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Characterization of cellulolytic actinobacteria from waste material of sugar factories in the South Gujarat and Maharashtra region

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Abstract
This study investigates the isolation, screening and characterization of cellulolytic actinobacteria from sugarcane press mud samples of sugar factories in South Gujarat. Heat and CaCO3 treatment enriched actinomycetes population. Out of 52 isolates, MSF9 and BSF22 exhibited significant CMCase production. Morphological, cultural, biochemical and molecular characteristics were examined. Both strains were citrate, indole, and urease positive. MSF9 was positive for sucrose and lactose, while both strains showed glucose and maltose positivity. Molecular analysis identified MSF9 as *S. celluloflavus* and BSF22 as *S. longisporoflavus*. Optimal conditions for cellulase activity and protein production were explored. Results revealed that both strains displayed maximum cellulase (CMCase) activity and total protein production at 45 °C. MSF9 exhibited the highest enzyme activity (1.89 U ml^{-1}) and protein production (2.42 mg ml^{-1}), while BSF22 showed significant activity (1.86 U ml^{-1}) and protein production (1.79 mg ml^{-1}) 45 °C temperature. Enzyme activity and protein production were lowest at 25 °C. The impact of pH on enzyme activity and protein production was studied, both strains showing peak CMCase activity at pH 7 (MSF9: 0.75 U ml^{-1}; BSF22: 0.93 U ml^{-1}) and lowest activity at pH 4. Varying carboxymethylcellulose (CMC) concentrations (0.5% to 3.0%) influenced enzyme activity and protein production, with optimal values at 2% CMC (MSF9: 1.38 U ml^{-1}, 3.18 mg ml^{-1}; BSF22: 1.41 U ml^{-1}, 3.53 mg ml^{-1}).

Keywords: CMC, sugarcane press mud, cellulase, actinomycetes, waste degradation

Introduction
The estimated global production of crystal sugar is approximately 354.95 million tons, and India alone operates nearly 704 sugar mills. These sugar mills generate approximately 8 million tons of sugarcane by-products known as press mud. Press mud is utilized as biocompost to enhance soil fertility and increase crop productivity due to its rich nutrient content, including cellulose, hemicellulose, fiber, organic carbon, nitrogen, phosphorus, potassium, magnesium, calcium, as well as essential micronutrients such as zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn). Moreover, it contains beneficial microorganisms that positively influence soil properties, particularly fertility, thereby enhancing crop productivity. Well-decomposed press mud possesses an odourless, dark brown, soft, and spongy texture, with abundant cellulosic and hemicellulosic materials, including fibers and organic aggregates. Cellulolytic actinomycetes are a specialized group of gram-positive, filamentous bacteria belonging to the phylum actinobacteria, known for their remarkable ability to degrade cellulose, the most abundant biopolymer on Earth. These microorganisms play a crucial role in carbon cycling and nutrient recycling in various environments, particularly in soil and plant litter ecosystems. With their unique enzymatic machinery, cellulolytic actinomycetes produce an array of cellulases, including endoglucanases, exoglucanases, and β-glucosidases, which work synergistically to hydrolyze the complex cellulose structure into simpler glucose units. Their proficiency in cellulose degradation makes them valuable contributors to biomass conversion for biofuel production and bioremediation processes. Moreover, their capacity to thrive in diverse habitats and adapt to varying environmental conditions underscores their significance in maintaining ecosystem balance and providing potential biotechnological applications. The study of cellulolytic actinomycetes continues to unveil their immense potential for sustainable resource utilization and environmental management. Cellulases are a group of enzymes that play a crucial role in the breakdown of cellulose, a complex polysaccharide found in the cell walls of plants.

