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Characterization and optimization of cellulase production from cellulolytic actinomycetes isolated from University farm and Dandi costal range of Gujarat

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Abstract
In this study, 48 actinomycetes isolates were obtained from Navsari Agricultural University farm soil and sediments of the Dandi coastal range, Navsari, Gujarat. Among these isolates, 10 were found to be positive for cellulase production and were further characterized based on their morphological features. Three potential isolates were selected for detailed biochemical assays, growth characteristics, and cellulase production. These isolates were identified as *Streptomyces rochei* B2II, *Streptomyces viridochromogenes* D1I, and *Streptomyces iakyrus* C1VI through 16S rDNA sequence analysis. The effect of various physico-chemical parameters on the growth and cellulase production of these isolates was investigated. Among the three isolates, *S. iakyrus* C1VI exhibited the highest cellulase production at an optimum temperature of 35 °C and pH 8. For *S. rochei* B2II and *S. viridochromogenes* D1I, the optimum cellulase production was observed at 35 °C and pH 10 and 8, respectively. Additionally, the concentration of 1% CMC was found to be optimal for the best CMCase production. Based on the optimum parameters, media were further optimized, and the enzyme activity, total protein, and dry biomass were estimated. Compared to the unoptimized conditions, the optimized cultural conditions for B2II, D1I, and C1VI resulted in an increase of 20.0%, 19%, and 22% in enzyme activity, 10.5%, 10%, and 14% in total protein, and 13%, 9%, and 19% in dry biomass, respectively. Furthermore, partial purification of the cellulase enzyme was carried out. Compared to the optimized conditions, the partially purified extracts of B2II, D1I, and C1VI showed an increase of 14%, 7%, and 18% in enzyme activity, and 10%, 9%, and 13% in total protein, respectively. These findings highlight the potential of these actinomycetes isolates for cellulase production and offer valuable insights into the optimization of cultural conditions for enhanced enzyme production.

Keywords: Actinomycetes, streptomycetes, carboxymethylcellulase, agricultural waste management

Introduction

Separate taxonomic group actinomycetes are the members of the order actinomycetales classified within domain bacteria. They are primarily aerobic and spore-forming Gram-positive bacteria with high G+C content. Some morphological features of fungi are similar to actinomycetes *viz*. production of aerial or substrate mycelium and filamentous growth. Actinomycetes produce geosmin volatile compounds and are responsible for the earthy smell of the soil. They are ubiquitous in nature, found both in aquatic and terrestrial habitats, including mangroves and sea sediments (Das *et al*., 2003) [2].

Enzymes are delicate protein molecules necessary for life. Cellulose is a fraction of plant matter and the most plentiful biomass on the earth (Venkata *et al.* 2013) [31]. Plant biomass comprises cellulose as the major component. 50% dry weight of plant biomass is cellulose and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta *et al*., 2003) [8]. Recently agricultural and industrial cellulosic wastes have been accumulating in huge amounts. Biobased products and bioenergy can be generated from cellulosic waste is a renewable resource that has attracted worldwide attention (Xing-hua *et al.*, 2009) [33]. Celluloses are perceived as the most vital renewable source for bioconversion. It has become an economic concern to improve an effective method to hydrolyze the cellulosic biomass or agricultural waste (Saraswati *et al*., 2012) [21].

Cellulose is the high molecular weight linear polymer of D-glucopyranose units linked together by (l→4) glycosidic bonds, with cellobiose dimer being the repeating unit. Bundle of microfibrils made by the cellulose chains which are joined with a hydrogen bond. These microfibrils further aggregate together to make cellulose fibrils. Cellulose is the most extensive polymer of plant cell walls (35–50%), followed by hemicelluloses (20–35%) and lignin (10–25%). The mechanisms of action and substrate specificities vary among different cellulases, but they are generally divided into exoglucanases (EC 3.2.1.74), endoglucanases (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and *β*glucosidases (EC 3.2.1.21) (Del-pulgar and Saadeddin, 2014). In aerobic bacteria including most of the actinomycetes, cellulases are free, non-complex and secreted extracellularly that break down cellulose using the different pathway. Exoglucanases act on reducing & nonreducing end of cellulose chain releasing glucose unit. Endoglucanases hydrolyze *β* (l→4) glycosidic bond randomly inside cellulose chain releasing dextrans of variable length. Cellobiohydrolase cleave glycosidic bond at the non-reducing end, release the cellobiose unit. *β* Glucosidase enzymes take part in hydrolysis of cellulose unit to monomeric glucose.

Materials and Methods

Collection of samples

Soil and sediment samples were collected from Navsari

Agricultural University farm soil and mangroves sediments of Dandi Coastal Range followed by the measurement of the pH. Various sample treatment strategies such as treatment of the samples with calcium carbonate and heat treatment were carried out prior to the isolation of the actinomycetes using the dilution and plate technique.

