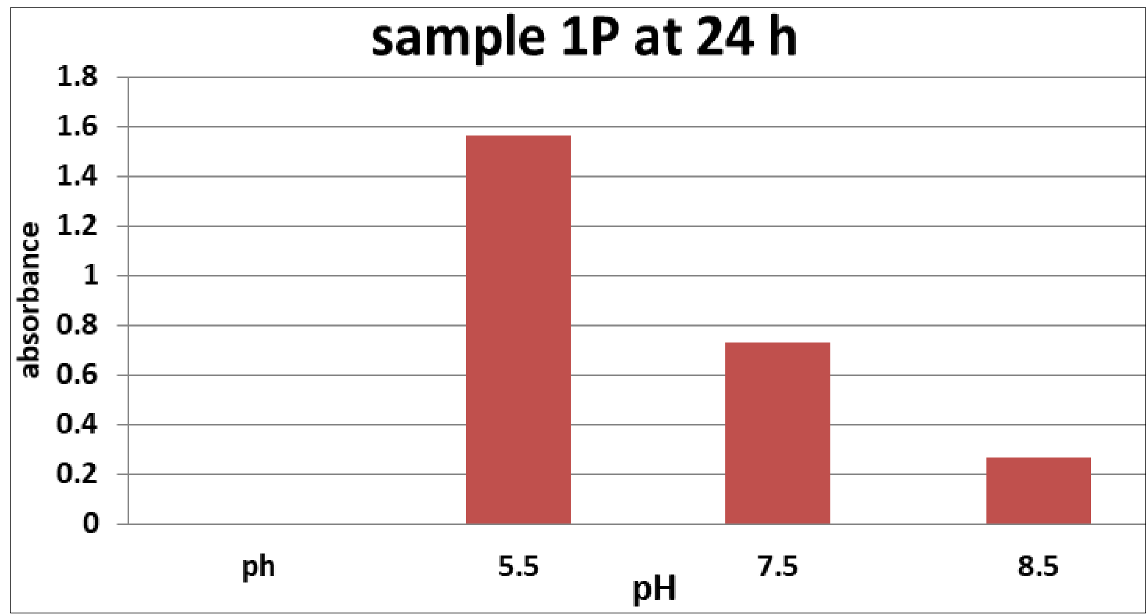
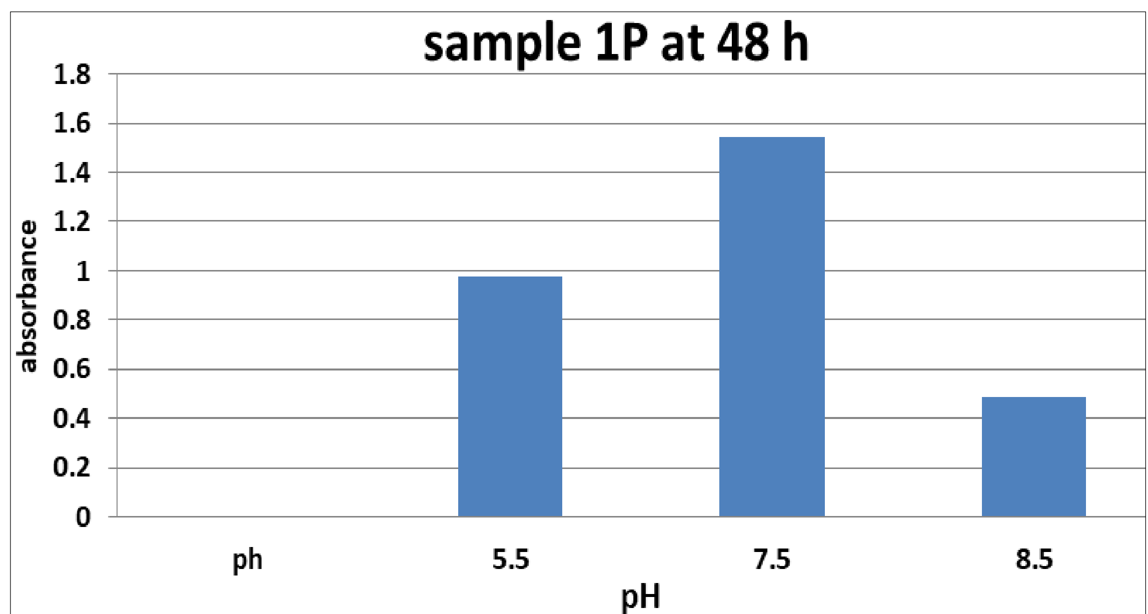
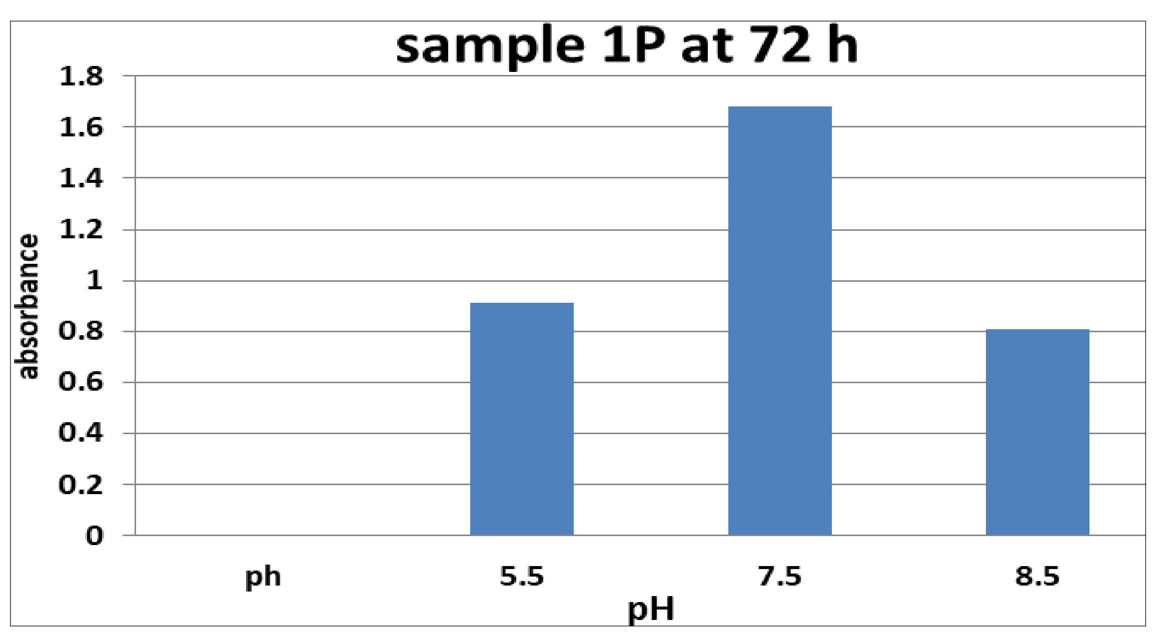


