Exploration of cellulose-degrading actinomycetes and immobilization

A Dissertation Report submitted

for the partial fulfillment of the Degree of Master of Science

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This is to certify that this dissertation work entitled "**Exploration of cellulose-degrading actinomycetes and immobilization**." was successfully carried out by Bhuva Shraddha A. towards the partial fulfilment of requirements for the degree of Master of Science in Microbiology of Atmiya University Rajkot. It is an authentic record of her own work, carried out by her under the guidance of Dr. Mousumi Das during the academic year of 2022-23 The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University..

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D E C L A R A T I O N

I hereby declare that the work incorporated in the present dissertation report entitled "**Exploration of cellulose-degrading actinomycetes and immobilization**." is my own work and is original. This work (in part or in full) has not been submitted to any University for the award of a any Degree or Diploma.

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Abbreviation

Short form	Full form
SCA agar	Starch casein Agar
KNO_3	Potassium nitrate
NaCl	Sodium chloride
K_2HPO_4	Dipotassium phosphate
$MgSO_4.7H_2O$	magnesium sulphate
$CaCO_3$	Calcium carbonate
$FeSO_4\ 7H_2O$	ferrous sulfate
CMC	carboxymethylcellulose
DNSA	3,5-Dinitrosalicylic acid
MR	Methyl red
VP	Vogus-Prosker
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
cm	Centimeter
µ g	Microgram
ml	Milliliter
pH	Potential of Hydrogen ion
OD	Optical Density
mm	Millimeter
ºC	Celsius
min	Minute
U	Unit
mg	Milligram

ABSTRACT

Cellulose is the most prevalent organic polysaccharide, produced by agricultural activity, paper and pulp industries, and textile industries. Actinomycetes are able to release enzymes that can break down organic material, such as cellulose. The aim of this study was to isolate actinomycetes from soil of Rajkot, Gujarat, India, and screen their cellulase enzyme activity and immobilization of enzyme by sodium alginate. A total of 148 isolates of actinomycetes have been isolated using starch casein agar (SCA).The abilities of actinomycetes to degrade cellulose were observed by clear zone on CMC (carboxymethylcellulose) agar medium and Cellulase assay. out of 148 isolates, 132 gave clear zone in CMC agar. 97 isolates gave < 15mm size zone, and 29 isolates gave 15 < 20 mm diameter zone. Among all 6 isolates gave the highest zone of degradation above the range of 30mm. The cellulase enzyme was immobilized by the encapsulated method with sodium alginate for more enzyme stability against unfavorable condition and to check enzyme activity at different temperatures, pH, time and concentration of NaCl. After cellulase assay and optimization studies, that enzyme was partially purified by ammonium sulphate precipitation and dialysis. After gel filtration by SDS-PAGE molecular weight of cellulase founded. By 16s rRNA sequencing SA31 was identified by *streptomyces spp*.

Keywords: actinomycetes, cellulose, cellulase CMC, enzyme purification, Immobilization, gel filtration, SDS-PAGE

1. INTRODUCTION:

Actinomycetes are filamentous Gram-positive microorganisms, they contain high G+C content, and they are aerobic and spore-forming bacteria (Chemoh, Bin-Ismail et al. 2021). Actinomycetes were first described in the late 19th century, and since then, they have been extensively studied for their potential applications in biotechnology and medicine. In Greek, "*atkis*" means ray, and "*mykes*" means fungus, so they have some similar features to fungi. They grow in filamentous-like structures and produce mycelium. (Deepthi, Sudhakar et al. 2012)Which can be aquatic or terrestrial. Actinomycetes are responsible for the earthy odour of soil. (Daquioag and Penuliar 2021) Cellulose is a part of the lignocellulose compound. Cellulose is a natural polysaccharide that is found in the cell walls of plants, algae, and certain bacteria. It is the most abundant organic compound on earth and is widely used in the production of paper, textiles, and various other products .On earth, cellulose is the most prevalent organic polysaccharide. It is produced from agricultural activity, paper and pulp industries, textile industries, etc . Every year, around 100 billion metric tonnes of cellulose are generated naturally, whereas the total biomass is approximately 280 billion metric tonnes. Cellulose is made up of D-glucose, which is linked via ß-1, 4-glycosidic linkages.(Islam and Roy 2018) . The cellulase enzyme plays an important role in the biodegradation of cellulose. This enzyme is produced by microorganisms like bacteria, fungi, actinomycetes, and protozoa. (Gautam, Bundela et al. 2012). This enzyme plays a major role at the industrial level and also plays role in bioremediation, wastewater treatment, etc.(Khan, Luna et al. 2016)There are three enzymes in cellulase: endoglucanase, exoglucanase (cellobiohydrolases), and ß -glucosidase(Jayasekara and Ratnayake 2019) Endoglucanase makes a random nick in cellulose. (Sukumaran, Singhania et al. 2005). For the conversion of cellulose into glucose, some steps are included:

Cellulos ⟶ cellobiose ⟶ glucose

Immobilization of enzymes refers to the process of attaching or confining enzymes onto a solid support or matrix, which allows them to retain their activity and stability for longer periods. The immobilization of enzymes has many benefits, including increased stability, reusability, and ease of separation from reaction products. (Almeida, Prata et al. 2022)There are several methods for immobilizing enzymes, including physical adsorption, covalent bonding, encapsulation, and entrapment.(Elakkiya, Prabhakaran et al. 2016) The

process of enzyme immobilization using calcium alginate involves the formation of a gel matrix around the enzyme, which protects the enzyme from environmental stresses and maintains its catalytic activity. The performance of immobilized enzymes can be affected by several factors, such as bead size, enzyme loading, cross-linking agent concentration, and reaction conditions. Optimization of these parameters can improve the activity and stability of the immobilized enzyme, making it more effective for various applications.(Sirisha, Jain et al. 2016) One of the common methods for purifying cellulase enzymes is ammonium sulfate precipitation. Ammonium sulfate is a salt that can be used to selectively precipitate proteins based on their solubility. Proteins can be selectively precipitated by adjusting the concentration of ammonium sulfate in the solution. At certain concentrations of ammonium sulfate, proteins with low solubility will precipitate out of the solution while leaving other proteins in the solution.Gel filtration chromatography, also known as size exclusion chromatography, is a type of liquid chromatography used to separate molecules based on their size and shape. It is a commonly used technique in biochemistry and molecular biology for the purification and analysis of proteins, nucleic acids, and other biomolecules.(Ó'Fágáin, Cummins et al. 2017)Cellulase SDS-PAGE is a technique used to analyze and separate cellulase enzymes based on their molecular weight. SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and it is a commonly used technique in biochemistry and molecular biology to separate proteins based on their size.(Hu, Heitmann Jr et al. 2010)

Aim :

To Explore cellulose degrading actinomycetes and immobilization.

Objectives :

- Isolation of actinomycetes from soil.
- Primary & secondary screening of cellulose-degrading actinomycetes.
- Immobilization of cellulase enzyme.
- Enzyme purification by ammonium sulphate precipitation method.
- Gel filtration chromatography and SDS-PAGE
- Molecular identification of potent cellulose-degrading actinomycetes.

2. Review of literature

2.1 Actinomycetes

Actinomycetes are a distinct group of microbes. Actinomycetes, like true bacteria, are prokaryotes. Actinomycetes are classified as actinobacteria. The taxon currently accommodates gram- positive bacteria that have DNA with high guanine-plus-cytosine content (G+C [69 to 73 mol %]) and that form extensive branching substrates and aerial mycelia.(Goodfellow and Williams 1983) Their growth is characterized by the formation of normally branching threads and rods, frequently giving rise to a typical mycelium, which is unicellular, especially during the early stages of growth.(AbdElgawad, Abuelsoud et al. 2020) Actinomycetes reproduce by special sporulating bodies or from parts of the vegetative mycelium (Mahajan and Balachandran 2012). They are a rich source of secondary metabolites with diverse biological activity (Jagannathan, Manemann et al. 2021)Actinomycetes can be isolated and identified from various natural habitats such as soils from various ecological units, marine water, pollen grain, sand, alkaline lake water.(Lin, Chen et al. 2011) Actinomycetes isolated from soil that secretes a range of extracellular enzymes and exhibits the capacity to metabolize recalcitrant molecules (McCarthy and Williams 1992). Actinomycetes centred mainly on their ability to form antibiotics, along with certain vitamins and enzymes. Actinomycin antibiotic produced by actinomycetes. Actinomycetes, particularly *Streptomyces,* play a major role in antagonistic interactions in soil (Dilip et al; 2013). Actinomycetes also produce lignocellulose enzymes which are useful in paper and pulp industry , textile industries , waste water treatment etc. several actinomycete strains that showed high cellulase activity. The study screened over 500 actinomycete strains and identified six strains that showed the highest cellulase activity. (De Simeis and Serra 2021)

2.2 Cellulose

Cellulose waste is a by-product generated by various industries, including pulp and paper, textile, food, and agriculture. It is composed of cellulose, hemicellulose, lignin, and other organic compounds, and its disposal can pose significant environmental challenges. Thus, the utilization of cellulose waste has gained significant interest in recent years. In this literature review, we will focus on the different strategies for the utilization of cellulose

waste produced from various industries.(Sundarraj and Ranganathan 2018)One of the major industries that generate cellulose waste is the pulp and paper industry. A study published in the Journal of Cleaner Production proposed the utilization of cellulose waste from the pulp and paper industry for the production of biofuels. The study demonstrated that cellulose waste could be hydrolysed into simple sugars, which could be used as a substrate for the production of biofuels such as ethanol (Bajpai et al., 2017).Another industry that generates a significant amount of cellulose waste is the textile industry. A study published in the Journal of Environmental Management proposed the utilization of cotton waste generated by the textile industry for the production of cellulose nanofibers. The study demonstrated that cotton waste could be chemically treated to produce cellulose nanofibers, which could be used in various applications such as packaging, papermaking, and bio composites (de Amorim, de Souza et al. 2020)The food industry is another major producer of cellulose waste, which is generated from various sources such as fruits, vegetables, and grains. A study published in the Journal of Food Science and Technology proposed the utilization of cellulose waste generated from fruits and vegetables for the production of dietary fibres. The study demonstrated that cellulose waste could be processed to produce dietary fibres, which could be used in various food applications such as bakery products, dairy products, and meat products (Baghel, Reddy et al. 2021)

2.3 cellulase

Cellulase enzymes play a crucial role in the degradation of cellulose, which is a major component of plant cell walls. Cellulases are produced by a wide range of microorganisms and are classified into three main categories: endoglucanases, exoglucanase, and β-glucosidases. Endoglucanases randomly cleave the internal β-1,4-glycosidic bonds in the cellulose chain, while exoglucanase cleave the ends of the cellulose chain. β-glucosidases hydrolyse cellobiose, which is a product of endoglucanase and exoglucanase activity, into glucose.(Begum and Absar 2009)A study by Horn et al. (2012) used electron microscopy and biochemical assays to investigate the degradation of crystalline cellulose by cellulase enzymes. The authors proposed a model for the synergistic action of endoglucanases and exoglucanase in the degradation of cellulose.(Horn, Vaaje-Kolstad et al. 2012)Another study by (Lynd, Weimer et al. 2002) discussed the challenges and opportunities of using cellulase enzymes for the production of biofuels. The authors highlighted the importance of improving the efficiency of cellulase enzymes and reducing the cost of their production

to make biofuel production more economically feasible.(Lynd, Weimer et al. 2002)a study by Zhao, Liu et al. 2018 investigated the regulation of cellulase production by Trichoderma reesei, a fungus that is widely used for industrial cellulase production. The authors identified several transcription factors that play a key role in the regulation of cellulase gene expression in this organism.(Zhao, Liu et al. 2018)