Heat and calcium carbonate treatment

Without any prior dilution, soil samples were subjected to heat treatment in a hot air oven at 60 ºC for 1 h to reduce the number of unicellular bacteria. After treatment, the samples were diluted and plated over cellulose basal media (CMC 10 g, sodium citrate 0.5 g, KH_2PO_4 2.0 g, K_2HPO_4 7.0 g, $MgSO4.7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1.0 g, Agar 10 g, PH 7.0, Distilled water 1 lit.), Nutrient agar, Starch casein agar (SCA) and Actinomycetes isolation agar (AIA) (Shirling and Gottlieb, 1966) [25]. Incubation was carried out at 28 ºC for 1week (Vijayakumar *et. al.*, 2007; Subramani *et. al.*, 2013) [32, 28] It is believed that the formation of aerial mycelia in several actinomycetes promoted with the treatment of samples with $CaCO_3$it enhancing the appearance of the actinomycetes on growth media (Natsume, *et. al.*, 1989) [18]. $CaCO_3$ was mixed with soil samples in a 1:1 ratio and incubated at 28 ºC for 10 days. After treatment samples were diluted up to 1:1000 and used for the isolation of actinomycetes. A typical chalky white colony with the rough surface was picked and subculture for getting pure isolates and maintained on their respective media slant at 4 °C and 20% (w/v) glycerol at -20 °C.

Qualitative screening of isolates by agar plate assay

All isolates were inoculated in Na salt of Carboxymethylcellulose (CMC) supplemented with BH agar by a single streak in the center of the plate and were incubated at 28 °C for 5 days, after which they were stained by flooding them with iodine solution (1% w/v) for 15 min.

The appearance of zone of clearance around growth indicated cellulose breakdown.

Relative enzyme activity (cm) = $(D^2 - d^2)/d^2$

Where, d= width of actinomycetes growth (cm)
D= width of activity in terms of hydrolysis (cm)

Morphological characterization of isolates

Actinomycetes positive isolates were characterized by their morphological and physiological features. Gram's staining of isolates was performed and slides were examined under a binocular optical microscope to observe for morphological characteristics *viz*, grams staining, shape, arrangement and cultural characteristics *viz*, colony size, shape, form, margin/edge, elevation, presence/absence of substrate mycelium, aerial mycelium colour, substrate mycelium colour and pigment production.

Quantitative screening for cellulase enzyme

Inoculum preparation

Selected isolates were inoculated in nutrient broth for 48 h at 35 °C and 120 RPM. After incubation period, these actinomycetes cells were used as inoculums.

Cellulase enzyme activity assay by the submerged fermentation process: 1% CMC supplemented with BH broth (50 ml), pH 7 was used for cellulase production. It was inoculated with 10% inoculums at 30 °C for 5 days. The culture was centrifuged at 4 °C and 10,000 rpm for 10 minutes. CMCase activity was determined in the crude supernatant. The activity was measured in terms of reducing sugars released which was quantified by dinitrosalicylic acid (DNS) method (Miller, 1959) [16]. The assay was performed by mixing 0.5 ml of the crude enzyme extract with 0.5 ml CMC substrate solution (prepared in 0.05 M sodium phosphate buffer, pH 7.0) and incubated in water bath at 50 °C for 30 minutes. DNS was added to the above mixture, and the reactions were terminated by boiling at 100 °C for 10 minutes. Blank was prepared by mixing substrate with boiled crude enzyme (Tilbeurgh, *et. al.*, 1982) [30]. Absorbance was measured at 540 nm. One unit (U) was defined as the amount of enzyme that releases 1 µg of glucose per minute under experimental conditions.

Estimation of total protein by Folin Lowry method

Total protein was measured in culture supernatants of isolates obtained same as in enzyme assay by Folin Lowry method of protein estimation (Lowry, *et. al.*, 1951) [14]. Bovine Serum Albumin (2 mg ml^{-1}) was used as standard. The assay was performed by using 1.0 ml of the crude extract and 1.0 ml distilled water to make 2.0 ml final reaction volume. Add 3 ml alkaline copper sulphate reagent and incubate for 10 minutes. Add 1N Folin Ciocaltue reagent and incubate for 30 minute in dark. Absorbance was measured at 660 nm. From the standard curve amount of protein produced were calculated.

Estimation of growth (dry biomass)

Total growth (Biomass) was measured using Dastager method (Dastager, *et. al.*, 2009) [3]. Incubated CMC broth was filtered through cellulose filter paper. Filtered biomass was oven dry at 80 °C and weighed. Biomass was expressed in terms of mg dry mass per 50 ml of culture medium.

Effect of Physico-chemical parameters on growth, enzyme activity and total protein production

Potential isolates were further studied for the effect of physicochemical parameters on growth, enzyme activity and total protein production in submerged fermentation method following the one factor at a time approach (OFAT) (Hegde, *et. al.*, 2011) [9]. Effect of temperature on growth, enzyme activity and total protein production was studied by varying temperature at25 °C, 30 °C, 35 °C, 40 °C, and 45 °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 6.0-10.0. 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.

Enzyme activity, total protein and biomass estimation in optimized culture condition

The optimum temperature, pH and substrate concentration so obtained was followed for this experimental step for determination

of growth, enzyme activity and total protein production in optimized culture condition.