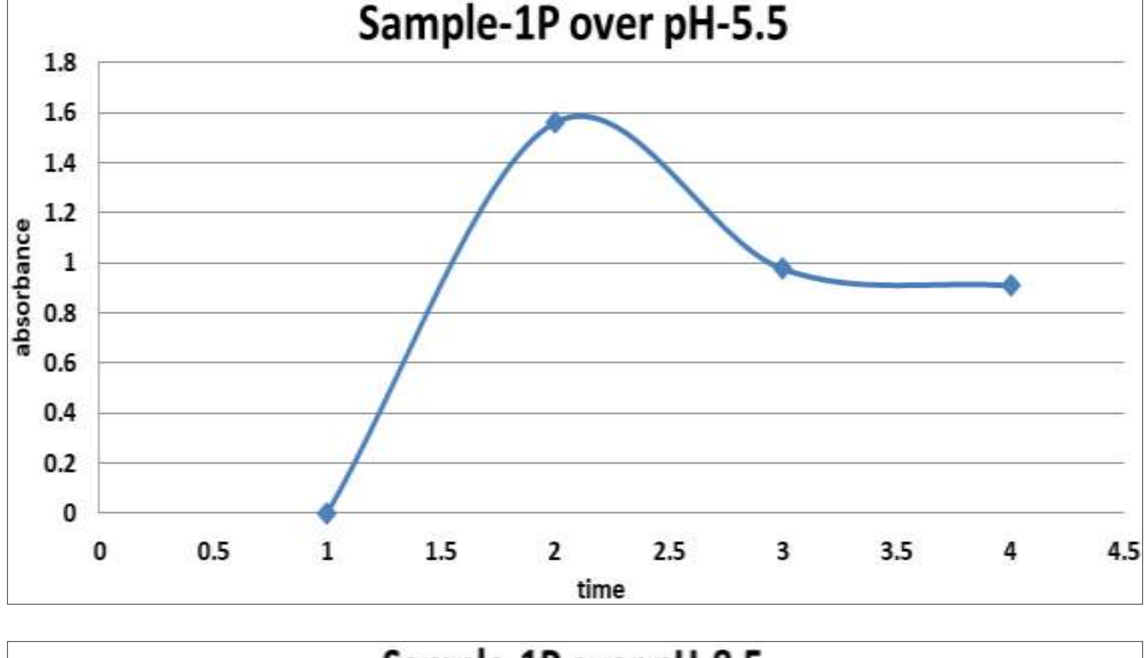
Fig 4: The 1st day growth shown by the 1A bacterial sample was good in pH 5.5 whereas the last day at pH 8.5 the growth was observed to be best