2.4 cellulase enzyme immobilization

Cellulase immobilization is a process of attaching enzymes to a solid support, which enhances their stability and reusability. This technique has been extensively studied in recent years, and a large number of research papers have been published on various aspects of cellulase immobilization.(Podrepšek, Primožič et al. 2012)One of the commonly used solid supports for cellulase immobilization is chitosan, which is a natural polysaccharide with a high affinity for enzymes. In a study published in the Journal of Molecular Catalysis B: Enzymatic, Chitosan beads were used to immobilize cellulase from Bacillus subtilis. The immobilized cellulase showed higher thermal stability and reusability compared to the free enzyme (Li, Li et al. 2018)Another study published in the Journal of Chemical Technology and Biotechnology explored the use of magnetic nanoparticles as a solid support for cellulase immobilization. The cellulase from Trichoderma reesei was immobilized on magnetic nanoparticles coated with chitosan. The immobilized enzyme showed a 90% retention of its activity after five cycles of reuse (Fang, Zhang et al. 2019).In addition to chitosan and magnetic nanoparticles, other solid supports such as alginate, silica, and graphene oxide have also been investigated for cellulase immobilization. For instance, in a study published in the Journal of Biotechnology, cellulase from Aspergillus niger was immobilized on alginate beads. The immobilized enzyme showed high stability at a wide range of pH and temperature conditions (Wang, Yu et al. 2019)Furthermore, some researchers have investigated the use of covalent bonding for cellulase immobilization. In a study published in the Journal of Industrial Microbiology and Biotechnology, cellulase from Aspergillus niger was covalently immobilized on functionalized silica. The immobilized enzyme showed higher activity and stability compared to the free enzyme (Bilal, Asgher et al. 2019)

3. MATERIALS & METHODS

3.1 Sample collection and pre-treatment:

Soil samples were collected from several locations in Rajkot, Gujarat (cattle grazing area, RMC dumping site etc which is shown in table No 1). Soil samples were collected in an airtight polyethylene bag. Soil samples were oven dried at 50℃ overnight.

3.2 Isolation of actinomycetes:

1 gram of soil samples was suspended in 10 ml of saline buffer (0.9% NaCl) and serially diluted until 10^{-8}, with 0.1 ml of the solution taken from each diluted sample and spread on Starch Casein Agar (SCA) (mg/l: starch 10; casein 0.3; KNO_3 2; NaCl $_2$; K_2HPO_4 2; $MgSO_4 7H_2O$ 0.05; $CaCO_3$ 0.02; $FeSO_4$ $7H_2O$ 0.01 Agar 18; pH7.2). For 5 to 7 days, the plates were incubated at 28 ± 2 °C. (Sapkota, Thapa et al. 2020)

3.3Screening of Cellulase-producing actinomycetes:

3.3.1 Qualitative assay:

After isolation, isolates were spot inoculated on carboxymethylcellulose (CMC) agar plate and incubated at 28 °C for 7 days. After incubation, the plate was flooded with 0.2% Congo red dye for 20 minutes, add 1N NaCl for 15 minutes and then wash. Measure the zone of degradation/zone of hydrolysis.(Shanmugapriya, Saravana et al. 2012)

3.3.2 Quantitative assay

The cellulase activity was measured using the 3.5-dinitro salicylic acid (DNSA) technique. A loopful culture was inoculated in 1% CMC broth and incubated at 28°C in shaking condition. Every day, bacterial culture was taken. 3 ml of culture was added in an Eppendorf tube and centrifuged for 10 minutes at 10,000 rpm. The pellet was separated from the centrifuged supernatant, and the supernatant was utilized as a crude extract enzyme to measure enzyme activity using the DNSA reagent.1% CMC was used as a substrate, then add 0.5M citrate buffer (pH 7) and incubate it. Add 3ml DNSA reagent and boil it for 5 min. Take OD at 540 nm.(Ghose 1987, Narkthewan and Makkapan 2019)

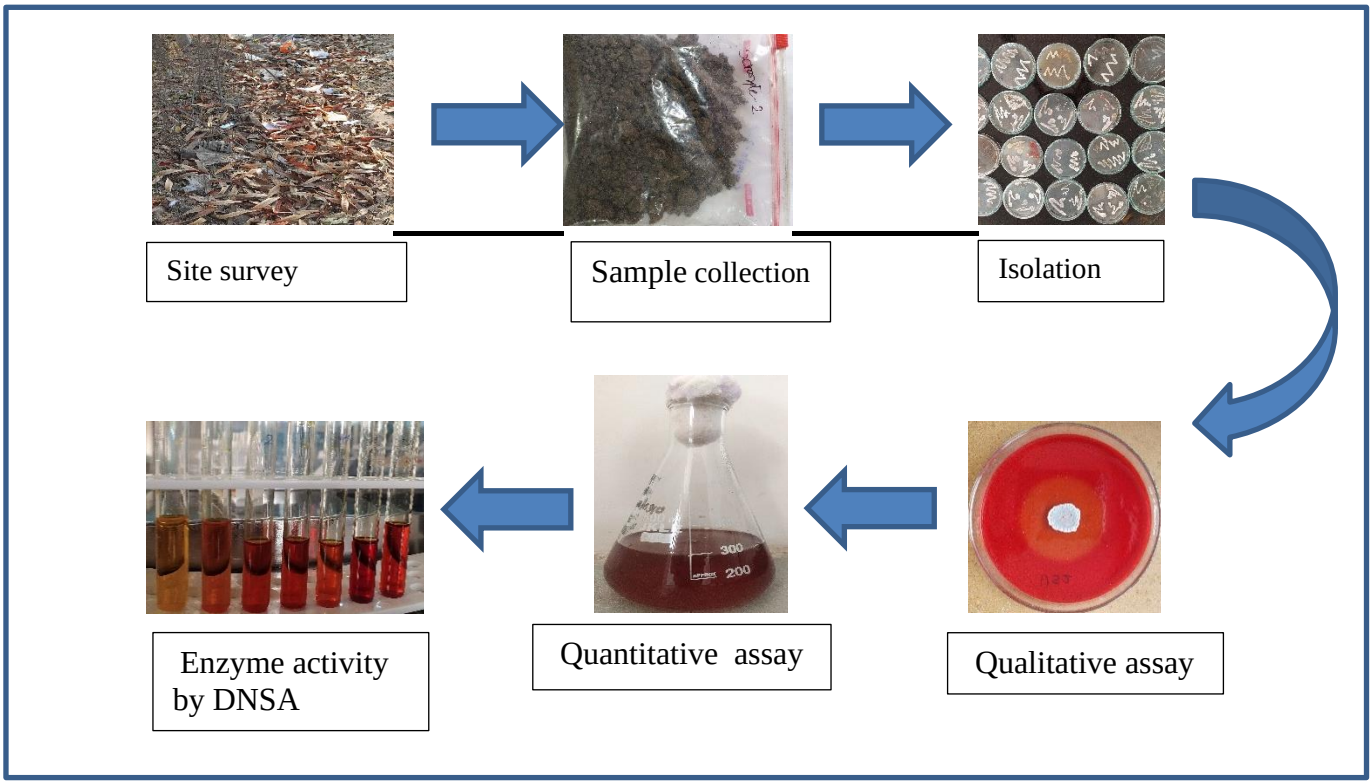

Fig .1 primary and secondary screening

Fig 1 shows site survey , sample collection , Isolation of actinomycetes , qualitative assay on CMC (carboxymethylcellulos) agar, then after quantitative assay in CMC broth and measure enzyme activity by DNSA method in crud enzyme .

3.4 Morphological and Biochemical Characteristics of potential Actinomycetes strain

Colony morphology , important microscopic features namely Gram staining, mycelial branching and sporulation pattern of the potent strain SA31(Malviya, Pandey et al. 2013).For confirmation and identification, there were biochemical tests like the Oxidase test, catalase test, MR, VP, indole, citrate test, (Berd 1973, Mehta and Jadeja 2022) were carried out to identify the genus of SA31comparing with criteria stated in Bergey's manual of systematic bacteriology.(Nordmark, Bakalinsky et al. 2007)

3.4.1 Methyl Red test

Prepare the MR broth tubes were autoclaved and sterilized then add the experimental bacterial culture using sterile techniques. Uninoculated MR broth is used as a control. Both

tubes were incubated at 28ºC for 5-7 days . After appropriate incubation adds 2-3 drops of methyl red indicator to observe the colour changes. After the addition of the indicator remaining the red colour is a positive test and the colour changed to yellow is a negative test

3.4.2 Voges-Proskauer test

The experimental organism was inoculated into VP broth by loop inoculation using sterile techniques. The uninoculated tube was kept as a control. Both tubes were incubated at 28ºC. After incubation adds alpha-naphthol and 40% KOH as an indicator. After adding the indicator the crimson-red colour formation indicates the positive result and colour change is the negative result of the

3.4.3 Citrate utilization test

Simmons citrate agar slant was prepared and autoclaved for sterilization. Then slant was inoculated with the test organism by stab and streak inoculation. An uninoculated tube was kept as control. Both tubes were incubated at 28ºC for 5-7 hours. After proper incubation observed the tubes for the growth and coloration of the medium. The colour of the medium if changed to blue indicates a positive result and green colour indicates a negative result .

3.4.4 Indole test

First, prepare the peptone water tubes were incubated with the bacterial culture broth culture using sterile needle techniques. The uninoculated broth was used as a control and both tubes were incubated at 28 ºC for 4-7 . After complete incubation, add 1 ml of Kovac's reagent in both tubes. After the addition of the reagent observed the cherry red colour on the top layer of the tube is a positive test and the absence of red coloration is indole negative

3.4.5 Oxidase test

For the oxidase test, the test organisms were rubbed over the oxidase disc and saw the colour changes of the disc. If the colour changes to purple that gives a positive result on the test.

3.4.6 Catalase test

For catalase test , take loopful culture on glass slide and add drops of hydrogen peroxide . If bubble formation occurs, it indicates positive test.

3.4.7 Starch hydrolysis

For starch hydrolysis test add iodine in culture containing starch agar plate . If it gave clear zone, it indicates positive result .