Partial purification of enzyme by ammonium sulphate saturation

Total of 1 L of crude culture extract was saturated up to70% by addition of 472g ammonium sulphate and the mixture was kept overnight at 4 °C for protein precipitation (Shaikh *et al*., 2013; Shanmugapriya *et. al*., 2012; Yassien *et. al.,* 2014) [23, 24, 34]. Precipitate was recovered by centrifugation at 10000 rpm, for10 min at 4°C and brought in 0.05 M phosphate buffer (pH 7.0). Enzyme activity was measured in the equalized protein samples by DNS method using glucose standard curve. Protein content in samples was determined by Folin Lowry method.

Biochemical characterization of potent isolates

The Actinomycetes strains which produce cellulase enzyme were further subjected to performed biochemical Characterization *viz.* MR VP test, Citrate utilization test, Starch hydrolysis test, Casein hydrolysis test, Gelatine hydrolysis test, Catalase test, Oxidase test, Indole test, Urea hydrolysis test, Carbohydrate fermentation test.

Molecular Characterization of potent isolates

The strain which shows maximum cellulase activity was further subjected to molecular identification by analysing 16S r DNA sequence.

Genomic DNA extraction from actinomycetes isolates

Total genomic DNA from the selected actinomycetes strains was extracted as per standard protocol (Fernando *et. al.*, 2002) [5] with some modifications. Suspension of the isolate was suspended in nutrient broth and incubated in an orbital incubator at 30 °C, 120 rpm for 48-72 hours to form a pellet of vegetative cells. The cells were harvested by centrifugation at 10000 RPM for 10 minutes and then suspended in TEN buffer (100 mM Tris-HCl, 10 mM EDTA, 250 mM NaCl, pH 7.2) having 1% sodium dodecyl sulfate (SDS). Proteinase-K (20 mg/ml) was then added to a final concentration of 100 µg $ml^{-1}$and mixed gently. The suspension of lysed cells was cooled to room temperature and an equal volume of phenol equilibrated with 0.5 M Tris-HCl (pH 8.0) was added and gently mixed. The third extraction with a 24:1 mixture of chloroform and isoamyl alcohol was carried out and the aqueous phase was collected carefully. DNA dissolved in solution was precipitated after the addition of 0.1 volume of 3.0 M sodium acetate (pH 7.5) and 0.6 volume of isopropanol. Incubating at -20 °C for 12 hours precipitated the DNA. The precipitated DNA was pelleted by centrifugation at 10,000 RPM for 15 minutes at room temperature. The excess salt was removed by washing the DNA pellets three times in 70% ethanol. The DNA samples were dried under vacuum and dissolved in appropriate volume of TE buffer (10 mM Tris; 1 mM EDTA, pH 7.5) and stored at -20 °C. Agarose gel electrophoresis was done to check the purity of DNA.

DNA concentration and purity were assessed spectrophotometrically by comparing absorbance at 260 and 280nm followed by 0.8% agarose gel electrophoresis. The concentration of DNA was found out from the following formula.

Conc. Of DNA (µg/ml) = OD at 260 nm ×50 × dilution factor

Identification of Actinomycetes by sequencing of the 16s rRNA

PCR amplification was performed using an Applied Biosystem Vertithermal cycler. The primers for PCR amplification were purchased from Sigma-Aldrich.

Universal Primer (Lane, 1991) [13] 27 forward 5' AGAGTTTCCTGGCTCAG 3' and 1492 reverse 5' ACGGCTACCTTGTTACGATT 3'. The PCR was performed in 20µl reaction mixture containing 2µl of 10X assay buffer, 1µl dNTP mix of 2.5 mM, 0.5µl of $MgCl_2$, 1µl each of forward and reverse primer (5pmol), 0.5µl of Taq polymerase,1µl of template DNA and 13.5µl of HPLC grade water with the following amplification for 16s rRNA initial denaturation at 95 ºC for 4 min followed by38 cycles of denaturation, annealing and extension (94 ºC for 1 min, 59.9 ºC for 2min and 72 ºC for 2 min) and final extension at 72 ºC for 20 min followed by hold for infinity at 4 ºC. The presence of PCR products was determined by 2.5% agarose gel electrophoresis and to analyse the size of amplified PCR product DNA markers of 100bp were used which was provided by the pure gene. The amplified product was sent for sequencing to Saffron life science Pvt. Ltd., Bilimora, Gujarat.

Statistical Analysis

The data for all experiments were expressed as mean ± standard deviation. The experiments for enzyme assays were set with three replicates and three aliquots were taken from each replicate for all parameters, except for biomass determination. The data was analysed by one-way analysis of variance (ANOVA) followed by LSD post hoc test. Where the analysis showed that a significant difference existed between treatments, the levels of significance were indicated as follows: $p< 0.001$ is designated by S^a (Considered as highly significant); $p< 0.01$ is designated by S^b (Considered as significant); $p< 0.05$ is designated by S^c (considered as statistically significant) and NS means not significant. Where the F test showed a significant difference in parameters, the means are significantly different ($p< 0.05$).