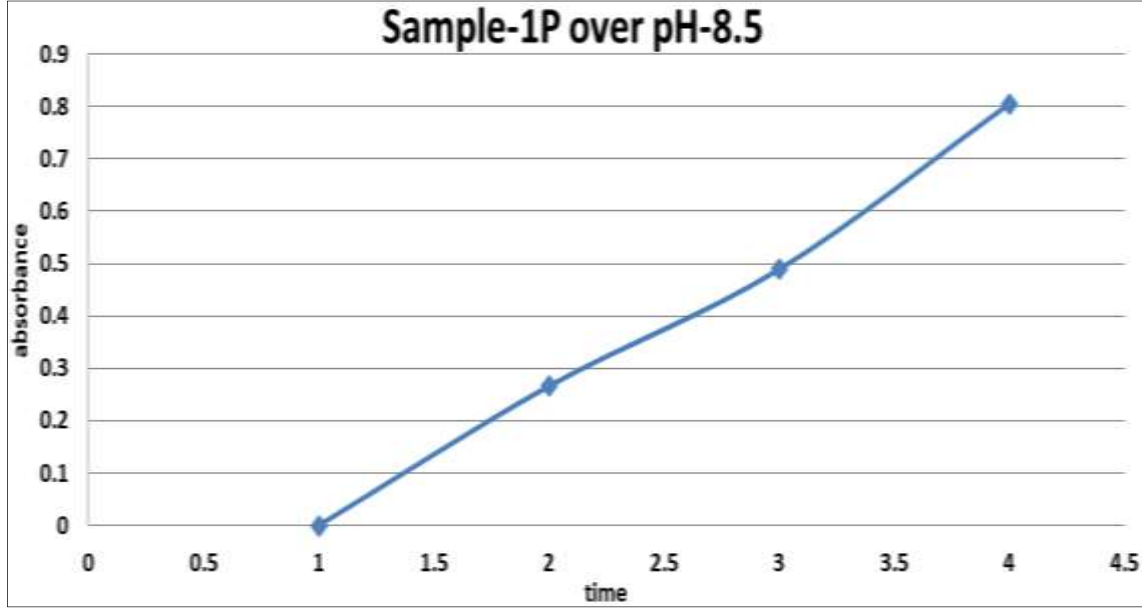
(c) Growth Pattern of 1P











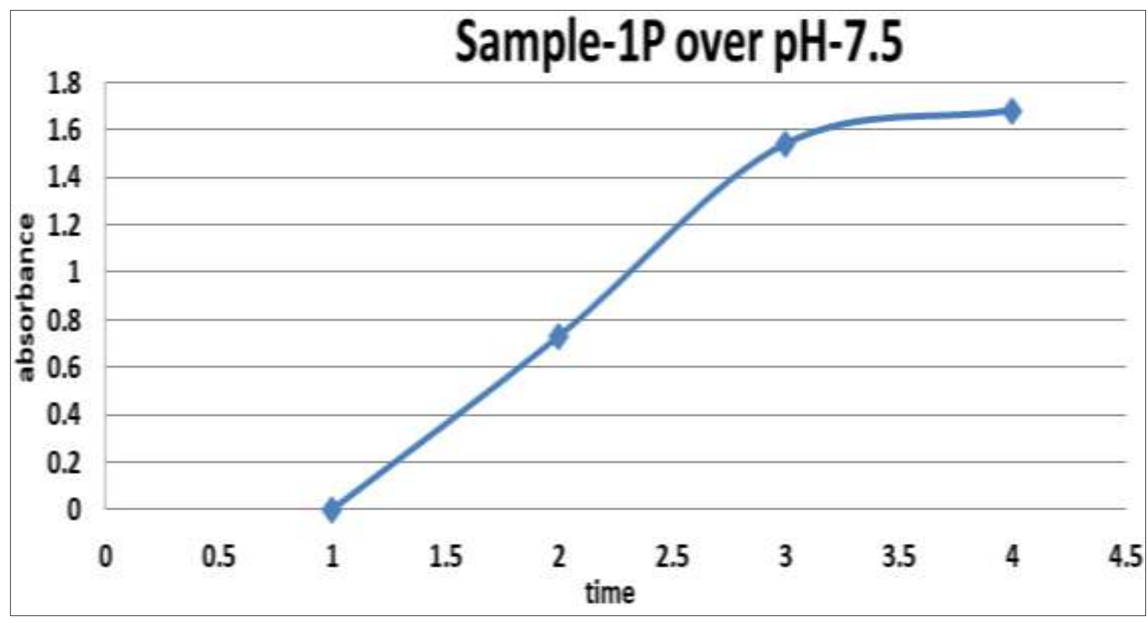