The cellulase enzyme system consists of several types of enzymes, each with specific functions in the hydrolysis of cellulose into simpler sugars like glucose (Bhimani and Sohaliya, 2021) [2]. Endoglucanases (EC 3.2.1.4): These enzymes randomly cleave internal bonds within the cellulose chain, creating shorter cellulose fragments. Exoglucanases or cellobiohydrolases (EC 3.2.1.91) work on the ends of cellulose chains, releasing cellobiose (A disaccharide of two glucose units) from the cellulose molecule. β-glucosidases (EC 3.2.1.21) further hydrolyze cellobiose into individual glucose molecules, making them available for microbial or enzymatic uptake. The cellulase enzyme system works synergistically, with endoglucanases initiating the process by breaking down internal bonds, making it easier for exoglucanases to access and act on the chain ends. The resulting cellobiose is then converted to glucose by β-glucosidases, which is a preferred substrate for many microorganisms. (Chuanjiao *et al.* 2021) [7]. Cellulases are produced by various actinomycetes genera, including *Streptomyces*, *Arthrobacter*, *Micromonospora*, *Nocardia*, *Actinoplanes* and *Cellulomonas*. They play a crucial role in the decomposition of plant material and are of significant importance in various biotechnological applications, such as biofuel production, paper and textile industries, and bioremediation of lignocellulosic waste.

The cellulase enzyme system's efficiency and specificity are influenced by factors such as temperature, pH, substrate concentration, and the presence of inhibitors or activators, making them subject to regulation and optimization in different applications (Roy *et al.* 2018) [21]. The study and application of cellulases are an essential aspect of green technology and sustainable resource utilization.

Materials and Methods

Sample collection and chemical characterization

Sugarcane pressmud samples were carefully collected from various sugarcane pressmud composting sites operated by sugar factories in the South Gujarat region. These sites were chosen to represent different composting processes and environmental conditions. Upon collection, the pressmud samples were transported to the laboratory where they underwent characterization to assess their chemical properties. The chemical parameters, including pH and electrical conductivity (EC), were determined following the procedure outlined by Rayment and Higginson (1992) [20]. The isolation process was promptly initiated within 8 hours of sample collection to minimize saprophytic developments.

Enrichment and isolation of cellulolytic actinomycetes

Unprocessed samples were subjected to heat treatment in a hot air oven at 60 ºC for 1 h to reduce the abundance of unicellular bacteria. The addition of $CaCO_3$ to soil samples in a 1:1 ratio and incubating them at 28 ºC for 10 days promoted the formation of aerial mycelia in several actinomycetes, enhancing their visibility on growth media. Following the treatment, the samples were diluted and plated on actinomycetes isolation agar (AIA). Incubation was carried out at 28 ºC for 1 week. Typical chalky white colonies with rough surfaces were selected and subcultured to obtain pure isolates, which were then preserved on their respective media slants at 4 ºC and in 20% (w/v) glycerol at -20 ºC for long-term storage. Table 1 shows various enrichment methods used for the isolation of novel actinobacteria.

Table 1: Various enrichment methods used for the isolation of novel actinobacteria

Samples	Enrichment Treatments	Isolation Medium	Incubation condition	References
Non-saline, arid, soil	120 ºCheat treatment for 15 minutes.	AIA (HiMedia), pH 7.3	45 ºC for up to 14 days	Kusuma *et al*. (2020) [12]
Desert soil	Heating at 55ºC for 6 minin a waterbath	Starch Casein Agar, withnystatin and cycloheximide 50 µg/mL	28 ºC for 14 days	Cortes-Albaya*et al*. (2019) [8]
Soil	Roomtemperature air dried for 7 days	Humic acid vitamin agar, withnalidixic acid 25 µg/mL and cycloheximide 50 µg/mL	30 ºC for 3 weeks	Moonmangmee *et al*. (2019) [17]
Soil	1 g of soil was diluted with 0.2 g/l $CaCO_3$. Suspension incubated at shaking condition 180 RPM for 1 hr at 30 ºC	Yeast Malt Extract Agar, with cycloheximide 50 µg/mL, calcium propionate 30 mg/mL	30 ºC for 14 days	Li *et al*. (2020) [13]

Qualitative screening for cellulase production

Cellulase producing actinomycetes were subjected to screening on selective carboxymethyl cellulose (CMC) agar plates. Pure cultures were spot inoculated on the plates using spore suspensions and then incubated at 30 ºC. After 3 days, the plates were flooded with a 1% Congo red solution for 15 minutes, followed by destaining with a 1M NaCl solution for another 15 minutes. The diameter of the zone of decolorization around each actinomycetes colony was measured. The colony exhibiting the largest zone of decolorization was selected for further characterizations.