Results and Discussion

Cellulose is the major fraction of organic carbon in soil and the main structural component of plants. Soil microbes are responsible for the recycling of this organic carbon into the environment (Mswaka and Magan, 1998) [17]. Cellulosic material degradation is a complex process and requires the involvement of microbial cellulolytic enzymes. Cellulosic substrates rich habitations are preeminent sources of cellulose degrading actinomycetes (Nutt *et. al.*, 1998) [19]. Several actinomycetes species have been discovered which have the capacity to alter cellulose to simple sugars (Kasana, *et. al*., 2008) [12] but the requirement for new isolated cellulolytic microorganisms still continues (Brander *et. al.*, 2000) [1]. With the aim of isolating cellulolytic actinomycetes a total of six soil samples (A1, A2, A3, C1, D1 and D2) were collected from rhizospheric soil of banana and sugarcane field, K. V. K. Farm, N. A. U., Navsari, Gujarat and four sediment samples (B1, B2, E1 and E2) from mangroves coastal range of Dandi, Gujarat.

Cellulose degrading actinomycetes were enriched by heat and $CaCO_3$ treatment. Enriched samples were dilution plated over nutrient agar (NA), and actinomycetes isolation agar (AIA). After dilution plate technique, well-isolated colonies showing distinct colony morphology were selected and further purified by sector streaking. Altogether, 48 isolates were obtained from different samples are listed in table: 1. All isolates maintained in pure culture and were preserved at 4 ºC temperature for further studies.

Table 1: Isolate numbers and sampling site with location

Sample	Isolates	Location
Rhizospheric soil of banana plant Sample No. (A1, A2 and A3)	A1I, A1II, A1III, A1IV, A1V, A1VI, A2I, A2II, A2III, A2IV, A2V, A3I, A3II, A3III, A3IV, A3V	20°55'44.8"N 72°53'22.3"E
Rhizospheric soil of Sugarcane plant Sample No. (C1, D1 and D2)	C1I, C1II, C1III, C1IV, C1V, C1VI, C1VII,C1III, D1I, D1II, D1III, D2I, D2II, D2III, D2IV, D2V, D2VI, D2VII	20°55'48.9"N 72°53'07.9"E
Sediment Sample from mangroves coastal range. Sample No. (B1, B2, E1 and E2)	B1I, B1II, B1III, B2I,B2II,B2III, B2IV, B2V, E1I, E1II, E2I, E2II, E2III, E2IV	20°53'28.1"N 72°47'43.6"E

Varying morphotypes were obtained using different techniques. Reports on various study revealed that the pretreatment of samples with heat can reduce undesirable bacteria and enhance the isolation of the actinobacteria. However, samples treated with $CaCO_3$ increase the chance of getting actinomycetes isolates from treated samples (Brander *et. al.*, 2000) [1]. Similar to the described pattern for other actinomycetes, the present findings indicated that, heat treatment of the samples appears to eliminate or significantly reduce the bacterial load in terms of CFU count as reflected in Table 2.

Table 2: Overall CFU counts of soil and sediment samples on AIA

Sample	CFU Count		
	Untreated	Heated	$CaCO_3$ Treated
Banana Rhizospheric Soil	1.6×10^7	6.9×10^5	1.8×10^7
Sugarcane Rhizospheric Soil	2.2×10^6	2.1×10^5	2.0×10^7
Sediments from Mangroves	2.6×10^6	2.9×10^5	2.5×10^7

Qualitative screening

All 48actinomycetes isolates were screened for CMCase production on agar plates containing (CMC 10 g, sodium citrate 0.5 g, KH_2PO_4 2.0 g, K_2HPO_4 7.0 g, $MgSO_4.7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1.0 g, agar 10 g, distilled water 1 lit. and pH 7.0). Isolate number A1II, A1V and A3III from banana soil, C1VI, C1IV, D2VII and D1I from sugarcane soil and B2V, B2II and B2IV from mangroves sediments formed a clear zone of hydrolysis on CMC agar plates when flooded with Grams iodine. Furthermore, all strains were found to completely degrade the filter paper with an incubation period of 7 days. Isolates C1VI show maximum CMCase production followed by B2II and D1Iwere selected as potent isolates for further study. Relative enzyme activity was calculated and results are as reflected in Table 3.

Table 3: Relative enzyme activity of actinomycetes measured in zone ratio

Banana Rhizospheric Soil		Sugarcane Rhizospheric Soil		Sediments from mangroves	
Isolates No.	Zone Ratio	Isolates No.	Zone Ratio	Isolates No.	Zone Ratio
A1II	2.3	C1VI	6.0	B2V	0.6
A1V	1.6	C1IV	0.9	B2II	5.0
A3III	2.0	D2VII	1.2	B2IV	2.2
		D1I	3.0		

Mean of three replications*

Morphological Characterization

Morphological and cultural characteristics of all cellulase positive isolates were carried out and observed that colonies ranged from pinpoint to large in size. The majority of the isolates showed the presence of aerial and substrate mycelium of white and brown colour respectively. Their colonies were tough, leathery or powdery in appearance. All the isolates were found to be Gram positive in nature which is a characteristic feature of actinomycetes.