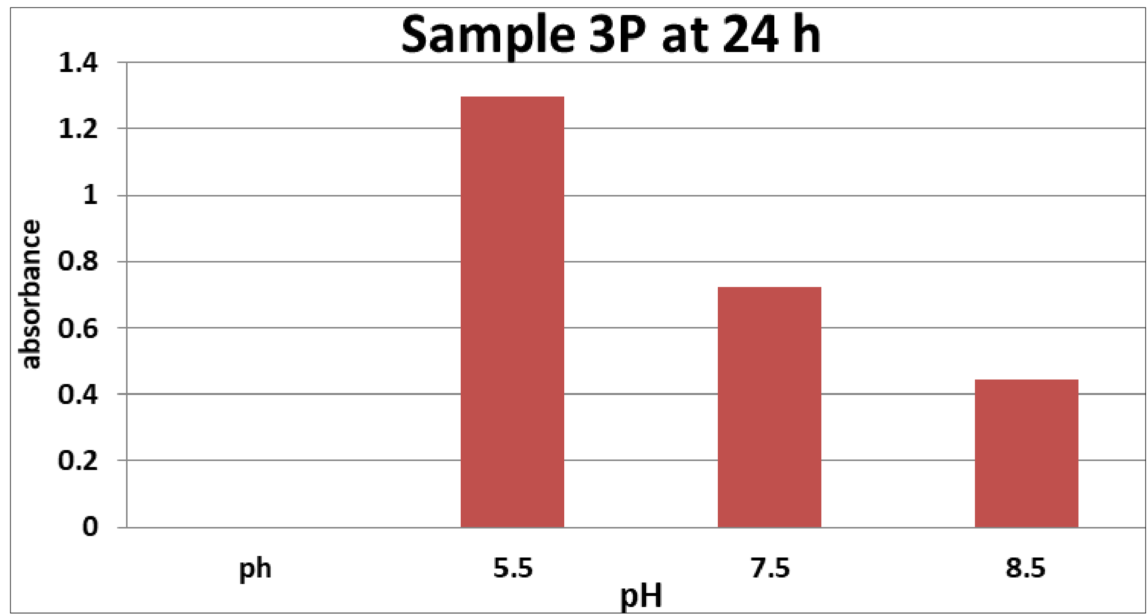
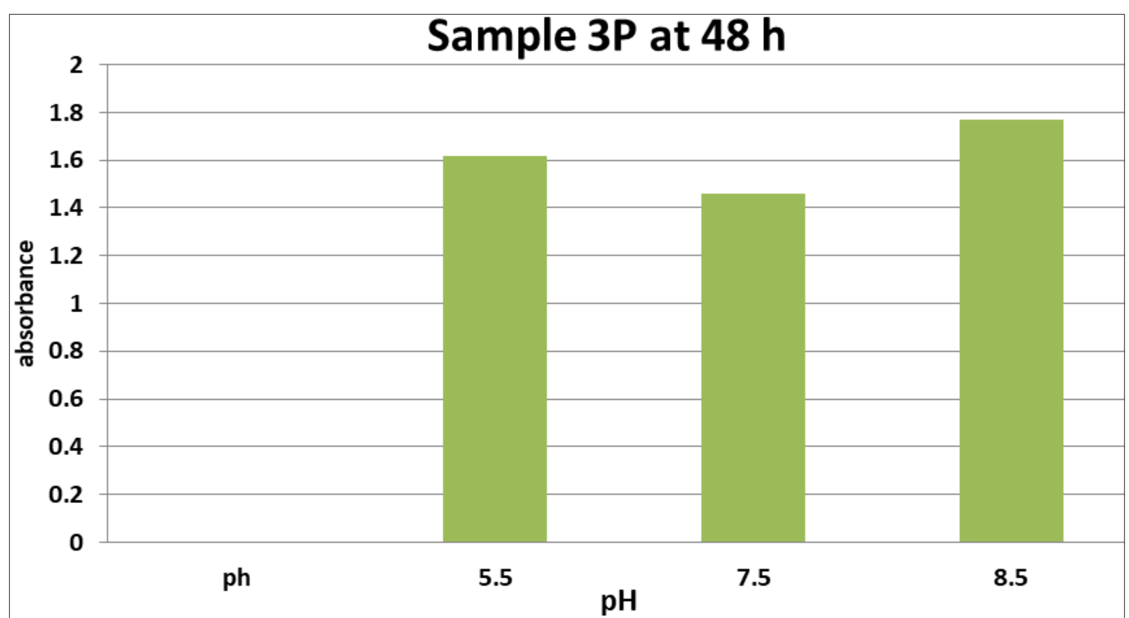
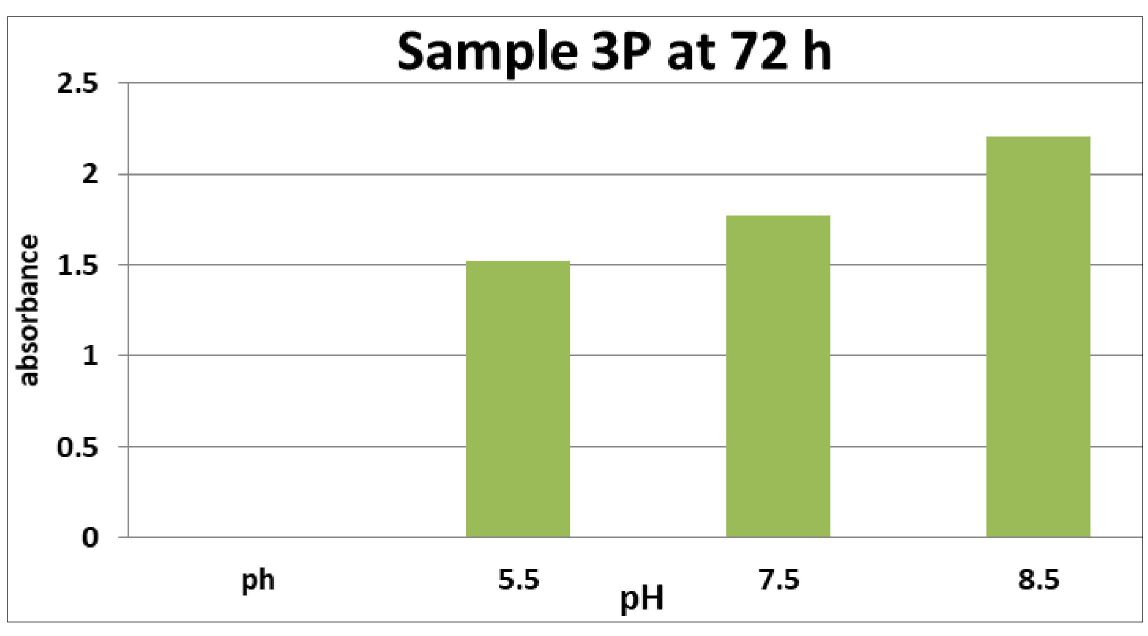


