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Exploration of Soil Microflora for Biosynthesis of Cellulases

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ABSTRACT
The soil contains various groups of fungi, bacteria, actinomycetes, archaea, and other organisms. The types and abundance of soil microorganisms, as well as the source of organic matter, influence the rate of decomposition of soil organic matter. The presence of a diverse array of microorganisms in soil enhances its capacity to decompose a broad spectrum of organic materials, which is particularly important given the substantial quantity of lignocellulosic material that is generated and requires degradation prior to utilization for other purposes. The exploration of soil microflora with the ability to produce cellulase and a comparison of various microorganisms with regard to their ability to degrade cellulose are the main foci of this study. From the collected sample, various 24 types of microorganisms were isolated and screened for their hydrolytic activity. The goal of the current study was to determine how various physico-chemical factors, such as pH (3-11), temperature (4-60 °C), incubation time (2–8 days), cellulose (1–5% w/v) concentration, affected the growth and production of the cellulase enzyme in the microorganisms in order to maximise the enzyme productivity. Bacteria are rapidly growing organisms with a higher capacity to degrade agricultural waste than fungi. Actinomycetes, in contrast, are more active than bacteria and fungi, but their rate of growth is slow. Further can be studied for hydrolytic enzyme in various industries.
Keywords; *Biodiversity, Cellulose, Cellulases, Biosynthesis*

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INTRODUCTION

The extensive availability of lignocellulosic biomass in nature necessitates the utilization of enzymes that possess exceptional catalytic performance and durability under conditions of elevated temperature and extreme pH for their effective biorefining. Cellulose is the primary structural component in lignocellulose, which is a linear homo-polysaccharide possessing 500 to 15,000 units of glucose linked with β-1,4-glycosidic bonds. The disaccharide of cellobiose is the smallest repetitive building block of this biopolymer [1]. The efficient degradation of the polysaccharides found in lignocellulose requires various enzymes working synergistically together. The extension of biocatalytic efficiency by enzyme cocktails in biodegradation processes is an interesting option to pursue [2]. The enzyme consortium of cellulases and hemicellulases comprising of endo-β-1,4-glucanase, exo-β-1,4-glucanase, β-1,4-glucosidase, endo-β-1,4-xylanase and endo-β-1,4-mannanase is required for its saccharification into free sugars. To make enzymes combination with commercially available enzyme becomes very expensive and make the hydrolysis of lignocellulosic material by that will be economically non-viable, it is required to produce these enzymes with suitable group of microorganisms. Among various microorganisms' aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa are better in terms of enzyme varieties and yields [3]. Mainly efficient cellulase activities are observed in fungi. But as it is time consuming recent interest is cellulase production by bacteria because bacteria have high growth rate as compared to fungi and has good potential to be used in cellulase production [4]. The degradation and utilization of cellulose play a vital role in the global carbon cycle, as they contribute

significantly to the carbon sources available on a global scale. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest. Solid-state fermentation (SSF) is carried out in absence or near absence of free water [8]. SSF for production of cellulases is rapidly gaining interest as a cost-effective technology as the microorganisms produce comparatively high titers of cellulase due to the conditions of fermentation which shows similarity to the natural environment. Filamentous fungi have been employed for cellulase production using solid-state fermentation where a basal mineral salts medium was used for moistening the substrate [6]. Production of cellulases from *P. citrinum* using brans of wheat and rice and rice straw as substrate; all these substrates supported the production of cellulases [7]. Cellulolytic enzymes have an extensive industrial application including in bio-ethanol production, pulp and paper, detergent, textile, food processing, wine and brewing, bioremediation, bio-refining, animal feed and agricultural industries which make these enzyme families, of high economic value comprising about 75% of the total demand of enzymes involved in biorefineries, textile, pulp and paper, and food and feed industries [1][5]. As cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem, the main component of plant biomass and it is also the dominant waste material from agricultural industry in the form of stalks, stems and husk [14]. Because plant's primary cell wall contains many macromolecules, including cellulose, hemicelluloses, pectin, and glycoproteins. The structure of cellulose microfibrils is characterized by the presence of linear glucan chains that are bound together by means of hydrogen bonds and other non-covalent interactions. Soil is reservoir of organic and inorganic nutrient components due to which the diversity is observed in microorganisms [15]. The microorganisms have also adapted the physical and biochemical parameter of soil and survive in those conditions; hence microorganisms constitute a huge and almost unexplained reservoir. Microorganisms represent richest of molecular and chemical diversity in nature. The inability of water to penetrate cellulose is due to insoluble nature of crystalline cellulose [16]. Amorphous cellulose allows the penetration of endoglucanase and another subgroup of cellulase that catalyses the hydrolysis of internal bonds. Cellulolytic enzymes are synthesized by several microorganisms and cellulose utilizing population includes several aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa [17]. The search for a novel and improved bacterial strain, having hyper cellulase productivity with more activity and high stability against temperature, pH and under non-aseptic conditions might make the process more economical [18]. Agro-based industry's function is to increase the value of raw agricultural products through downstream so that products are marketable, consumable, and used to generate income and provide profit to the producer [19]. Cellulose degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest. Solid-state fermentation (SSF) is carried out in absence or near absence of free water [20]. SSF for production of cellulases is rapidly gaining interest as a cost-effective technology as the microorganisms, especially fungal cultures produce comparatively high titers of cellulase due to the conditions of fermentation which shows similarity to the natural environment [21]. Filamentous fungi have been employed for cellulase production using solid-state fermentation where a basal mineral salts medium was used for moistening the substrate. [24] studied the production of cellulases from *P. citrinum* using brans of wheat and rice and rice straw as substrate; all these substrates supported the production of cellulases. Higher yield of cellulases and 10-fold reduction in the production cost have been reported with SSF cultures compared to SMF cultures. It offers many advantages over SMF, including high volumetric productivity, higher concentration of products, less effluent generation, and low catabolic repression which makes it a promising technology in near future [22].