Morphological and cultural characterization

Purified colonies of actinomycete isolates were subjected to a comprehensive characterization based on their morphological and physiological features, following the protocols outlined in the International Streptomyces Project (ISP) by Shirling and Gottlieb (1970) [24]. To study their micro-morphological features, the cover slip culture method was employed, as described by Williams and Cross (1971) [29]. In this technique, individual isolates were transferred to cover slips embedded in AIA medium and incubated at 28 °C for 1-4 weeks. The cover slips were then observed under a binocular optical microscope to examine the presence of mycelium, its fragmentation, and spore chain morphology. Gram's staining of the isolates was also performed. Subsequently, the isolates were cultured on different ISP media, including ISP 2, ISP 3, ISP 4, ISP 5, and ISP 6, and observed after 8-30 days of incubation to assess their growth characteristics and distinctive features. The observed structures were compared with those described in Bergey's manual (Sneath *et al.*, 1986) [25].

Biochemical characterization

Biochemical characterization of the actinomycetes were performed as per the standard protocol. The biochemical tests performed were Indole test, Methyl red test, Vogues - Proskauer test, Citrate utilization test, Catalase test, Oxidase

test, Sugar fermentation test, Starch hydrolysis test, Urease test (Daquioag and Penuliar, 2021) [9].

Molecular characterization

DNA isolation was performed its quality was assessed on a 1.0% Agarose Gel, revealing a single band of high-molecular-weight DNA. Subsequently, a specific gene fragment was amplified via PCR, resulting in a single, distinct PCR amplicon band observed on Agarose Gel. The PCR amplicon was then purified using column purification to eliminate any contaminants. For further analysis, the DNA sequencing reaction of the PCR amplicon was carried out with the 27F primer, utilizing the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer. The obtained gene sequence was subjected to BLAST against the NCBI Gene bank database to find similar sequences. The first ten sequences with the highest identity scores were selected and aligned using multiple alignment software programs for comparative analysis and identification of similarities and variations.

Inoculum preparation and enzyme production

For the inoculum preparation, the chosen isolates were first cultivated in nutrient broth for a period of 3-4 days. Following this, the culture were individually inoculated into separate 150 ml fresh flasks containing BH broth supplemented with 1% CMC, designated as the medium for cellulase enzyme production. These flasks were then incubated for a period of 5 days at a temperature of 30 °C. Once the incubation period was completed, the cell-free supernatant was collected by subjecting the cultures to centrifugation at 10,000 rpm for 10 minutes. This resulting cell-free supernatant served as the crude enzyme extract. (Saffari *et al*., 2016) [23].

Quantitative estimation of cellulase production

The cellulase activity, of the crude enzyme extracts from the final cultures was determined using the DNSA method (Ghose, 1987) [10]. The enzyme reaction mixture was prepared by combining 0.5 ml of the crude enzyme extract from each sample with 0.5 ml of the substrate in separate test tubes. The substrate solution consisted of 1% CMC prepared in 50 mM citrate buffer. The substrate blank contained 0.5 ml of the substrate solution and 0.5 ml of 0.05 M citrate buffer. The reagent blank consisted of 1 ml of 0.05 M citrate buffer in test tubes.

Protein estimation in crude extract

The protein content of the crude culture broth was determined using the standard protocols of Lowry's method (Lowry *et al*., 1951) [14]. The reaction mixture consisted 1.0 ml of the crude extract and 1.0 ml distilled water, 3 ml alkaline copper sulphate reagent and 1N Folin Ciocaltue reagent. The crude extract of media without fungal inoculation was used as blank throughout the estimation.

Effect of temperature, pH and substrate concentration

Potential isolates were further studied for the effect of physicochemical parameters on growth, enzyme activity and total protein productionin submerged fermentation method following the one factor at a time approach (OFAT). Effect of temperature on growth,enzyme activity and total protein production was studied by varying temperature at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C. Substrate concentration 1% CMC, pH 7, and 5 days shaking incubation were taken constant. Effect of pH was studied by varying pH of the culture broth in the range of pH 4.0-10.0. Temperature 35 °C, substrate concentration 1% CMC and 5 days shaking incubation were taken constant. Effect of CMC substrate concentration were studied by varying substrate concentration at 0.5%, 1.0%, 1.5%, 2.0% and 2.5% (w/v) in culture broth. Temperature 35 °C, pH 7, and 5 days shaking incubation were taken constant. After 5 days of incubation enzyme activities, total protein and growth were determined as per standard methods described above.