3.5 Enzyme immobilization

Prepare 40 ml of 2 % sodium alginate slurry by autoclaving and then cool it at room temperature,10 ml enzyme solution is mixed with the sodium alginate solution to form a homogeneous mixture. The mixture is then dropped into a cross-linking agent such as calcium chloride, which causes the formation of sodium alginate beads that encapsulate the enzyme. The beads are allowed to harden for a period of time, typically 30-60 minutes, to form a stable matrix. The beads are washed with distilled water to remove any residual cross-linking agent and then stored in a suitable buffer solution.(Abdel-Sater, Hussein et al. 2019)

3.6 Optimization of different conditions for free enzyme and immobilized enzyme

3.6.1 Effect of temperature on the activity of free enzyme and immobilized enzyme

Respectively free enzyme and immobilized enzyme (beads) were added in 1% CMC broth and then added to a buffer after that incubated at various temperatures (35, 40, 45 ,50 , 55, 60 ℃) for 30 min. Add 3 ml DNSA reagent and boil it for 5 min and measure O.D. at 540 nm. (Abdel-Sater, Hussein et al. 2019, Sulyman, Igunnu et al. 2020)

3.6.2. Effect of buffer pH on the activity of free enzyme and immobilized enzyme

The activity of enzyme is determined by adding enzyme (free enzyme and immobilized enzyme) substrate into buffer which contains various pH (4, 5, 6, 7, 8) then incubating it for 5 min and adding 3ml DNSA reagent, boiling it and measure O.D. at 540 nm. (Sulyman, Igunnu et al. 2020)

3.6.3 Effect of incubation time on the activity of the free enzyme and immobilized enzyme

For optimization of incubation time for free enzyme and immobilized enzyme, add enzyme in 1% CMC and buffer then incubate for various time periods (20,40,60,80 min). Add 3 ml DNSA reagent and boil it for 5 min and measure O.D. at 540 nm.(Bellaouchi, Abouloifa et al. 2021)

3.6.4 Effect of NaCl concentration on the activity of the free enzyme and immobilized enzyme

Respectively free enzyme and immobilized enzyme (beads) were added in 1% CMC (which contain various NaCl concentration 1% ,2%, 3% , 4%), add a buffer, and then incubate it . Add 3 ml DNSA reagent and boil it and measure O.D. at 540 nm. (Tamilanban, Velayudhan et al. 2017)

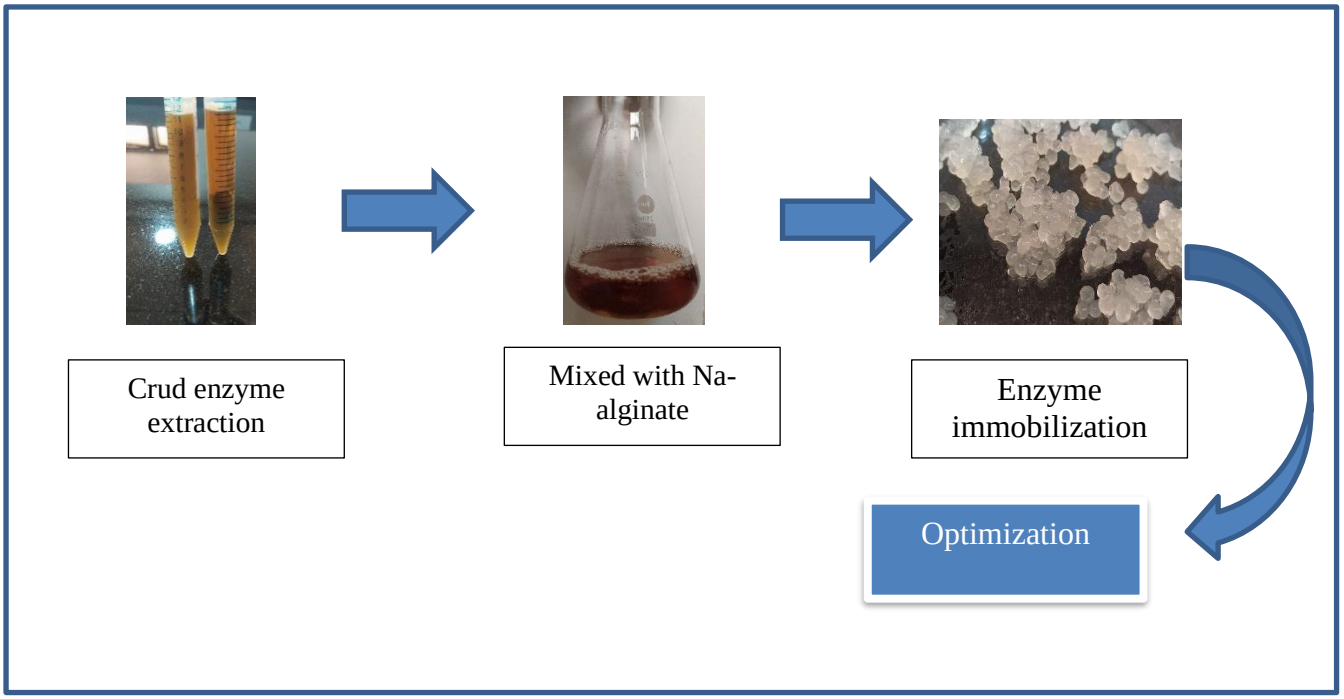

Fig. 2 enzyme immobilization and optimization

Fig.2 shows crud enzyme extraction from cultured broth by centrifugation after that mix it with sodium alginate and then dropped it in calcium alginate solution . After immobilization ,optimize both immobilized and free enzyme at different condition like temperature , pH , incubation period and NaCl concentration . It is measured by DNSA method and calculate enzyme activity

3.7 Purification of cellulase enzyme by ammonium sulphate precipitation

3.7.1 Ammonium sulphate precipitation

Take 7 days of incubated 1% CMC broth and centrifuge it at10000 rpm for 10 min and take supernatant as crud enzyme. 70% ammonium sulphate was slowly added to the crud enzyme gently stair it and kept under cooling condition for precipitation, centrifuge it at 5000 rpm for 15 min and the pallet was dissolved in citrate buffer for dialysis. (Dar, Pawar et al. 2019, Sulyman, Igunnu et al. 2020)

3.7.2 Dialysis

In the dialysis process, the precipitated enzyme is placed in a dialysis bag and dialyzed against a citrate buffer overnight in cooling conditions.(Abdel-Sater, Hussein et al. 2019)

3.8 Gel filtration chromatography

Sephardex G-25 slurry was packed into column and equilibrate with buffer , dialyzed enzyme was appeared to column and fractions were collected and use for enzyme activity.(Tamilanban, Velayudhan et al. 2017)

3.9 SDS-PAGE

SDS-PAGE was performed to determine the molecular weight of protein . (He 2011) For enzyme separation 12 % separating gel (30%Acrylamide-bisacrylamide Solution 6ml ; distilled water 3ml; 2.5X Tris-SDS Buffer pH 8.8 6ml ; 10% APS solution 10 µl ; TEMED 18 µl) and 5% stacking gel (30%Acrylamide-bisacrylamide Solution 1.3ml ; distilled water 5.1ml; 5X Tris-SDS Buffer pH 6.8 1.6ml ; 10% APS solution 75 µl ; TEMED 10 µl) were prepared. (Sulyman, Igunnu et al. 2020) Then add pertained protein ladder and enzyme sample in the wells. Electrophorese at 100 volts and 10 mA as per manual of HiPer SDS-PAGE techning kit , after electrophoresis gel wash with distilled water and then add stain solution and de-stain solution. Visualize the gel for protein bands. (Deka, Jawed et al. 2013)

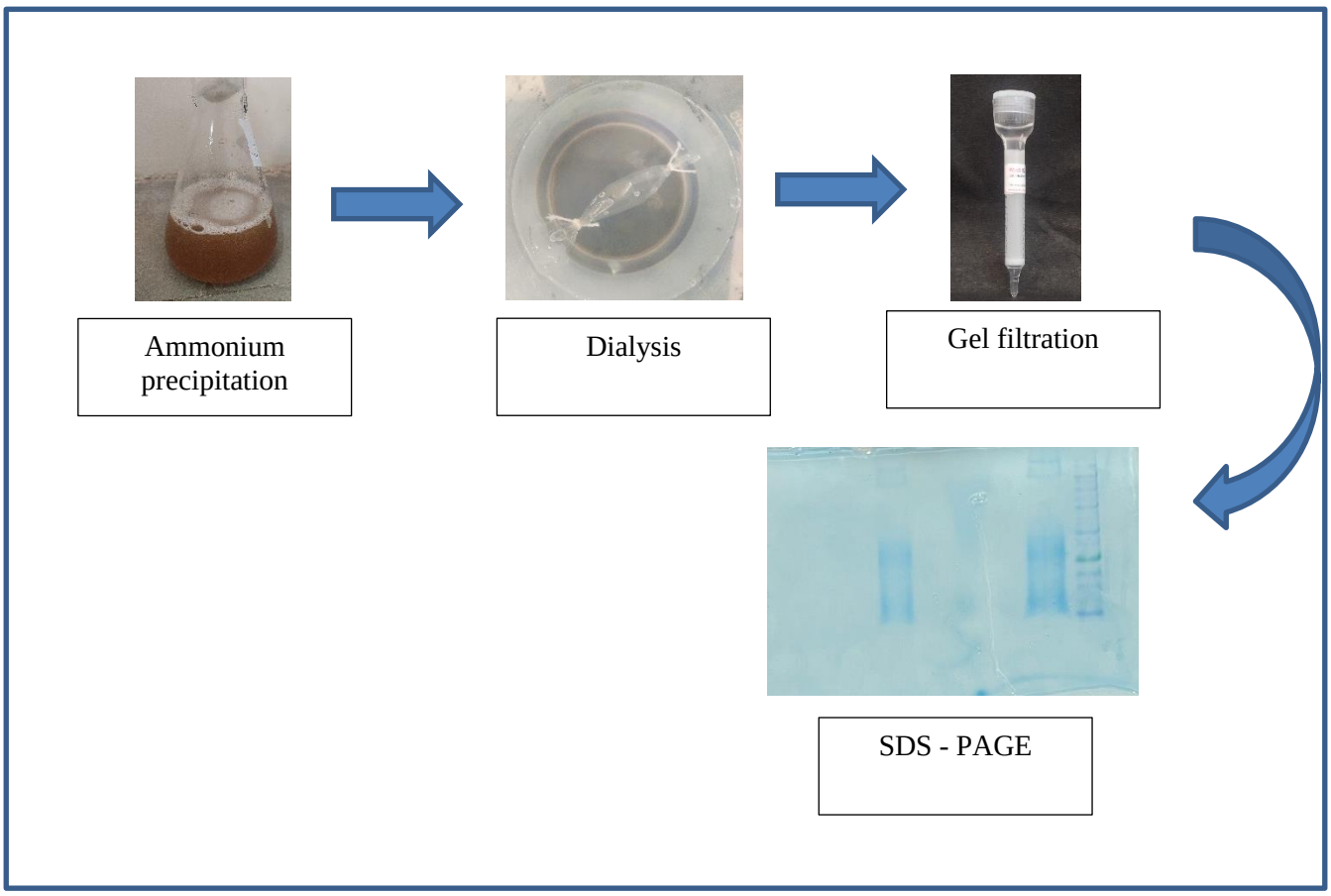

Fig 3 . enzyme purification and characterization

Fig.3 indicate enzyme purification by ammonium sulphate precipitation method. In this method ammonium sulphate add in crud enzyme and precipitate it and centrifuge it. After centrifuge precipitate dissolve in citrate buffer and dialyse by dialysis bag in same buffer. Purify it with gel filtration and proceed for SDS-PAGE .