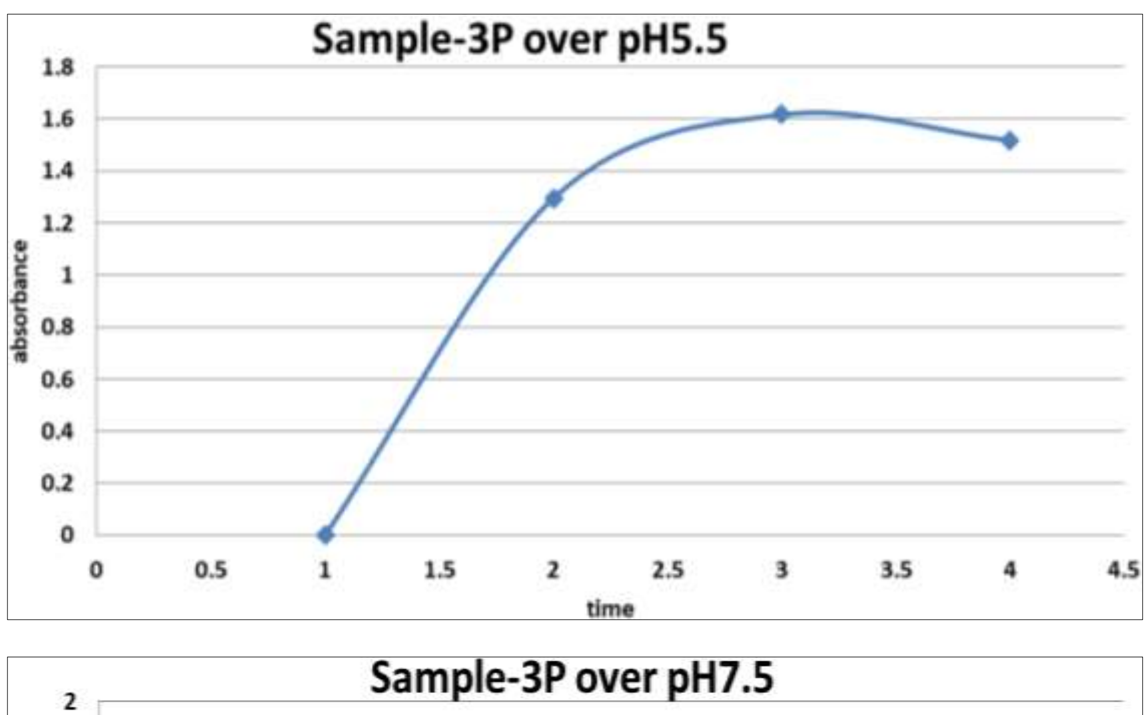
Fig 5: The bacteria of the sample 1P initially grows better on pH 5.5 but in the later stage it shows growth mostly on 7.5 (neutral)