Results and Discussion

Characterization of pressmud samples

Pressmud samples from sugarcane pressmud composting sites, which are operated by sugar factories in the South Gujarat region, were collected for this study. The physico-chemical properties of the collected samples were reported as per table 2.

Table 2: Physico-chemical properties of collected samples

Samples	Sample collection site	Ec (dS/m)	pH
Sample 1	Navapur Sugar Factory, Maharashtra	3.52	7.46
Sample 2	Mahuva Sugar Factory, Di. Surat, Gujarat	3.24	7.32
Sample 3	Bardoli Sugar Factory, Di. Surat, Gujarat	3.87	7.67

The data related to the physico-chemical parameters showed that the electrical conductivity (EC) of the pressmud samples ranged from 3.24 to 3.87 dS/m. The highest EC value was recorded in sample 3 (3.87 dS/m), while the lowest EC value was observed in sample 2 (3.24 dS/m). Another crucial parameter, pH, was found to be within the range of 7.32 to 7.67. The highest pH value was recorded in sample 3 (7.67), and the lowest pH value was observed in sample 2 (7.32). Generally, the Ec of press mud samples can range from 2 to 6 (dS/m). However, it's important to note that the "normal" EC value can differ significantly based on local conditions, the type of soil or organic matter present, and the presence of other substances or additives in the press mud. A high Ec value in press mud could indicate an increased concentration of dissolved salts and ions, which might have an impact on the growth and survival of microorganisms, including actinomycetes. High EC levels may create osmotic stress and affect microbial activity, leading to alterations in microbial community structure (Jiang *et al.* 2019) [11].

Enrichment and isolation of cellulolytic actinomycetes

Heat and $CaCO_3$ treatment were used to enrich cellulose-degrading actinomycetes. The enriched samples were then subjected to dilution plating on actinomycetes isolation agar (AIA). Distinct colonies with well-isolated and unique morphology were selected from the dilution plate technique and further purified through sector streaking. In total, 52 isolates were successfully obtained from various samples, and each isolate was maintained in pure culture, preserved at a temperature of 4 ºC for subsequent studies.

Qualitative screening of actinomycetes isolates for cellulase production

A total of 52 actinomycetes isolates were subjected to screening for CMCase production using agar plates containing 1% CMC supplemented with BH agar. Among these isolates, NSF8, NSF11, NSF12, NSF16, and NSF17

from Navapur Sugar Factory, Maharashtra, MSF1, MSF8, MSF9, MSF11, MSF12, and MSF18 from Mahuva Sugar Factory, Dist. Surat, Gujarat, andBSF6, BSF17, BSF19, and BSF22 from Bardoli Sugar Factory, Dist. Surat, and Gujarat exhibited a clear zone of hydrolysis on CMC agar plates when flooded with Gram's iodine. Moreover, all strains showed complete degradation of filter paper within a 7-day incubation period. Notably, isolate MSF9 demonstrated the highest CMCase production, followed by BSF22 making those potent isolates for further investigations. The relative enzyme activity was calculated as described by Bhimani and Sohaliya (2021) [2], and the findings are outlined in Table 3. In support to present investigation Daquioag and Penuliar (2021) [9] employed enrichment techniques to isolate 235 actinobacteria. Initial screening revealed cellulolytic activity on CMC-MSM agar plates in 48.51% (114/235) of the isolates. Furthermore, Putri and Setiawan (2019) [19] documented the isolation of 57 strains of actinomycetes, among which 17 isolates exhibited extracellular cellulase enzyme activity, with clear zone ratio ranging from 1.2 to 3.1 mm.