Biochemical Characterization of Isolates

Biochemical characterizations of three potent isolates were studied on different types of media and the results indicated that all the isolates were producing catalase and oxidase enzymes. The positive utilization of gelatine was observed only in isolate B2II. Casein hydrolysis was shown by isolate C1VI and B2II. All three isolates were citrate positive, whereas indole and methyl red negative. Starch was utilized by isolate D1I. Isolate C1VI shows urease and VP test positive. The majority of the isolates were found to grow on different carbon sources provided in the carbohydrate utilization broth. Four carbon source glucose, maltose, sucrose and lactose were tested and observed that isolate D1I was positive for all sugar while C1VI was positive for glucose and maltose and B2II was positive for glucose and lactose. The results of biochemical characterization are presented in Table: 5.

Table 5: Biochemical characterization of actinomycetes isolates

Isolates	Biochemical Test / Sugar Fermentation Test													
	Catalase	Oxidase	Gelatine Hydrolysis	Casein Hydrolysis	Starch Hydrolysis	Citrate Utilization	Urease	Indole	Methyl Red	Vogues Proskauer	Glucose	Maltose	Sucrose	Lactose
B2II	+	+	+	+	-	+	-	-	-	-	+	-	-	+
D1I	+	+	-	-	+	+	-	-	-	+	+	+	+	+
C1VI	+	+	-	-	-	+	+	-	-	+	+	+	-	-

Identification of selected isolates by 16S rDNA sequencing

16S rDNA analysis was carried out for three isolates. Amplification of 16S rDNA gene by PCR resulted in the generation of a single discrete PCR amplicon band (1500 bp) on agarose gel for all isolates. Actinomycetes that were closely related to the isolates were identified by carrying out BLAST of 16S rDNA gene sequence with the database of NCBI gene bank.

Based on the sequence identity of the 16S rDNA gene against gene bank database isolates B2II, D1I and C1VIwere found to be closely related to the members of genus *Streptomyces*. Isolate B2II showed 99% identity with *S. rochei* strain NBRC 1559 (Accession number NR_116078.1) and *S. enissocaesilis* strain NBRC 1636 (Accession number NR_115668.1) hence it was identified as *S. rochei*. The highest similarity 99% of D1I was observed with *S. viridochromogenes* strain NBRC 3113 (Accession number NR_112526.1) and *S. viridochromogenes* strain NBRC 3113 (Accession number 112482.1) respectively. The 16S rRNA gene sequences of D1I isolates was submitted to NCBI and the assigned Genebank accession numbers to the isolated D1I strains were MZ577212. Isolate C1VI was found to be related to genus *Streptomyces* showing 96% identity with *S. iakyrus* strain NBRC 13401 (Accession number NR_041231.1) and *S. violaceochromogenes* strain NBRC 13100 (Accession number NR_112369.1).

Effect of physico-chemical parameters on growth, total protein and enzyme production: Effect of Temperature: The sodium salt of CMC supplemented with Bushnell and Hans Broth was prepared and inoculated with 10% inoculums. Flasks were incubated at different temperatures for 120 hours and enzyme activity was calculated. Maximum CMCase enzyme activity (1.34 U ml^{-1}) and total protein (1.84 mg ml^{-1}) was observed in isolate C1VIat 35 °C, while maximum biomass (189.86 mg $50ml^{-1}$) was observed in isolateB2IIat 30 °C which is also the optimum growth temperature for actinomycetes (Table 6), Data analysis revealed a highly significant difference between all the temperatures tested for CMCase activity, as the temperature was increased from 40 °C to 45 °C enzyme activity declined significantly. A considerable amount of work published on different *Streptomyces* sp. report 45 °C as an optimum temperature for CMCase activity (McCarthy and Cross, 1984) [15]. In a recent study on *Streptomyces griseorubens* St-1 which was isolated from Indian soil, maximum cellulase activity was found at 45 °C (Prashad *et. al*., 2013) [20]. Contradictory to the described pattern for other actinomycetes, the present findings of the study indicated that optimum enzyme production, biomass and total protein occurs at 35 °C by *Streptomyces iakyrus* C1VI, *Streptomyces rochei* B2IIand *Streptomyces viridochromogenes* D1I. In the present study, the optimum temperature for biomass and enzyme productions was found to be in the range of 30 to 35 °C. Similar results also published on *Streptomyces* suggested that most of the actinomycetes behave as mesophilic and have an optimum growth temperature ranging from 30-37 °C (Goodfellow and Williams, 1983) [6].