3.8 Molecular identification

A better cellulose degrading isolate was chosen for molecular characterisation by 16s rRNA gene sequencing based on both hydrolytic value on CMC and enzymatic activity test results. Molecular identification done by gene explore diagnostic and research centre Pvt. Ltd. For molecular identification DNA was isolated on 1% agarose gel , after that fragment of 16S rRNA gene was amplified by PCR . A single discrete PCR amplicon band was observed when resolved on Agarose and purified by column purification. 16S rRNA was used to carry out BLAST with NCBI GenBank database.

4.RESULT

4.1 Isolation of actinomycetes

Based on the morphology on the SCA plate, 148 actinomycetes were isolated from the soil. They have a chalky powder colony. Some isolate have white colour chalky powdery colony , some have Gray colour colony. Many have various reverse pigments like yellow , orange , red ,pink black ,Gray etc.

Table .1 Over view of isolated actinomycete strains from different site

Site name	Number of isolates
Grazing grasslands used by cattle	19
Garden near big bazaar	6
Garden near Gunatit Nagar	11
Munjaka dumping site	16
Site near Uma bhavan road	23
Anandpar Village (site 1)	19
Anandpar Village (site 2)	33
RMC dumping site ,Sheetal Park (bio degradable waste)	13
RMC dumping site ,Sheetal Park (House hold waste)	8

Table 1 contain site name of soil sample collection and number of isolates which gave Powdery colony on SCA plate. This shows that 19 isolates are from Grazing grasslands used by cattle , 6 isolates from Garden near big bazaar, 11 isolates Garden near Gunatit Nagar , 16 isolates fro Munjaka dumping site Munjaka dumping site , 23 isolates from site near uma bhavan ,42 isolates were anand per village site 1 and 2 . 13 isolates were isolated from RMC dumping site .

SA5

SA21

SA23

SA31

SA52

SA83

Fig.4 Potent actinomycete on SCA plate

Fig.4 shows the potent actinomycetes strain SA5 ,SA21,SA 23, SA31 , SA52, SA83 on Starch casein agar medium . Incubated at 30 °C for 5 -7 days . SA5 and SA31 have white powder colony and yellow reverse pigment, SA21 gave grey colour colony , SA23 gave white powdery colony with black reverse pigment . SA52 gave greyish white colny , when SA83 gave orange reverse pigmented colony.

4.2 Qualitative Assay

In the quantitative assay out of 148 isolates, 132 gave clear zone in CMC agar. 97 isolates gave < 15mm size zone, and 29 isolates gave < 20 mm diameter zone after incubated at 30°C for 7 days . Among all 6 isolates, they gave the highest zone of degradation above the range of 30mm. The zone of hydrolysis of potent strain is mentioned in table No. 1

Table .2 Zone of hydrolysis of potent actinomycete strain in CMC agar

Sample number	**Zone of hydrolysis (mm)**
SA5	30
SA21	35.7
SA23	30
SA31	37.5
SA52	30
SA85	32

Table 2 Refer zone of hydrolysis by potent actinomycete strain ,on CMC agar organism spot inoculated and incubate at 30°C for 7 days ,then it stains with Congo red dye . culture SA5 , SA21, SA23 , SA31 , SA52, SA85 respectively gave 30mm , 35.7mm , 30mm, 37mm, 30mm and 32mm diameter zone of hydrolysis . from this result SA31 gave biggest zone of hydrolysis , so it is selected for further study .

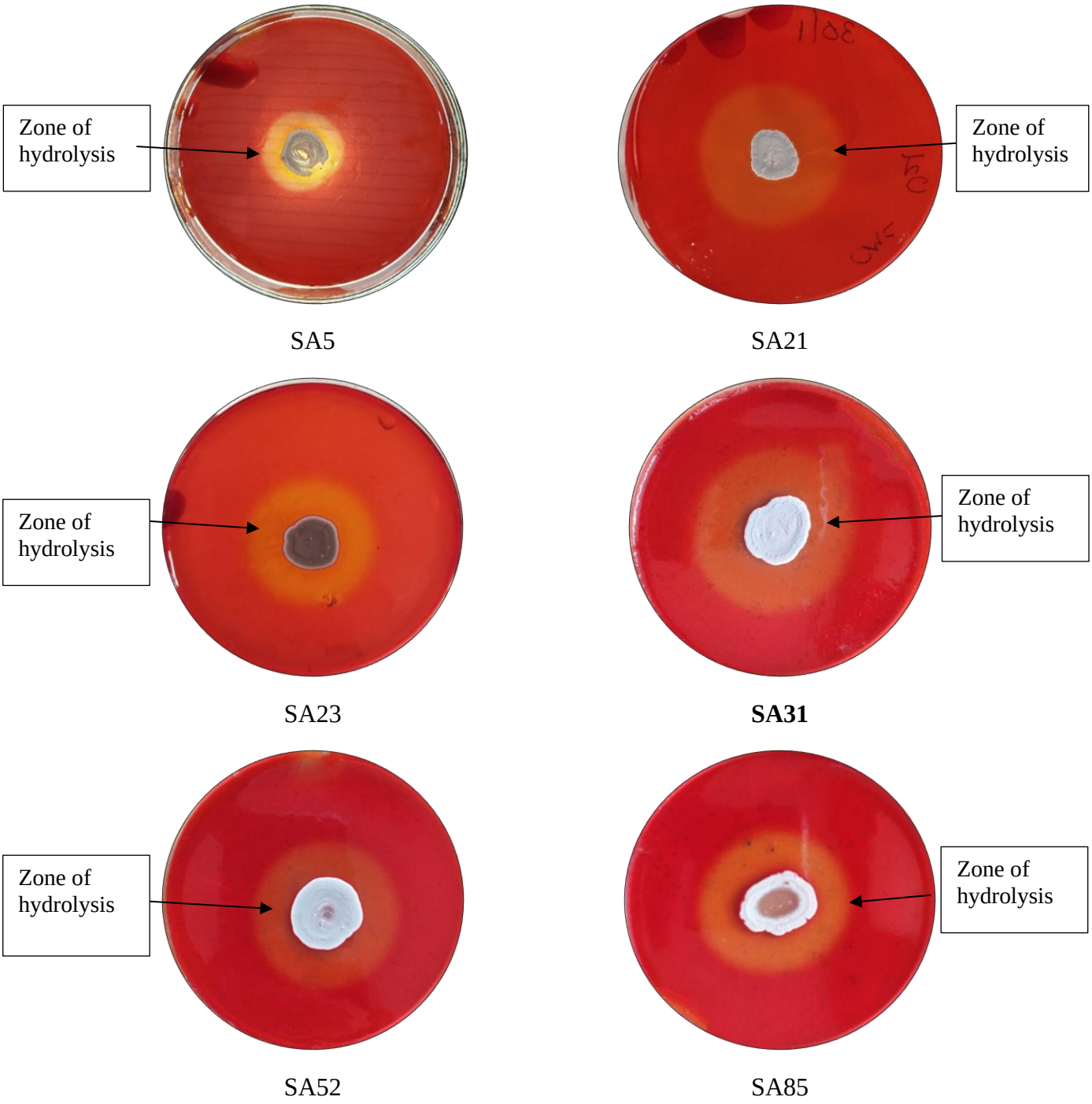

Fig. 5 Zone of hydrolysis by potent actinomycetes on CMC

Fig .5 shows the zone of hydrolysis by actinomycetes on CMC agar plate after incubation of 30°C for 7 days . stain by Congo red dye which gave red colour with non-degraded part and gave yellow color to degraded part , which is hydrolyse by cellulase enzyme produce by actinomycetes .

4.3 Quantitative assay

In quantitative assay every 24 hour take culture from CMC broth and separate crud enzyme by centrifuge and perform DNSA method . The CMCase activity of SA31 was the highest from all isolated cultures. The enzyme activity of potent microorganisms is mentioned in graph.

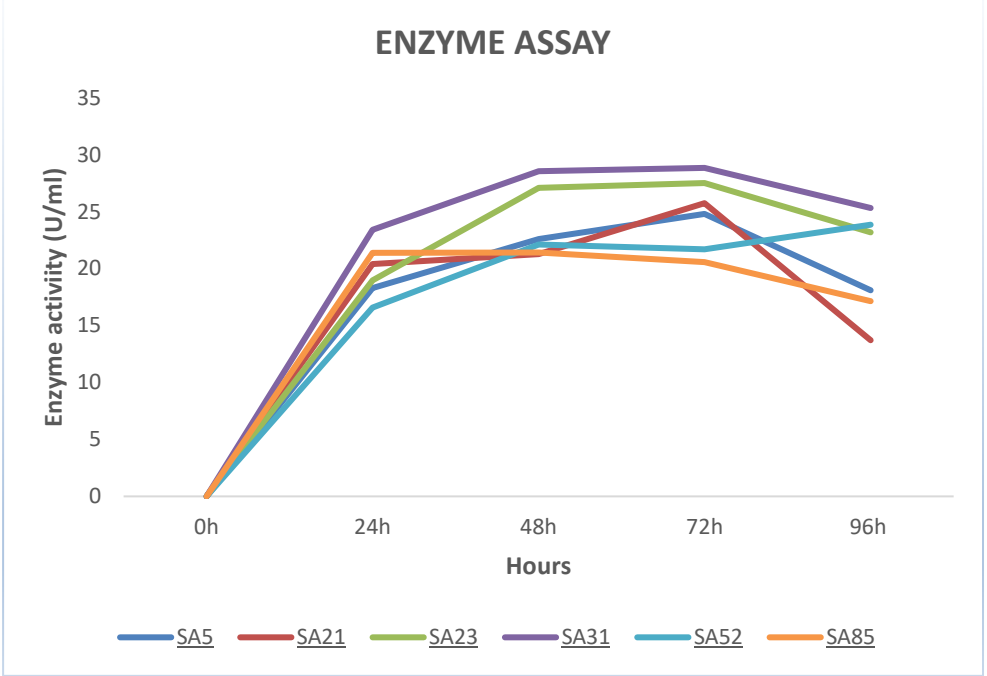

Fig 6. Comparative enzyme activity of potent actinomycetes

This figure shows the comparative enzyme activity of potent actinomycetes strain , actinomycete strain which gave high result in qualitative assay which are selected for quantitative assay . SA5 , SA21 ,SA23 ,SA31 , SA52 ,SA 85 at different time 0h, 24h , 48h, 72h , 96h. SA 31 strain gave highest enzyme activity compare to other culture , then after SA23 gave second highest enzyme activity at 72-hour incubation . The amount of cellulase activity in the crude enzyme extract is quantified by comparing the colour or fluorescence produced by the reducing sugars, which is develop by reaction with DNSA .

4.4 Morphological and Biochemical Characteristics of potential Actinomycetes strain

Morphological characteristics of potent isolated strain is observe on Starch casein agar plate after incubation at 30°C temperature for 7 days . Characteristics like size, shape , colour , reverse pigment , margine , texture , elevation ,opacity ,gram staining.