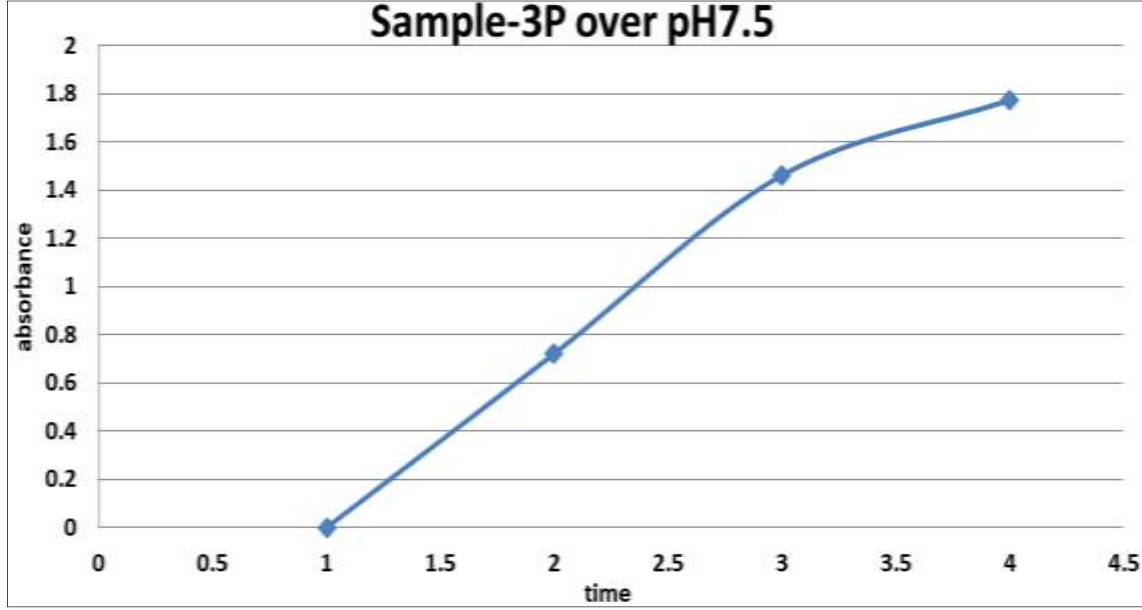
(d) Growth Pattern of 3P











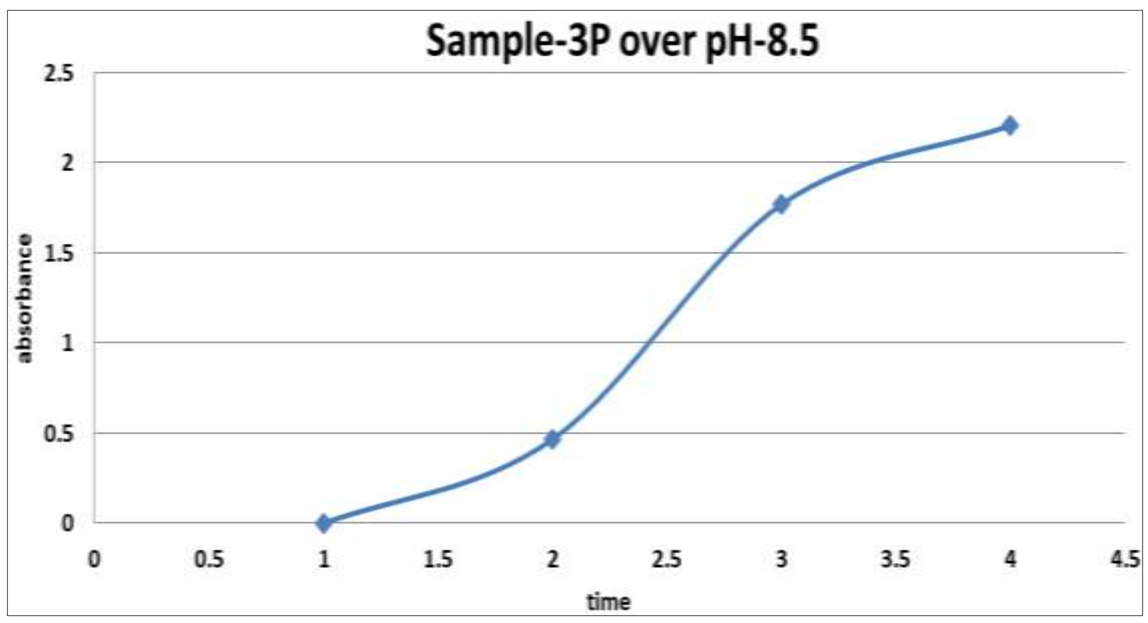
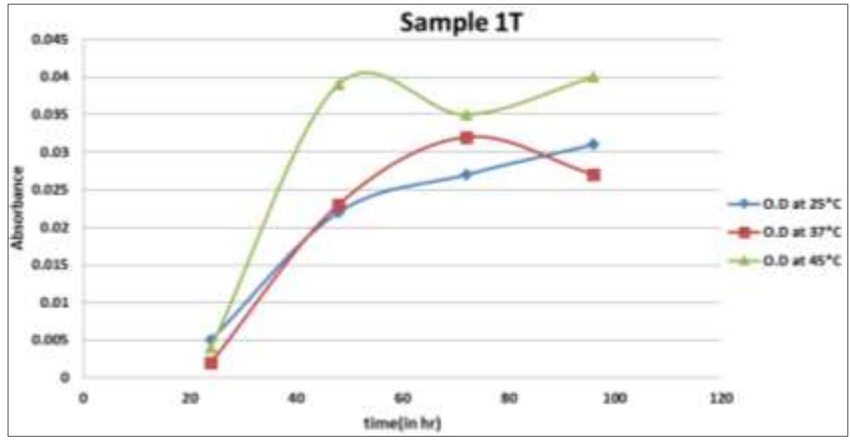
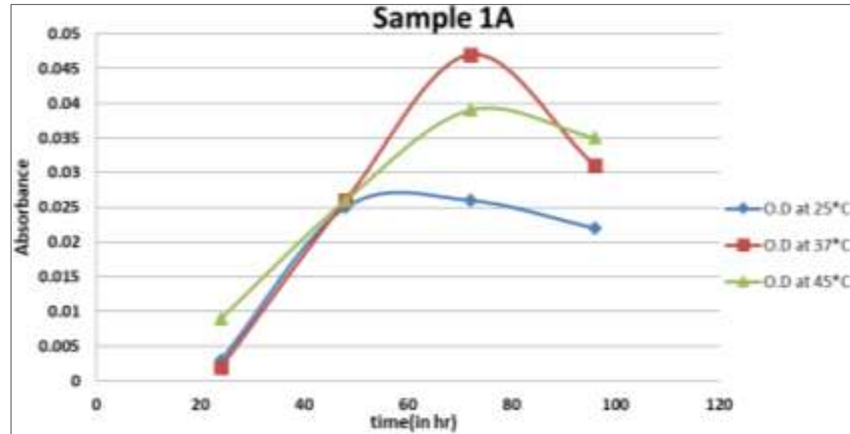
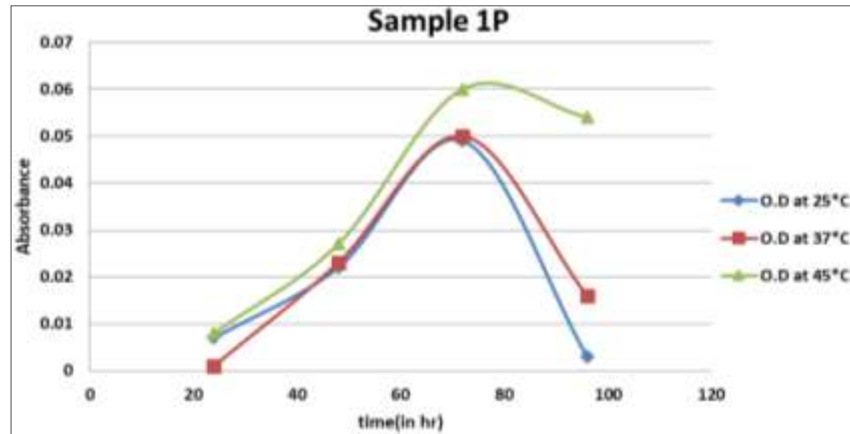