Table 6: Enzyme activity, Total protein and Dry biomass of isolates at different temperatures

	Isolates	Temperature (°C)					S. Em. ±	C. D. 5%	C. V. (%)
		25	30	35	40	45			
CMCase activity (U ml^{-1})	B2II	0.55	0.64	0.81	0.50	0.35	0.007	0.028	3.28
	D1I	0.33	0.41	0.68	0.33	0.26	0.009	0.022	3.56
	C1VI	0.76	0.89	1.34	0.72	0.63	0.014	0.043	3.26
Total protein (mg ml^{-1})	B2II	0.96	1.13	1.42	0.87	0.59	0.011	0.032	2.14
	D1I	0.82	1.03	1.72	0.82	0.65	0.019	0.057	3.72
	C1VI	1.04	1.22	1.84	0.99	0.84	0.017	0.052	2.91
Dry biomass (mg $50ml^{-1}$)	B2II	138.82	189.86	176.87	90.94	43.46	2.295	6.918	3.59
	D1I	137.57	167.22	188.76	72.74	39.95	0.902	2.720	1.49
	C1VI	151.93	160.76	182.14	86.48	45.32	0.820	2.462	1.30

Effect of pH

A culture medium pH was varied from pH 6.0 to 10.0 for studying the effect of pH on enzyme productions. Least enzyme activity was observed at pH 6.0 and then it increased with the increase of pH of the medium. Maximum cellulase activity was observed at pH 8.0 in isolate D1I and C1VI whereas at pH 10.0 for isolate B2II (Table 7). Maximum CMCase activity (0.80 U ml^{-1}), total protein (1.95 mg ml^{-1}) and biomass (197.8 mg $50mg^{-1}$) occurred at pH 10.0 in isolate B2II. Whereas maximum CMCase activity (0.67 and 1.33 U ml^{-1}), total protein (1.84 and 1.90 mg ml^{-1}) and biomass (187.85 and 186.11 mg $50ml^{-1}$) observed in isolatesD1I and C1VIrespectively at pH 8.0. Maximum CMCase production occurred at pH 8.0 and 10.0 respectively; hence other parameters were optimized at the same conditions. These observations also revealed the alkaliphilic nature of isolates B2II, D1I and C1VI and their enzymes. In actinomycetes, most of the studies are available on *Streptomyces* species that reported maximum CMCase production over a broad pH range from 5.5 in *S. lividans* (Theberge *et al*., 1992) [29], to pH 6.0 in *Streptomyces* sp. J2 (Jaradat *et al*., 2008) [11], pH 7.0 in *S. griseorubens* (Prashad *et al*., 2013) [20] and *S. drozdowiczii* (Semedo, *et al*., 2000) [22]. In a study on *Thermonospora curvata* optimum cellulase production occurred at pH 8.0 (Stutzenberger, 1971) [27]. An alkaline novel *Streptomyces* species from east African soda lakes showed an optimum enzyme production at pH 8.0 (Solingen *et. al*., 2001) [26]. CMCase production by *Streptomyces rochei*B2IIwas found to be highest at pH 10.0 shows the alkaline nature of these actinomycetes and their enzyme. It is a well-known fact that enzymes are not stable at very high or very low pH values as they get denatured and thus highly basic and highly acidic pH values adversely affects enzyme production by microorganisms (Haltrich *et. al*., 1996) [7].

Table 7: Enzyme activity, Total protein and Dry biomass of isolates at different pH

	Isolates	pH					S. Em. ±	C. D. 5%	C. V. (%)
		6.0	7.0	8.0	9.0	10.0			
CMCase activity (U ml^{-1})	B2II	0.07	0.42	0.54	0.75	0.80	0.007	0.021	2.75
	D1I	0.03	0.48	0.67	0.48	0.25	0.006	0.019	3.29
	C1VI	0.16	0.99	1.33	1.01	0.60	0.012	0.037	2.97
Total protein (mgml^{-1})	B2II	0.18	1.02	1.33	1.83	1.95	0.019	0.058	3.04
	D1I	0.07	1.31	1.84	1.34	0.68	0.020	0.060	3.78
	C1VI	0.23	1.42	1.90	1.45	0.89	0.022	0.065	3.68
Dry biomass (mg 50ml^{-1})	B2II	40.22	181.61	151.34	190.23	197.80	0.738	2.226	0.97
	D1I	52.24	161.00	187.85	176.34	158.52	0.572	1.725	0.78
	C1VI	48.56	169.20	186.11	170.10	150.00	0.839	2.530	1.16

Effect of substrate concentration

From the different substrate concentration, 0.5% to 2.5% (CMC) as sole carbon sources tested on isolates B2II, D1I and C1VI. Maximum cellulase activity (1.35 U ml^{-1}), Total protein (2.56 mgml^{-1}) and biomass (202.2 mg50mg^{-1}) was obtained in isolate C1VI when the medium was supplemented with 1% CMC as sole carbon source followed by 1.5%, 2% and 0.5% and 2.5% (Table 8). Data analysis reviled a significant difference between cellulase activity, total protein and biomass when the medium was supplemented with different substrate concentrations. In the present study, five different concentrations of CMC% were tested and observed that treatment of 1% CMC produced maximum enzyme activity. Thus CMC was easily metabolized by *Streptomyces* and also acted as an inducer for CMCase production. More or less similar results were found in a study on *Streptomyces* sp. They observe that treatment 1% CMC concentration was better for endoglucanase production (Jang and Chen, 2003) [10].