Table 3. morphological characteristics of potent isolated actinomycetes

Colony characteristics	**Colony Name**					
	SA5	**SA21**	**SA23**	**SA31**	**SA52**	**SA85**
SIZE	Small	Small	Small	Medium	Medium	Medium
SHAPE	Circular	Circular	Circular	Circular	Circular	Circular
COLOUR	White	White	White	White	White	White
REVERSE PIGMENT	Yellow	Gray	Black	Yellow	Dark yellow	Orange
MARGIN	Entire	Entire	Entire	Entire	Entire	Entire
TEXTURE	Powdery	powdery	powdery	Powdery	Powdery	Powdery
ELEVATION	Raised	Raised	Flat	Raised	Raised	Raised
OPACITY	Opaque	Opaque	opaque	Opaque	Opaque	Opaque
GRAM STAINING	Positive	Positive	positive	Positive	Positive	Positive

Table 3 shows the morphological characteristics of isolated actinomycetes by review literature conclude that actinomycetes shown small to medium size colony , circular shape , powdery colony which have different color but mostly they have white colour with different reverse pigment like yellow , red , orange , black ,blue , grey ,greenish yellow etc. they contain entire margin , raised or flate elevation with opaque .

Table 4 biochemical test of potent strain

Test	Colony Name					
	SA5	**SA21**	**SA23**	**SA31**	**SA52**	**SA85**
MR	-VE	-VE	-VE	-VE	-VE	-VE
VP	-VE	+VE	-VE	-VE	-VE	+VE
INDOLE	-VE	-VE	-VE	-VE	-VE	-VE
CITRATE	+VE	+VE	+VE	+VE	-VE	+VE
STARCH HYDROLYSIS	+VE	+VE	+VE	+VE	+VE	+VE
OXIDASE	-VE	-VE	-VE	+VE	-VE	-VE
CATALASE	-VE	+VE	-VE	+VE	+VE	-VE

Table 4 shows the biochemical test of isolated actinomycetes strain SA5, SA21, SA23 ,SA31, SA52 , SA85 . Biochemical test like Methyl Red test, Voges-Proskauer test , Indole test , citrate test , starch hydrolysis test, oxidase test , catalase test was performed . SA 31 isolate gave MR negative , VP negative , indole negative , citrate positive , starch hydrolysis positive , oxidase positive and catalase positive .

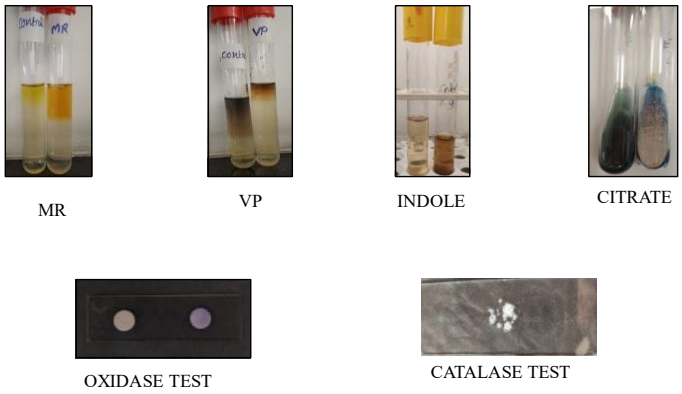

Fig .7 biochemical test of potent strain SA31

Fig .7 shows the images of biochemical test , MR test gave yellow colour it indicate negative test , VP also negative , indole negative , citrate slant change colour green to blue it indicate citrate positive , oxidase disk change in blue color it indicate positive result , in catalase test organism form bubble after add hydrogen peroxide solution it indicate positive test.

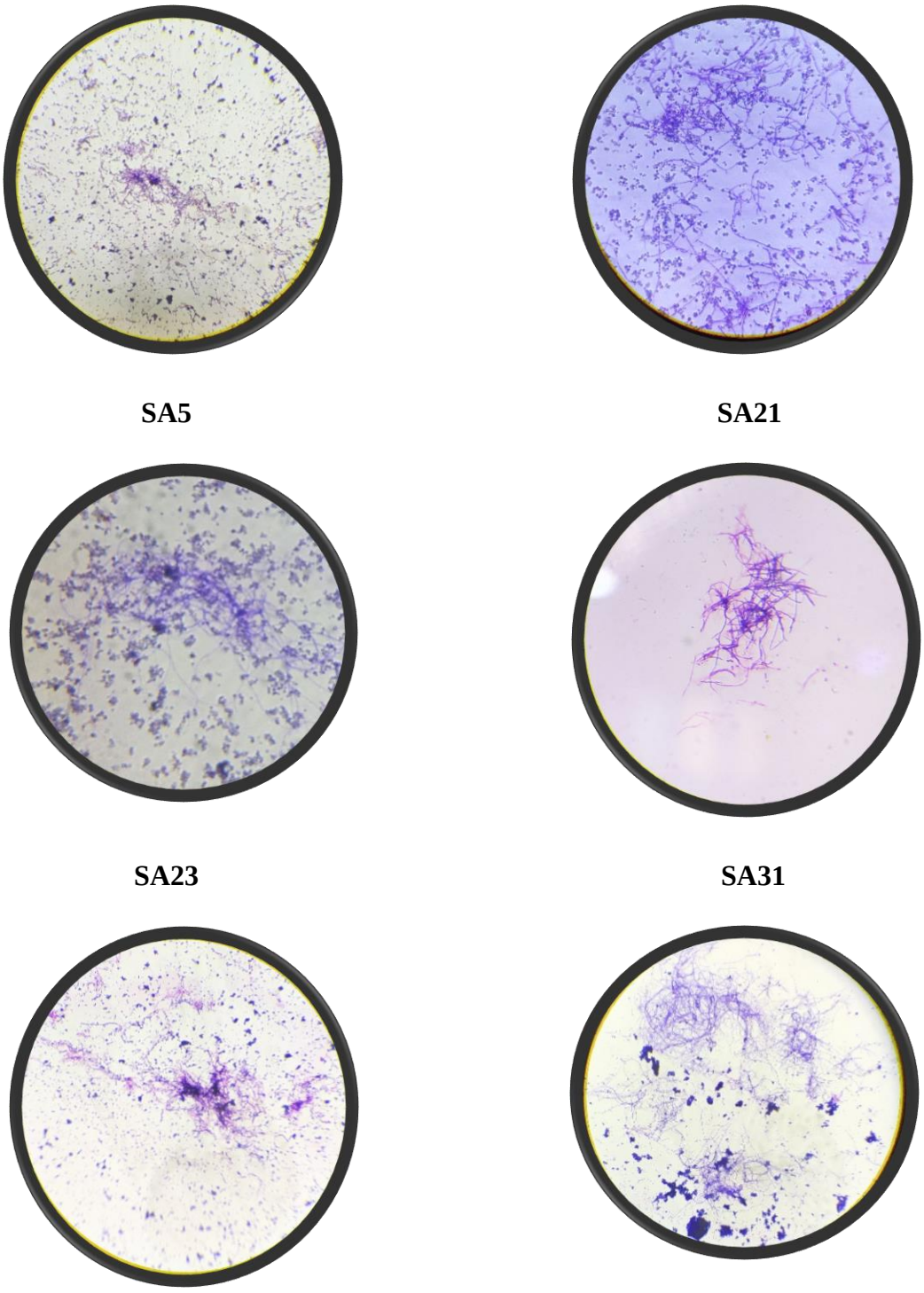

Fig. 8 Gram staining of potent actinomycete strain

Fig .7 contains gram staining of actinomycetes strain SA5, SA21, SA23 ,SA31, SA52 , SA85 under oil immersion lanes , Gram staining showing long , filamentous like structure , branched gram-positive bacteria .

4.5 Enzyme immobilization

After immobilization by entrapment method by sodium alginate the enzyme activity is increase compare to free enzyme .

4.6 Optimization of different conditions for free enzyme and immobilized enzyme

4.6.1 Effect of temperature on the activity of free enzyme and immobilized enzyme

By performing enzyme activity at different temperature using DNSA method . At different temperature 30 , 35, 40, 45, 50, 55, 60 °C. At 50 °C temperature enzyme gave highest activity . the activity of free enzyme and immobilized enzyme is mention in graph.

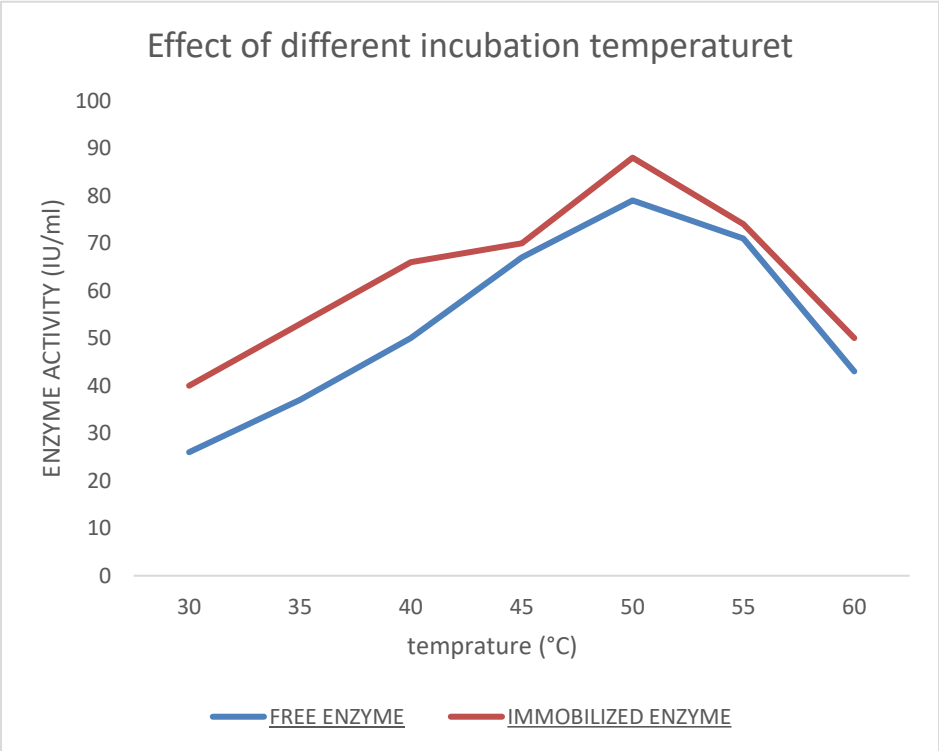

Fig. 9 Effect of temperature on activity of enzyme

Fig . 9 shows enzyme activity of free and immobilized enzyme at different temperature 30 , 35, 40, 45, 50, 55, 60 °C . The highest enzyme activity of free enzyme and immobilized enzyme at 50 °C is respectively 79 U/ml and 88 U/ml.

4.6.2Effect of pH on the activity of free enzyme and immobilized enzyme

By performing enzyme activity of free and immobilized enzyme which is produce by potent actinomycete strain SA31 at different pH (4 ,5 , 6 ,7, 8) with DNSA method. At 5 pH both free and immobilized enzyme gave high activity. The activity of free enzyme and immobilized enzyme is mention in graph.