Fig 6: Initially at pH 5.5 the growth of the bacteria of sample 3P was high, then the growth showed mostly at pH 8.5 (it should be basic)

Optimization of temperature for bacteria producing cellulase
Growth curves of bacterial samples on CMC broth







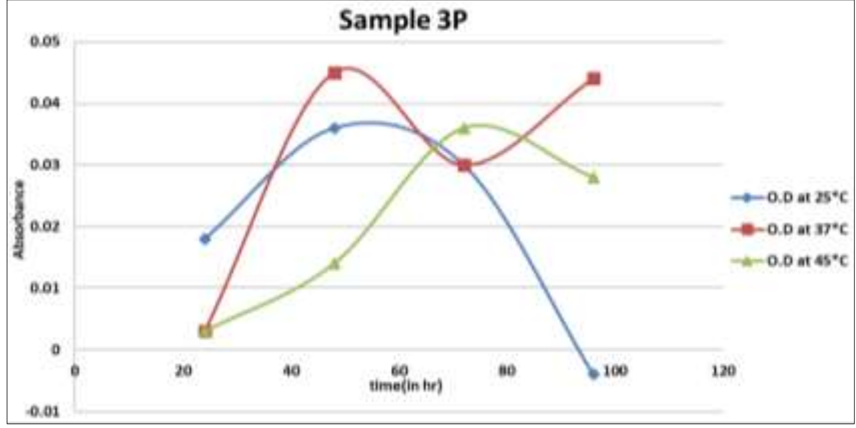


Fig 7: The optimum temperature for growth of cellulose producing bacteria

Molecular Identification

Isolation of genomic DNA from select bacterial isolates

Agarose Gel Electrophoresis of DNA isolates run on 1.5% Agarose Gel

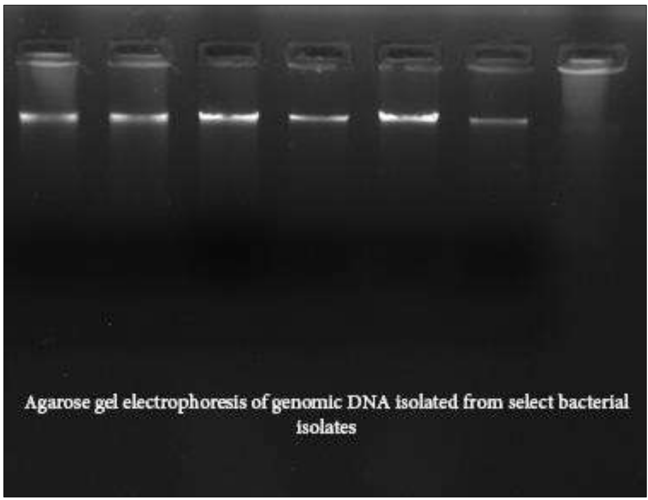


Fig 8: Agarose gel electrophoresis of genomic DNA isolated from selected bacterial isolates