Table 8: Enzyme activity, Total protein and Dry biomass of isolates at different substrate concentration

	Isolates	CMC (%)					S. Em. ±	C. D. 5%	C. V. (%)
		0.5	1.0	1.5	2.0	2.5			
CMCase activity (U ml^{-1})	B2II	0.45	0.78	0.62	0.59	0.24	0.0052	0.016	2.03
	D1I	0.29	0.66	0.52	0.42	0.18	0.0050	0.015	2.24
	C1VI	0.98	1.35	1.01	0.99	0.51	0.0190	0.057	3.93
Total protein (mg ml^{-1})	B2II	1.45	2.53	1.97	1.88	0.45	0.013	0.038	1.52
	D1I	1.62	2.45	1.54	1.94	0.64	0.017	0.052	2.09
	C1VI	1.87	2.56	1.93	1.89	0.98	0.035	0.105	3.77
Dry biomass (mg 50ml^{-1})	B2II	154.9	198	103.9	177.2	108.2	1.336	4.028	1.80
	D1I	145.6	193.2	89.7	167.6	103	0.902	2.718	1.29
	C1VI	152.8	201.2	93.6	183	109.2	0.908	2.947	1.32

Enzyme activity, total protein and biomass estimation in optimized culture condition

Based on the effect of physicochemical parameters on the growth of potential isolates growth media were optimized with the sodium salt of 1% CMC (Medium viscosity) supplemented with Bushnell and Hans Broth was prepared and inoculated with 10% inoculums at pH 10 for B2II, pH 8 for D1Iand C1VI. Temperature maintains for all three isolates at 35 °C. Enzyme activity, total protein and biomass of isolates were estimated after incubation of 5 days. Enzyme activity 0.99, 0.87 and 1.75 Uml^{-1}, total protein2.70, 2.57 and 2.82 mg ml^{-1} and 223, 211 and 239 mg 50 ml^{-1} dry biomass of isolates were found respectively (Table 9). Comparisons between unoptimized (Control) and optimized culture condition resulted higher enzyme activity, total protein and biomass were found in optimized compare to control condition. In optimized culture conditionof B2 IIresulted in an increase of 20.0% enzyme activity, 10.5% total protein and 13% dry biomass. While, inD1Iincrease of 19% enzyme activity, 10% total protein and 9% dry biomass and inC1VIincrease of 22% enzyme activity, 14% total protein and 19% dry biomass were observed.

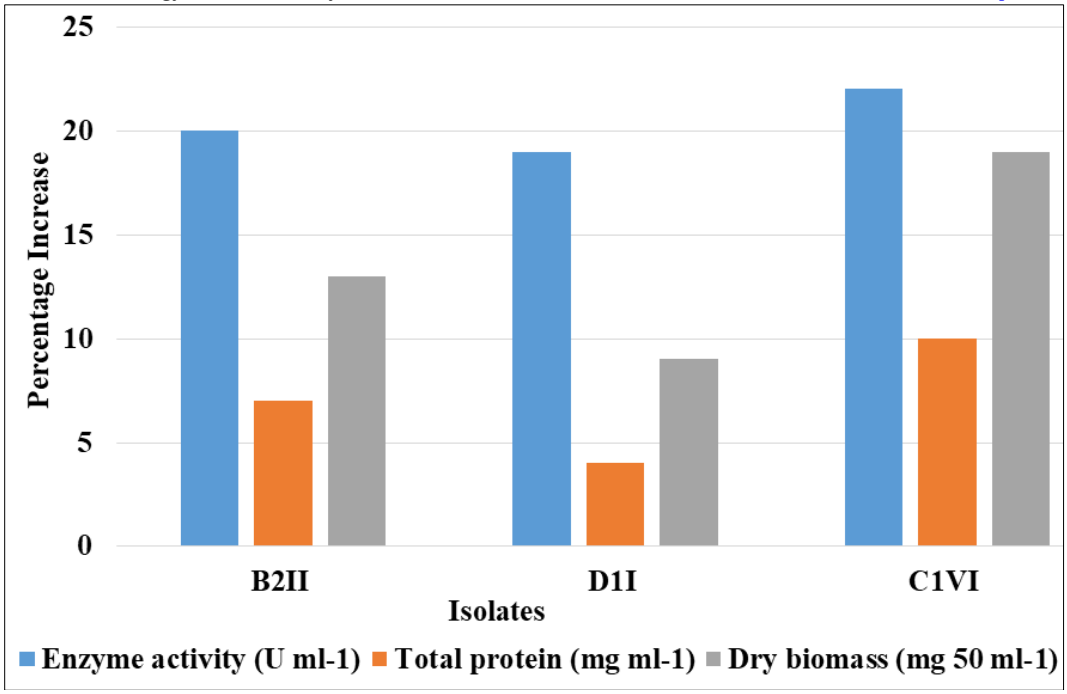

Fig 1: Comparison of Enzyme Activity, Total Protein, and Dry Biomass in Optimized Culture Conditions for B2II, D1I, and C1VI Isolates