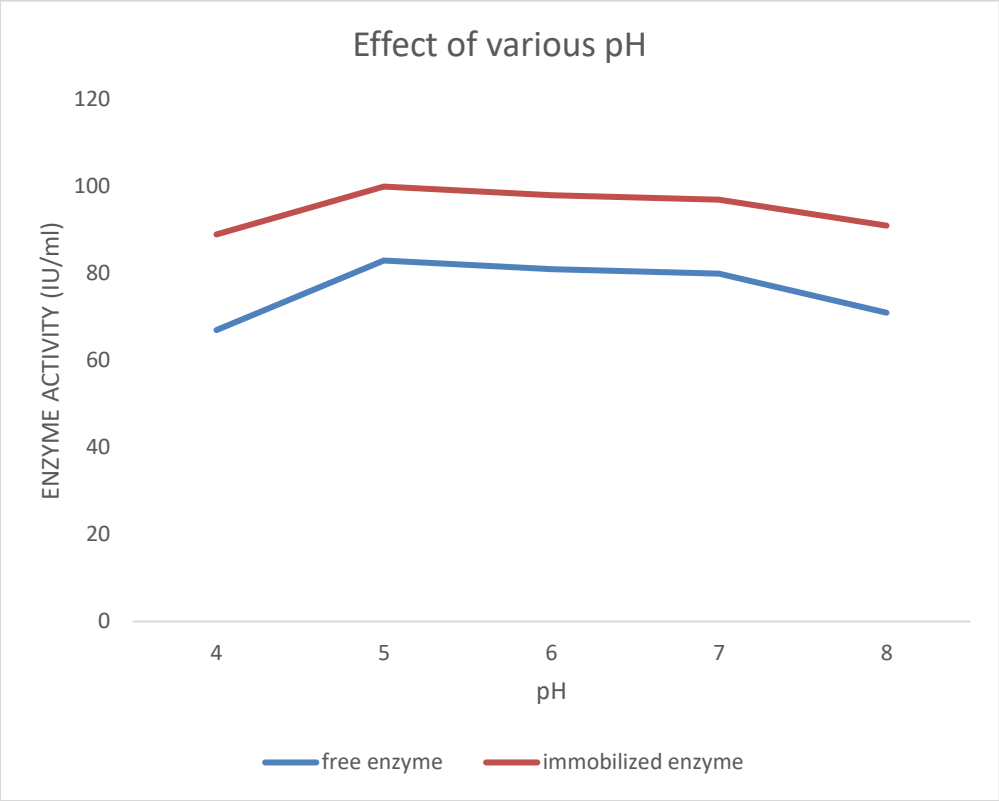

Fig. 10. Effect of pH on activity of enzyme

Fig . 10 shows enzyme activity of free and immobilized enzyme at different pH like 4 , 5 ,6, 7, 8. Which is measure by DNSA method . Both free enzyme and immobilized enzyme gave activity at 5 pH is respectively 83 U/ml and 100 U/ml.

4.6.3. Effect of incubation time on the activity of free enzyme and immobilized enzyme

Enzyme activity of free and immobilized enzyme which is produce by potent actinomycete strain SA31 measured by DNSA method at different incubation time period 20min , 40 min ,60min ,80min . Highest enzyme activity of free enzyme and immobilized is at 40min , the activity of free enzyme and immobilized enzyme is mention in graph.

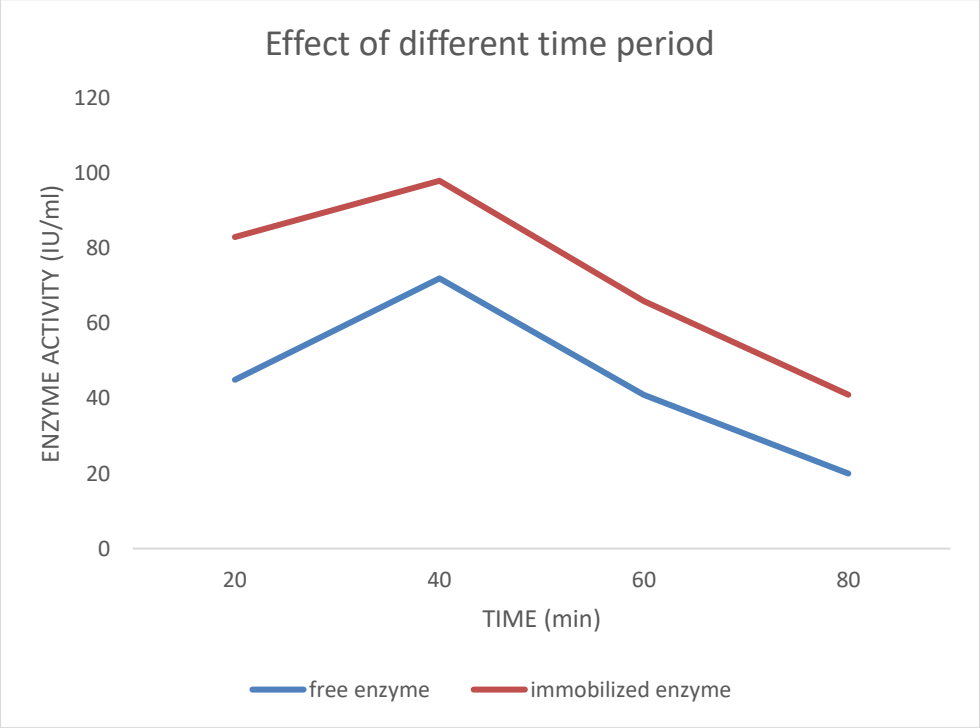

Fig .11 Effect of temperature on activity of enzyme

Fig.11 shows enzyme activity of free and immobilized enzyme at different incubation time period 20min , 40min, 60min , 80 min . Which is measure by DNSA method . The highest activity of free and immobilized enzyme at 40 min is respectively 72 U/ml and 98 U/ml.

4.6.4 Effect of NaCl concentration in substrate on the activity of free enzyme and immobilized enzyme

Enzyme activity of free and immobilized enzyme which is produce by potent actinomycete strain SA31 measured by DNSA method at different concentration of NaCl in substrate 1 % , 2% , 3% , 4% . . Highest enzyme activity is at 3 % concentration of NaCl .

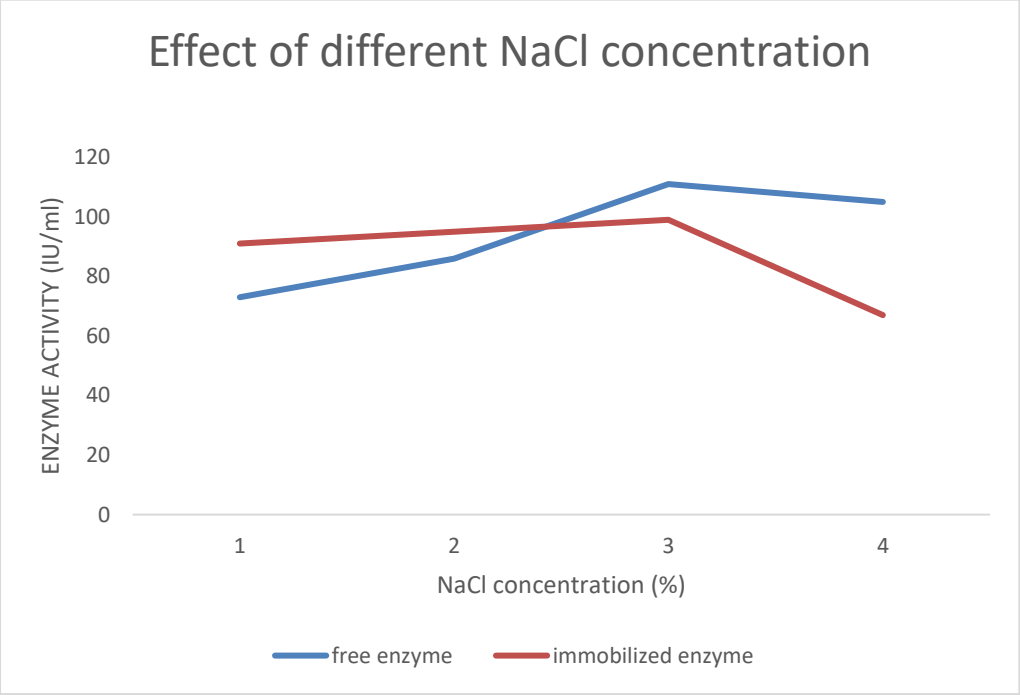

Fig .12 Effect of NaCl concentration in substrate on activity of enzyme

Fig .12 shows enzyme activity of free and immobilized enzyme at different concentration of NaCl in substrate 1 % , 2% , 3% , 4% . Which is measure by DNSA method . The highest activity of free and immobilized enzyme at 3% NaCl is respectively 111 U/ml and 99 U/ml.

4.8 Ammonium sulphate precipitation

By performing ammonium sulphate precipitation protein concentration is increase compare to crud enzyme there are 3.8-fold enzyme concentration is increase by ammonium sulphate precipitation . enzyme activity is checked by DNSA method and protein estimation done by Bronsted Lowry method . the enzyme summary is mentioned in table 4.

Table 4 .Summary of crud , ammonium sulphate precipitated enzyme and gel filtrated enzyme

	Crud enzyme	**ammonium sulphate precipitated enzyme**	**Gel filtrated enzyme**
Volume of fraction (ml)	**250**	**90**	**2**
Protein concentration (mg/ml)	**70**	**37**	**26**
Total amount (mg)	**17500**	**3330**	**52**
Enzyme activity (U/ml)	**40**	**72**	**97**
Specific activity (U/mg)	**0.5**	**1.9**	**3.7**
Total amount	**10000**	**6480**	**194**
Yield (%)	**100**	**64.8**	**1.94**
Purification fold	**1**	**3.8**	**7.4**

Table 4 shows the protein concentration ,enzyme activity , specific activity , yeid (%), and purification fold of crud enzyme , ammonium sulphate precipitated enzyme and gel filtrated enzyme . The purification fold of enzyme is respectively 1 ,3.8 and 7.4 . It shows that when enzyme became purify it show high purification fold .

4.7 SDS-PAGE

By performing SDS-PAGE of cellulase enzyme and electrophoresis. it compares with ladder. By comparing enzyme with ladder ,The molecular weight of cellulase was found to be 23 kDa comparing with marker .