Table 3: Primary screening of isolates for enzyme production

Isolates	Zone Diameter/ Colony Diameter (mm)	Zone Ratio	Isolates	Zone Diameter/ Colony Diameter (mm)	Zone Ratio
NSF8	15/12	1.25	MSF11	22/13	1.69
NSF11	17/13	1.30	MSF12	18/15	1.2
NSF12	21/13	1.61	MSF18	21/13	1.61
NSF16	27/21	1.28	BSF6	12/9	1.33
NSF17	19/13	1.46	BSF17	19/8	2.37
MSF1	25/12	2.08	BSF19	22/11	2
MSF8	27/14	1.92	BSF22	15/6	2.5
MSF9	36/12	3			

Morphological characterization

Morphological and cultural characteristics of all cellulase-positive isolates were thoroughly examined, revealing a wide range of colony sizes from pinpoint to large. The majority of the isolates displayed the presence of both aerial and substrate mycelium, with white and brown colours, respectively. The colonies exhibited tough, leathery, or powdery appearances. Additionally, all isolates exhibited the Gram-positive nature, which is a characteristic feature of actinomycetes. Detailed results are summarized in Table 4. In a similar study Putri and Setiawan (2019) [19] identified that out of the 57 isolates, 17 (29.9%) exhibited the production of soluble pigments in the medium. The majority of these isolates were attributed to the *Streptomyces* genus, characterized by fungi-like branching and the formation of both aerial and substrate mycelia.

Biochemical characterizations

The biochemical characterization of the actinomycete isolates was conducted on different types of media, revealing that both isolates, MSF9 and BSF22, were citrate positive, indole positive, and urease positive. However, isolate BSF22 was negative for methyl red and Voges-Proskauer tests. The majority of the isolates showed growth on various carbon sources provided in the carbohydrate utilization broth. Among the four tested carbon sources (Glucose, sucrose, maltose, and lactose), isolate MSF9 and BSF22 were positive for glucose and maltose, while isolate MSF9 was positive for sucrose and lactose. The detailed results of the biochemical characterizations are presented in Table 5. In a study conducted by Lwin *et al*. (2020) [15], similar positive results were reported for *Streptomyces sp*., which showed positive results for glucose, maltose, sucrose, lactose, methyl red test, citrate utilization test, and urease test. However, the VP test was found to be negative. Similar results also found by Daquioag and Penuliar (2021) [9] identified *Streptomyces sp*. through biochemical testing, showing negative results for indole and methyl red, and positive results for catalase, citrate, and Voges-Proskauer tests.

Table 4: Biochemical characterization of isolates

Sr. No.	Name of the Test	MSF 9	BSF 22
1	Catalase test	Positive	Positive
2	Indole test	Negative	Positive
3	Methyl Red test	Negative	Negative
4	Voges-Proskauer test	Positive	Negative
5	Citrate utilization test	Positive	Positive
6	Urease test	Positive	Positive
7	**Sugar fermentation test**		
	Glucose	Positive	Positive
	Maltose	Positive	Positive
	Sucrose	Positive	Negative
	Lactose	Positive	Negative

Identification of selected isolates by 16S rDNA sequencing

In our study, analysis of the 16S rDNA gene sequences and comparison with the gene bank database revealed that isolates MSF9 and BSF22 are closely related to *Streptomyces* genus members. Isolate MSF9 exhibited 96.05% sequence identity with *Streptomyces celluloflavus* strain CSSP694 (Accession number NR_115419.1), classifying it as *S. celluloflavus*. Isolate BSF22 shared 82.89% similarity with *Streptomyces longisporoflavus* strain NRRL ISP-5165 (Accession number NR_115963.1), identifying it as *S. longisporoflavus*. Among these,

Streptomycetes, comprising nearly 70% of the soil population, are essential for secondary metabolite production (Malviya *et al*., 2018) [16]. A similar study by Daquioag and Penuliar (2021) [9] focused on the molecular identification of *Streptomyces olivaceus*, *Streptomyces sp*., and *Streptomyces exfoliates* isolated from soil and compost.

Media optimization study

Actinomycetes, a prevalent soil microorganism across diverse soil types (Celaya-Herrera *et al*., 2021) [6], serve as crucial saprophytes that actively decompose organic matter, thereby enriching soil fertility. Notably, actinomycetes possess cellulose-degrading capabilities, positioning them as key players in cellulose breakdown, alongside bacteria, molds, and yeast. Enzyme production employing alternative raw materials, precise strain selection and meticulous attention to environmental variables. Fundamental parameters, such as incubation time, substrate concentration, temperature, pH, and the utilization of metal ions as activators or inhibitors, significantly influence the process. These factors collectively shape enzyme production efficiency and yield.