Table 9: Enzyme activity, total protein and biomass of isolates under unoptimized and optimized culture condition

	B2II- Unoptimized	B2II-Optimized	D1I- Unoptimized	D1I- Optimized	C1VI- Unoptimized	C1VI-Optimized
Enzyme activity (U ml^{-1})	0.80	0.96	0.68	0.81	1.35	1.65
Total protein (mg ml^{-1})	2.53	2.70	2.46	2.57	2.56	2.82
Dry biomass (mg 50 ml^{-1})	198	223	193	211	201	239

Partial purification of cellulase enzyme by ammonium sulphate saturation

Crude enzyme was partially purified by 70% ammonium sulphate saturation (21-22). Protein content was determined in the partially purified protein samples by the earlier mentioned procedure. For enzyme activity comparison in partially purified extract, protein contents in all samples were equalized. Cellulase enzyme activity and total protein was found maximum in isolate *S. iakyrus*C1VI (1.79U ml^{-1}) and (2.97 $mgml^{-1}$) respectively followed by *S. rochei* B2II (1.2U ml^{-1}) and (2.86 $mgml^{-1}$) and *S. viridochromogenes* D1I (0.97U ml^{-1}) (2.7 $mgml^{-1}$) respectively (Table 10). During primary screening *S. iakyrus* C1VI showed maximum zone of clearance followed by *S. rochei B*2II and *S. viridochromogenes* D1I. The results of primary screening were confirmed during secondary screening. The enzyme activity was found maximum in C1VI followed by B2II and B2II both in case of optimized crude extract as well in partially purified extract of samples (Table 9).

Table 10: Enzyme activity and total protein of isolates in partially purified extract and optimized crude extract

	B2II-Optimized	B2II–Partially Purified	D1I- Optimized	D1I- Partially Purified	C1VI-Optimized	C1VI- Partially Purified
Enzyme activity (U ml^{-1})	0.96	1.2	0.81	0.97	1.65	1.79
Total protein (mg ml^{-1})	2.70	2.86	2.57	2.7	2.82	2.97

In comparison with the optimized condition, a partially purified extract of B2II, D1I and C1VIresulted in an increase of 14%, 7% and 18% enzyme activity and 10%, 9% and 13% total protein in respective isolates were observed. Further work needs to be done for purification of cellulase enzyme by column chromatography, determining the enzyme activity and protein content in purified culture extracts followed by protein profiling on SDS PAGE.

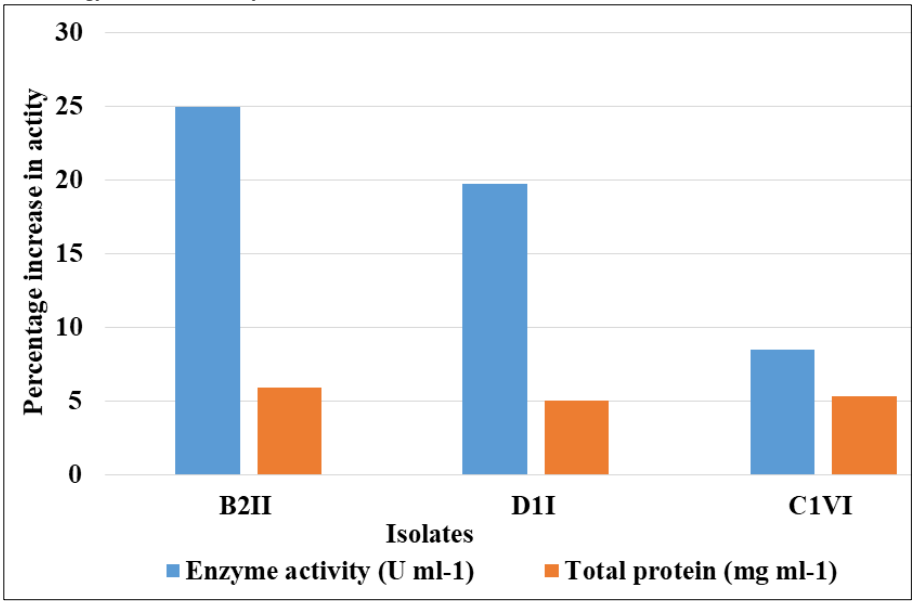

Fig 2: Percentage Increase in Enzyme Activity and Total Protein for B2II, D1I, and C1VI Isolates in Optimized Culture Conditions

Conclusion

Present work was carried out for the isolation of cellulolytic actinobacteria from Navsari habitats. Isolates were subjected to qualitative screening and based on the results isolates B2II, D1I and C1VI representing habitats *S. rochei*, *S. viridochromogenes* and *S. iakyrus* were selected for quantitative screening. Isolates were morphologically and biochemically characterized. The 16S rRNA gene sequencing and phylogenetic analyses showed that isolates belong to the genus *Streptomyces*. Enzyme activity and protein content in crude culture extract was estimated. The cell free extract was also subjected to partial purification, followed by determination of enzyme activity and protein content. Optimum enzyme activity was found in C1VI followed by B2II and D1I.

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