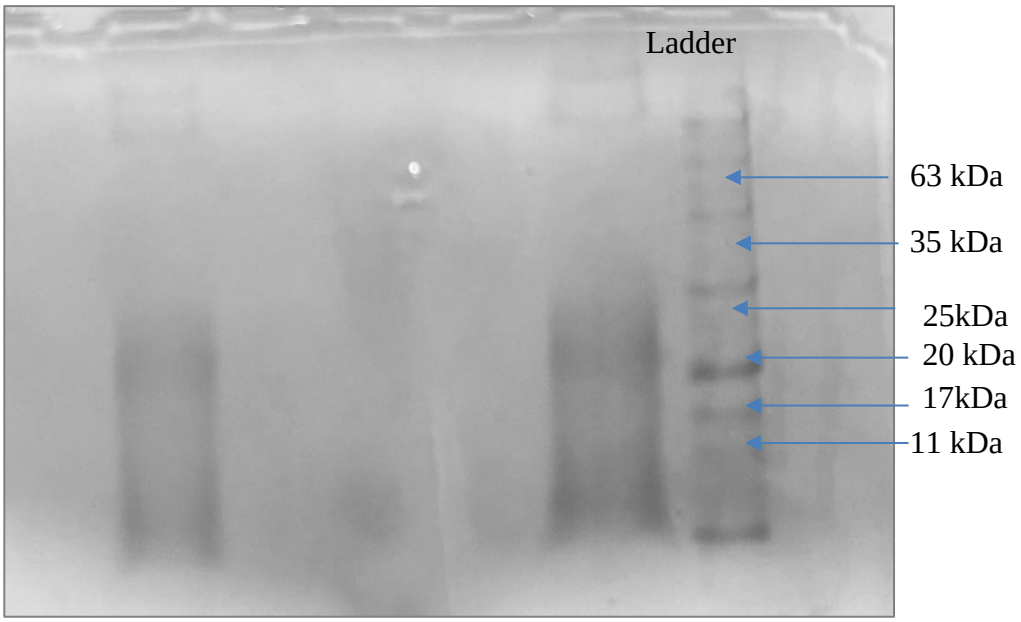

Fig .13 SDS-PAGE of purified enzyme

This figure shows the SDS-PAGE of cellulase enzyme . In ladder shows the band on the basis of molecular weight of protein like 245 , 180, 135, 100, 75 , 63 ,48 , 35 , 25 , 20, 17, 11 kDa . The enzyme sample band is found between 20 -25 kDa. By observation the molecular weight of cellulase enzyme is found 23 kDa.

4.8 Molecular identification

The sample SA31 is potent organism concluded by qualitative and quantitative assay. By molecular identification SA 31 is closely related to ***Streptomyces sp***. based on nucleotide homology analysis. After molecular identification sequence submitted in NCBI (The National Center for Biotechnology Information), accession number is **OQ660493.1**

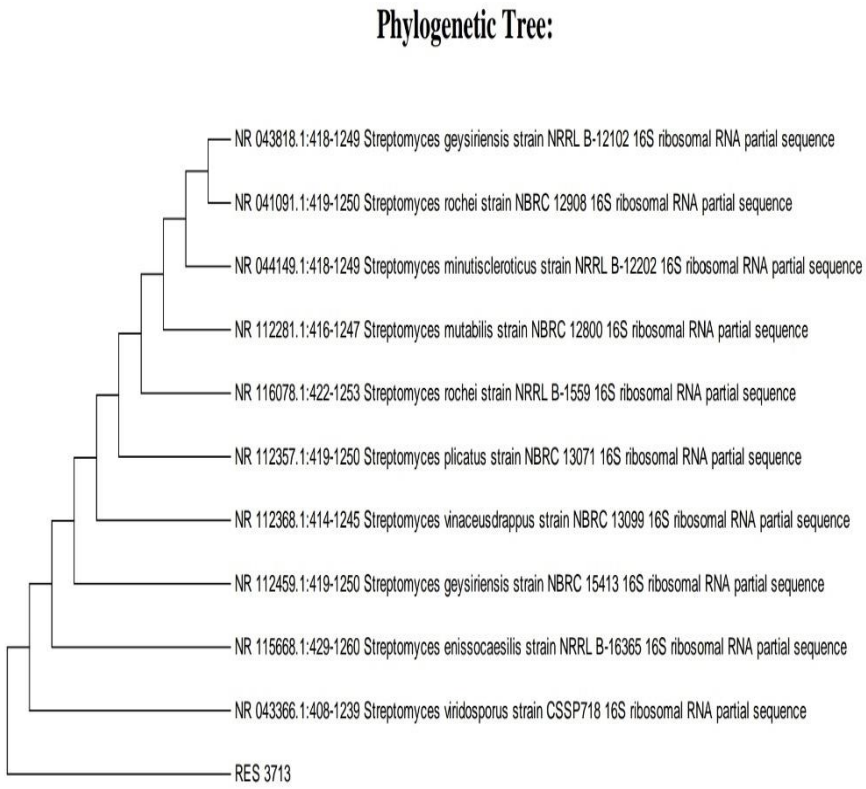

Fig.14 Molecular Phylogenetic analysis by Neighbour -Joining method

This figure shows Molecular Phylogenetic analysis by Neighbour -Joining method the sample RES 3713 is closely related with *streptomyces* strain . The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

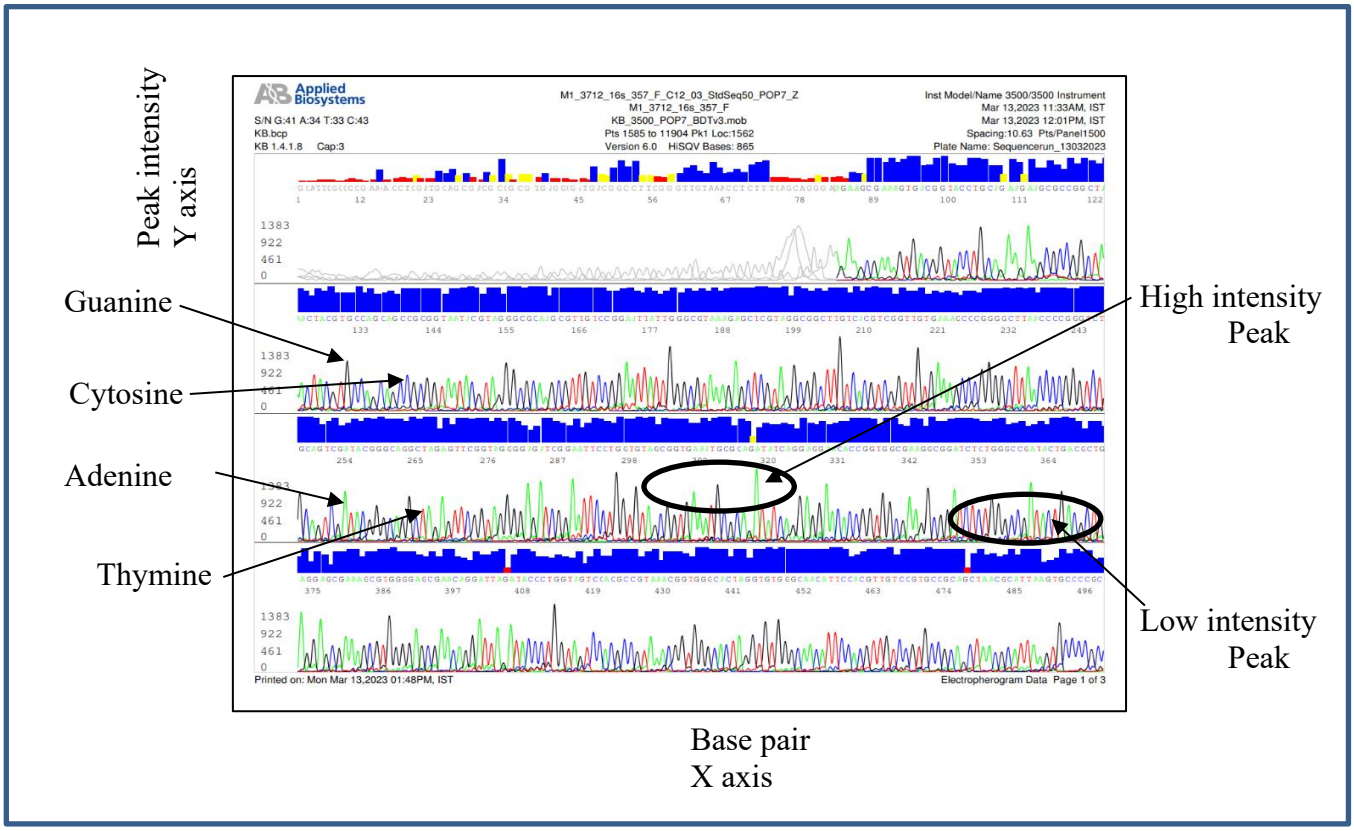

Fig . 15 Sanger sequencing chromatogram (page 1)

Fig. 14 show the Sanger sequencing chromatogram which show the A,T,G,C contain in gene. This chromatogram shows high G + C contain, this match with actinomycetes. Blue colour pic shows Cytosine , Black colour pic shows Guanine, Red colour shows Thymine, Green colour pic Adenine .On x axis it shows base pair and on Y axis it shows the peak intensity of peak . fig. 14 shows the 4 to 496 bp sequence .

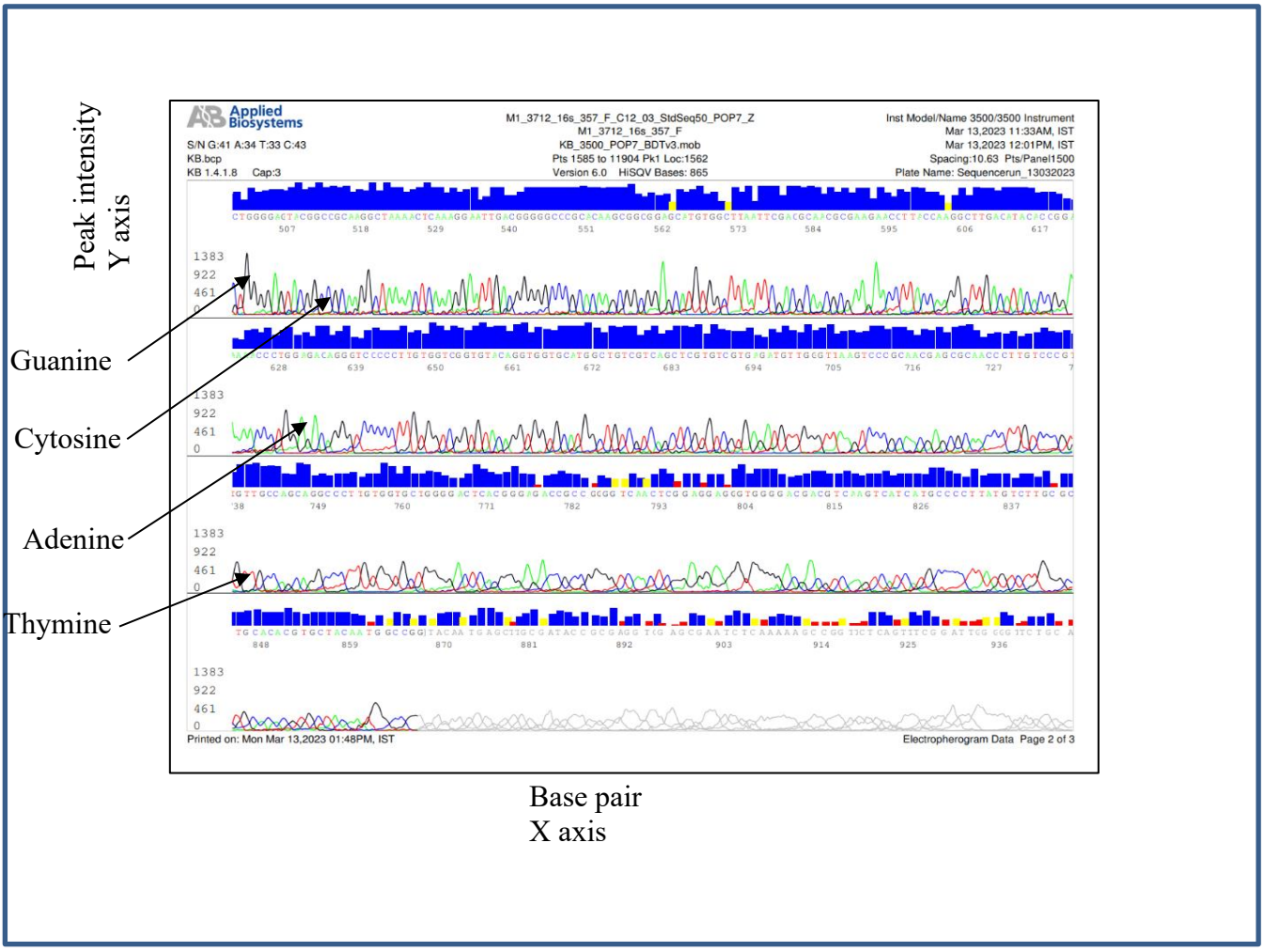

Fig. 16 Sanger sequencing chromatogram (page 2)

Fig. 16 show the Sanger sequencing chromatogram which show the A,T,G,C contain in gene. This chromatogram shows high G + C contain, this match with actinomycetes. Blue colour pic shows Cytosine , Black colour pic shows Guanine, Red colour shows Thymine, Green colour pic Adenine .On x axis it shows base pair and on Y axis it shows the peak intensity of peak . fig. 15 shows the 507 to 936 bp sequence .