Polyacrylamide Gel Electrophoresis of PCR products

The 16s rRNA amplicons were resolved in polyacrylamide gel. The resolved bands of each bacterial isolate were seen in the image below

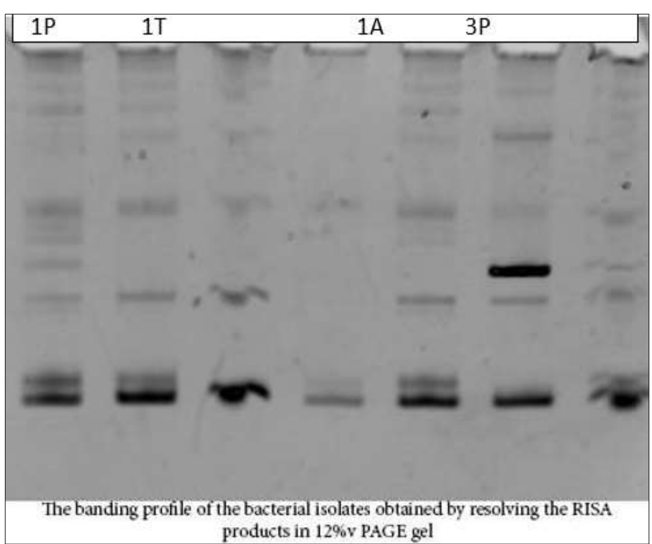


Fig 9: Polyacrylamide gel electrophoresis of PCR amplicon

In order to characterise the strain, the nucleotide sequences of the 16S rDNA of the strain was determined. The 16S rRNA gene sequencing analysis evidenced that the strains had highest homology with *Paenibacillus* and *Pseudomonas*. According to Wang *et al*. (2008) [22], a thermophilic, cellulose-degrading bacterium called *Paenibacillus* sp. strain B39 was identified as endoglucanase based on its high activity on CMC substrate activity. The bacterium was isolated from the compost made from chicken dung. Moreover Islam *et al*., 2019 [4] some beneficial bacteria that produce cellulase from the molasses leftover from the sugar industry. *Paenibacillus* sp. showed the greatest potential for maximum cellulase synthesis under ideal conditions among the isolated strains, and the purified enzyme's molecular weight was determined to be 66.9 kDa. The purified cellulase demonstrated great specificity on CMC based on the substrate specificity test, indicating that it is an endo-β-1, 4-glucanase.
In previous study Vimal *et al*., (2016) [21] reported that uging the CMC coated plates as a growing medium, three bacteria were identified from the waste paper sector. Gram iodine was added to the mixture to validate the strains potential cellulase-producing capability by forming a clear zone. *Bacillus* subtilis, *Bacillus* cereus, and CB3, CB4, and CB8 strains were determined by their morphological, cultural, and biochemical traits. Using 16s r RNA sequence analysis, the isolates were run through a molecular identity process. Identifying and differentiating closely related bacterial species is made possible by 16s rRNA sequencing. The evolutionary relationships of the isolates are also revealed by the phylogenetic tree that was built using the sequence analysis.
To obtain cellulase with a high yield, stability, and purity, more research is underway.

Conclusion

In this study, cellulase producing bacteria from different source was isolated. The aim was to isolate bacterial cellulase producers as they grow faster as compared to fungi and their requirements are quite simpler. Several isolates could be recovered by spread plate from different sources, producing cellulase. For those isolates displaying cellulase activity on the CMC containing four different genera of isolates including (1A), (1T), (1P), (3P). When Congo red agar test was carried out, the sample 3P showed the largest area of decolourization among all the four sample. The morphological characterization study of the sample using gram staining method the sample 1A showed gram positive filamentous and the sample 1T, 3P and 1P showed gram negative morphology. Different substrates are used in the present study as a carbon source to produce good yield of cellulase enzyme. It has been reported that, physico-chemical factors influence the growth of the organisms and also the cellulase producing microorganisms depend on many factors. In the estimation of temperature and pH, all the samples were found to be basic in nature except 1P (it was observed as mostly acidic). The sample 3P showed best growth in the pH 8.5 suggesting it to be highly basic. Upon molecular identification the 16S rRNA gene sequencing analysis evidenced that the strains had highest homology with *Paenibacillus* and *Pseudomonas.* The present work was a qualitative assay to screen for maximum cellulase producing bacteria amongst the isolates; morphological and genetic analyses of all the isolates were done. The optimum parameters for growth and production of enzyme were studied. Further improvement in the performance of cellulase can be impacted by optimizing various parameters for individual isolates.

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