Effect of temperature on enzyme activity and protein production

The study evaluated the enzyme activity and total protein production of two isolated actinomycetes, MSF9 and BSF22, at different temperatures. Results showed that both strains achieved maximum CMCase enzyme activity and total protein production at 45 °C. MSF9 displayed the highest enzyme activity of 1.89 U ml^{-1} and total protein production of 2.42 mg ml^{-1}, while BSF22 showed significant enzyme activity of 1.86 U ml^{-1} and total protein production of 1.79 mg ml^{-1} at 45 °C temperature. Conversely, the lowest enzyme activity and total protein production were observed at 25 °C for both strains. MSF9 recorded 0.79 U ml^{-1} enzyme activity and 1.37 mg ml^{-1} total protein production, while BSF22 had 0.81 U ml^{-1} enzyme activity and 1.48 mg ml^{-1} total protein production. This highlights the temperature-dependent response of MSF9 and BSF22, with optimal enzyme activity and protein production at 45 °C and reduced levels at 25 °C Table 5.

Table 5: Effect of temperature on enzyme activity and protein production

	Isolates	Temperature (°C)						S. Em. ±	C. D. 5%	C. V. (%)
		25	30	35	40	45	50			
CMCase activity (U ml^{-1})	MSF9	0.79	1.07	1.73	1.77	1.89	1.85	0.02	0.08	3.06
	BSF22	0.81	1.77	1.71	1.75	1.86	1.84	0.02	0.08	2.91
Total protein (mg ml^{-1})	MSF9	1.37	1.38	2.19	2.23	2.42	2.03	0.03	0.012	3.34
	BSF22	1.48	1.57	1.51	1.68	1.79	1.76	0.03	0.10	3.39

The investigation unveiled significant variances in all parameters across different temperature treatments, revealing intercorrelations among the assessed variables. Bispo *et al*. (2018) [5] reported maximal endo-β-glucanase production (approx. 740 U L^{-1}) in *Streptomyces diastaticus* PA-01 under submerged fermentation, achieved after 120 h of growth at pH 4.8 and 50 °C. Correspondingly, Sugumaran *et al.* (2013) [27] explored five actinomycetes, observing enzyme production ability at 28 °C and 50 °C, aligning with our findings. In comparison to other investigations, Prasad *et al.* (2013) [18] documented higher cellulase enzyme production in actinomycetes at 45 °C following a 6-day incubation period. Furthermore, Bhoomi *et al*. (2020) [4] observed that their comprehensive analysis of various incubation temperatures revealed 35 °C to be the optimal condition, effectively enhancing both enzyme production and activity within the actinobacterial strain AB4.

Effect of pH on enzyme activity and protein production

The study investigated the impact of pH on enzyme activity and total protein production in two actinomycetes, MSF9 and BSF 22. pH levels ranging from 4.0 to 10.0 were tested. MSF9 displayed its highest CMCase enzyme activity (0.75 U ml^{-1}) and total protein production (0.90 mg ml^{-1}) at pH 7, while BSF22 exhibited its maximum CMCase enzyme activity (0.93 U ml^{-1}) and total protein production (0.66 mg ml^{-1}) at pH 8. Both strains demonstrated their lowest enzyme activity and protein production at pH 4, with MSF9 recording 0.27 U ml^{-1} and 0.25 mg ml^{-1}, and BSF22 recording 0.25 U ml^{-1} and 0.44 mg ml^{-1}, respectively Table 6.

Table 6: Effect of pH on enzyme activity and protein production

	Isolates	pH							S. Em. ±	C. D. 5%	C. V. (%)
		4	5	6	7	8	9	10			
CMCase activity (U ml^{-1})	MSF9	0.27	0.34	0.41	0.75	0.66	0.50	0.43	0.01	0.03	3.98
	BSF22	0.25	0.43	0.55	0.90	0.45	0.51	0.49	0.01	0.04	4.70
Total protein (mg ml^{-1})	MSF9	0.25	0.38	0.31	0.40	0.93	0.76	0.32	0.01	0.03	3.69
	BSF22	0.44	0.47	0.48	0.57	0.66	0.60	0.49	0.01	0.03	3.85

In comparison to existing studies, our investigation aligns with several optimization findings in the field. This observation underscores the relevance of pH in influencing cellulase synthesis. Additionally, Bhoomi *et al.* (2020) [4] determined that pH 7 emerged as the optimal condition for fostering enzyme production and activity within the isolated species during their investigation of various incubation pH levels.