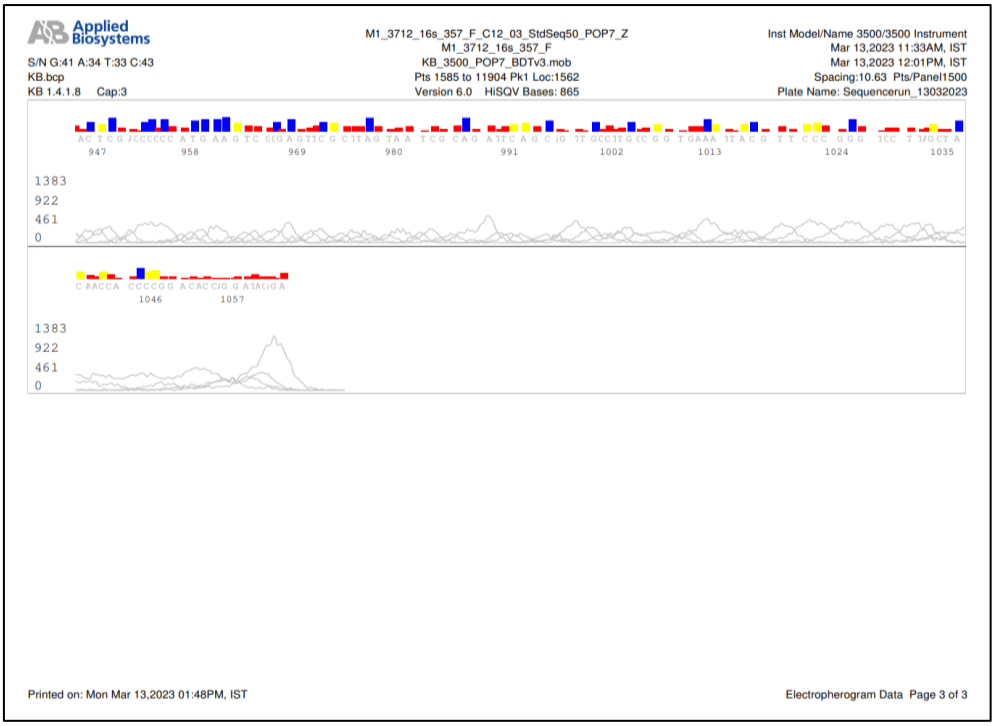

Fig. 16 Sanger sequencing chromatogram (page 3)

Fig. 16 show the Sanger sequencing chromatogram which show the A,T,G,C contain in gene. This chromatogram shows high G + C contain, this match with actinomycetes. Blue colour pic shows Cytosine , Black colour pic shows Guanine, Red colour shows Thymine, Green colour pic Adenine .On x axis it shows base pair and on Y axis it shows the peak intensity of peak . fig. 15 shows the 507 to 936 bp sequence .

5 . DISCUSSION

This study has covered major concerns about isolation of cellulose degrading actinomycetes and immobilization. To isolate cellulose degrading actinomycetes, samples were collected from soil and compost. The samples were plated onto a selective medium containing carboxymethyl cellulose (CMC) as the sole carbon source. The plates were incubated at 30°C for 5-7 days, then screened for cellulase activity using the Congo red assay, which detects the hydrolysis of CMC by the formation of a clear zone around the colony.

5.1 Enzyme activity

In the study of one paper ,the highest cellulase activity was displayed by *S. albidoflavus* (1.165 U/ml/min),(Das, Solanki et al. 2014).According to Daba Gudeta Guder and M.S.R. Krishna cellulase enzyme was extracted from each isolate and enzyme activity assay was performed based on the DNSA method. Enzyme activity ranged from 0.225u/ml to 1.652u/ml in which maximum result was obtained in bacterial isolate, which are belong to belong to *Bacillus speci Bacteroides species, Enterobacter species.*(Guder and Krishna 2019)

5.2 Enzyme immobilization

The immobilized enzymes showed higher stability and reusability than the free enzymes. The immobilized enzymes retained 80% of their activity after five cycles of reuse, whereas the free enzymes showed a rapid decline in activity after the first cycle. The immobilized enzymes also showed higher thermal stability than the free enzymes, retaining 70% of their activity after incubation at 60°C for 1 hour. The article by Tsegaye et al. (2018) The study involved sampling compost soil from different locations and screening the samples for cellulase activity using the CMC (carboxymethyl cellulose) agar method. The bacterial and fungal isolates were then identified based on their 16S rRNA and its gene sequences, respectively. (Tsegaye et al. 2018)According to Mohmad et al The most effective method for keeping cellulas active was to immobilize them by encapsulating them. The immobilized enzyme also maintained activity up to 80 degrees Celsius, reaching its peak at 50 degrees Celsius and pH 5.5. Due to its simplicity in formulation, gentle gelation conditions, non-toxicity, biocompatibility, low cost, and resistance to microbial attack, enzymes encapsulated within alginate beads are favoured.

5.3 enzyme optimization

In a study by Siddiqui et al. (2020), cellulase-producing bacteria were isolated from soil samples and screened for their ability to degrade cellulose. The researchers identified several bacterial strains with high cellulase activity and characterized the purified enzymes. They found that the enzymes had optimal activity at pH 5.5-6.5 and 45-55°C, and could efficiently degrade cellulose in various forms….. Another study by Nair et al. (2020) focused on the optimization of cellulase production by Streptomyces sp. VITBSS2 isolated from soil. The researchers optimized the fermentation conditions to maximize enzyme production and found that the optimal pH was 6.0 and the optimal temperature was 37°C. They also evaluated the potential of the purified enzyme for industrial applications such as biofuel production and found that it had high activity against various cellulose substrates.

5.3 Enzyme purification by ammonium sulphate precipitation

In a study by Kowalska et al. (2019), cellulase enzyme produced by Actinomycetes was purified using ammonium sulfate precipitation. The purified cellulase enzyme was then analyzed for its enzyme activity, protein concentration, and purity. The results showed that the cellulase enzyme was purified to 2-fold, and the enzyme activity was increased by approximately 3-fold compared to the crude enzyme extract. The protein purity was also increased to approximately 80%. everal studies have reported successful cellulase purification using ammonium sulfate precipitation. For example, a study by Kumar and Singh (2017) used this method to purify cellulase enzyme produced by a strain of Bacillus subtilis. The researchers reported a 6-fold increase in the specific activity of the enzyme after purification, indicating successful removal of impurities.

5.4 SDS-PAGE

The results showed that out of the 60 bacterial and 20 fungal isolates screened, 27 bacterial and 8 fungal isolates exhibited cellulase activity. The most promising isolates were identified as Bacillus sp. (bacteria) and Aspergillus sp. (fungi), which showed high cellulase activity on CMC agar plates. SDS-PAGE analysis of the crude cellulase extracts from selected bacterial and fungal isolates. The analysis revealed the presence of multiple protein bands, indicating that the cellulase enzymes were composed of multiple subunits.(Islam and Roy 2018)

Conclusion

Initiating with 148 out of 132 best strains of actinomycetes and culminating to 6 strains through a series of experiments designed and one potent isolate carried Forward for exploring significant cellulose degradation and enzyme immobilization. In this study successfully isolated cellulose degrading actinomycetes from soil and compost samples and demonstrated the immobilization of their cellulolytic enzymes on sodium alginate beads. The immobilized enzymes showed higher stability at temperature (50 C) , pH (5), incubation time (40min) and concentration of NaCl (3%) in substrate and reusability than the free enzymes, indicating their potential application in the production of bioenergy and bioproducts from cellulose.

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Achievement

Serial no.	Date	Certificate
1.	3-9 February, 2023	Attend online certificate course “MICROSCOPY – AN OVERVIEW’
2.	11 February, 2023	Poster presented in national conference “ MICROBIOMES TO MECROMOLECULES : CONNECTING THE DOTES ” organized by department of microbiology & biotechnology ,Gujarat university , Ahmadabad , Gujarat.
3.	22 & 23 February, 2023	Attend national conference “ EMERGING PARADIGM AGRICULTURAL MICROBIOLOGY ” organized by Department of Microbiology , Atmiya University , Rajkot, Gujarat.
4.	21 March ,2023	Sequence submission in NCBI (National Centre for Biotechnology Information)

Poster presented in national conference

“ MICROBIOMES TO MECROMOLECULES: CONNECTING THE DOTES”

organized by department of microbiology & biotechnology ,

Gujarat university , Ahmadabad , Gujarat.

NATIONAL CONFERENCE

MICROBIOMES TO MACROMOLECULES: CONNECTING THE DOTS

February 22 & 23, 2023

CERTIFICATE

This is to certify that

Dr./Mr./Ms. Bhuva Shraddha Ajitbhai

has actively participated in **National Conference** on "MICROBIOMES TO MACROMOLECULES: CONNECTING THE DOTS" organized by Department of Microbiology & Biotechnology, University School of Sciences, Gujarat University, Ahmedabad – 380 009 on 22nd & 23rd February 2023.

He / She presented paper **Oral** / **Poster** / only **Attended** under **P.G.** / **Ph.D.** / **Faculty** category.

Prof. (Dr.) Meenu Saraf
Convenor
Director, School of Sciences
Professor and Head,
Department of Microbiology and Biotechnology
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Dr. Rakeshkumar R. Panchal
Organizing Secretory
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Department of Microbiology and Biotechnology
Gujarat University

Prof. (Dr.) H. A. Pandya
Patron
Hon. Vice-chancellor
Gujarat University

Department of Science & Technology
Government of Gujarat

GSBTM
TRANSCRIPTING BRIGHTER BIO FUTURE

Streptomyces sp. strain SMUA31 16S ribosomal RNA gene, partial sequence

GenBank: OQ660493.1

FASTA Graphics

Go to:

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  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAR-2023) Microbiology, Atmiya university,
Kalawad
            road, Rajkot, Gujarat 360005, India
COMMENT     Sequences were screened for chimeras by the submitter using
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Sequence submission in NCBI (National Centre for Biotechnology Information)