Ruparelia *et al*. (2020) [22] conducted an optimization study, unveiling optimal enzyme activity levels for actinomycete isolates JRC1 and JRC2 at a substrate concentration of 3%, a temperature of 35 °C, and a pH of 7.0. These findings closely correlate with our exploration of substrate concentration, temperature, and pH effects on enzyme production. Similar trends were reported by Soeka *et al.* (2019) [26] in their investigation of Indonesian soil-isolated *Streptomyces* strains. Optimal cellulase activity for *S. macrosporeus* BB 32 and *S. macrosporeus* KRC 21 D

occurred after 2 and 5 days of incubation, respectively, at a pH of 8.0 and a temperature of 35 °C. These findings substantiate our own regarding incubation duration, pH, and temperature. Furthermore, Akond *et al.* (2016) [1] discovered optimal cellulase production at pH 7 for *Streptomyces* JUBM-35-NS-1 and at pH 8 for *Nocardia* JUBM-35-NS-2. Prasad *et al.* (2013) [18] also reported *S. Griseorubens* St-1's highest cellulase enzyme production at pH 7 on the 6th day of incubation.

Effect of CMC substrate concentration on enzyme activity and protein production: The study investigated the enzyme activity and total protein production of two actinomycetes, MSF9 and BSF22, at different carboxymethyl cellulose (CMC) concentrations (ranging from 0.5% to 3.0%). MSF9 displayed its highest CMCase enzyme activity (1.38 U ml-1) and total protein production (3.18 mg ml^{-1}) at 2% CMC, while BSF22 showed the highest CMCase enzyme activity (1.41 U ml^{-1}) and total protein production (3.53 mg ml^{-1}) at 2.5% CMC. Conversely, both strains exhibited their lowest enzyme activity and total protein production at 0.5% CMC, with MSF9 recording 0.99 U ml^{-1} and 1.40 mg ml^{-1}, and BSF22 showing 0.85 U ml^{-1} and 1.09 mg ml^{-1}, respectively Table 7.

Table 7: Effect of CMC concentration on enzyme activity and protein production

	Isolates	CMC Concentration (%)						S. Em. ±	C. D. 5%	C. V. (%)
		0.5	1	1.5	2	2.5	3			
CMCase activity (U ml^{-1})	MSF9	0.99	1.04	1.27	1.38	1.15	1.09	0.02	0.08	4.07
	BSF22	0.85	0.97	1.02	1.16	1.41	1.03	0.02	0.09	4.68
Total protein (mg ml^{-1})	MSF9	1.40	1.67	2.54	3.18	2.79	1.94	0.03	0.11	2.80
	BSF22	1.09	1.27	1.48	1.92	3.53	2.57	0.03	0.10	3.00

In enzyme reactions, factors like enzyme concentration, existing enzyme activity, and substrate availability are crucial, as highlighted by Vepsäläinen *et al.* (2012) [28]. When it comes to making cellulase with carboximethylcellulose, each microorganism's performance varies due to its unique environmental conditions, as indicated by Prasad *et al.* (2013) [18]. In a study by Soeka *et al.* (2019) [26] on *Streptomyces* isolates from Indonesian soil, they observed distinct cellulase enzyme activity patterns. Specifically, *S. macrosporeus* BB 32 displayed optimal cellulase production at 2 days of incubation with a CMC concentration of 1.75% and a temperature of 35 °C. On the other hand, *S. macrosporeus* KRC 21 D exhibited peak cellulase activity at 5 days of incubation, utilizing a CMC concentration of 2% and a higher temperature of 50 °C. This underscores the strain-specific and environmental influences on cellulase optimization.

Conclusion

This study elucidates the isolation, screening, and characterization of cellulolytic actinobacteria from sugarcane press mud samples in South Gujarat's sugar factories, with MSF9 and BSF22 showcasing significant CMCase production. Through comprehensive analyses, MSF9 was identified as *S. celluloflavus* and BSF22 as *S. longisporoflavus*. Optimal conditions for cellulase activity and protein production were determined at 45 °C, pH 7, and 2% CMC concentration. These findings contribute insights into cellulase-producing microorganisms, offering potential for biotechnological applications in cellulose degradation and utilization.

Conflict of Interest

Not available.

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Not available.

Reference

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