MATERIALS AND METHODS

SAMPLE COLLECTION AND ISOLATION

Sample was collected from different Agricultural waste disposal site of Ahmedabad, Gujarat, India. Sample was collected in sterile zip-lock plastic bag and was stored at 4°C. The collected waste samples were brought to the laboratory for further study with respect to cellulolytic activity. About 1.0 g of sample added into 9.0 mL of sterilized normal saline. 0.1 mL of each aliquot was spread from prepared 10^{-1} to 10^{-6} dilutions onto isolation agar medium with the help of sterile glass spreader. Plates were incubated at room temperature for 72 hours [9]. Nutrient Agar medium, Potato Dextrose Agar medium and Actinomycetes Isolate Agar medium are used for the isolation of Bacteria, Fungi and Actinomycetes.

SCREENING FOR CELLULASE PRODUCING MICROORGANISMS

QUALITATIVE STUDY FOR CELLULASES

Qualitative study of cellulase enzyme was done by spot plate method. Each isolate of Bacteria, Fungi and Actinomycetes were individually spotted on the center of CMC agar plate (CMC 10.0 g/L, KH_2PO_4 0.5 g/L,

$MgSO_4$ 0.25 g/L, gelatine 2.0 g/L). After incubation of 72 hours plates were stained with 0.1 % Congo red for 20 mins and followed by 1 N NaCl counter stain for an hour [10]. The cellulolytic potential of the positive isolates was evaluated by calculating the cellulolytic index (CI), which is the ratio of the diameter of the zone of hydrolysis to the diameter of the colony [28].

QUANTITATIVE STUDY FOR CELLULASES

One loop full of the culture of bacteria, spores of fungi and actinomycetes were added into 25 mL CMC media and incubated at room temperature for 24, 48 and 144 hours respectively. 15 mL of the prepared inoculum was added to 100 mL of production medium and incubated at room temperature on 125 rpm for 72 hours. After incubation period growth was measured at 600 nm OD and number of cells calculated by McFarland standard accordingly for bacteria. Dry weight were measured for fungi and actinomycetes; then culture broth subjected to centrifugation at 10000 rpm for 10 min at 4°C and supernatant was collected for enzyme assays and stored at 4°C for further characterization of enzyme. The cellulose-degrading potential or ability of all positive isolates was qualitatively evaluated by measuring hydrolysis capacity (HC) according to [12]. After each incubation interval amount of sugar released during the enzymatic reaction was measured using dinitrosalicylic acid (DNS). A mixture containing 1.0 mL of 2 % (w/v) carboxymethylcellulose (CMC)/ filter paper (FP), 0.5 mL of 0.05 M acetate phosphate buffer (pH 7.0), and 0.5 mL of crude enzyme was incubated for 15 min at 50°C. The reaction was stopped by adding 3.0 mL of DNS reagent. The mixture was boiled for 10 min for colour development which was measured by a spectrophotometer at 540 nm (absorbance). One unit (U) of cellulase activity was defined as the amount of enzyme required to release 1.0 µ mole of glucose/min/mL of crude supernatant under the assay conditions.

PRODUCTION OF ENZYME

Media composition described by Usman *et al.* [12] was used for enzyme production. For preparation of inoculum, the isolate showing maximum activity in secondary screening was used. 10% inoculum added to production medium and kept at room temperature in orbital shaker at 110-120 rpm. Culture was harvested after 24 hours interval and centrifuged at 8,000 rpm for 10 min. The supernatant was collected and used as the crude extracellular enzyme source for CMCase assay [13].

EFFECT OF CMC CONCENTRATION ON ENZYME PRODUCTION

Cellulases synthesis was influenced by the change in concentration of substrate CMC in the medium. There was increase in the cellulase production as the CMC concentration increases in the production medium. Bacteria, Fungus and Actinomycetes were grown in same production medium with varying concentrations of CMC that was 1 %, 2 %, 3 %, 4 % and 5 % under shaking condition [23]. After each interval of incubation period enzyme was extracted by centrifugation and crude enzyme activity was measured by DNSA method. Thus, the effect of CMC concentration was determined by comparing the concentration of sugar released with the standard graph.

EFFECT OF TEMPERATURE ON ENZYME PRODUCTION AND ENZYME ACTIVITY

To check effect of temperature on enzyme production; Organism was inoculated in the flask containing 100 mL production medium and incubated at 28, 37, 45, 55 and 65°C and samples withdraw after 4 days of incubation. To determine thermal stability, the extracted enzyme was kept at 30, 40, 50, 60 and 70 °C for 30 min. After that, the substrate was added and the enzyme activity was assayed by DNSA method.

EFFECT OF PH ON ENZYME PRODUCTION AND ENZYME ACTIVITY

To check effect of pH on enzyme production; Organism was inoculated in the flask containing 100 mL production medium having pH 3, 5, 7, 9, 11 and incubated at room temperature and samples withdraw after 4 days of incubation. Cellulase activity was assayed in different reaction buffers 50mM (sodium citrate buffer for pH 3.0 and pH 5.0; phosphate buffer for pH 7.0; boric acid-borax buffer for pH 9.0; Sodium Orthophosphate buffer for pH 11.0) to determine the effect of pH on activity. Each system has its separate blank containing respective pH buffer solution and incubated at different temperatures. After incubation DNS reagent is added and absorbance is measured at 540 nm. Enzyme activity was calculated by comparing with standard glucose curve and estimating residual glucose concentration.

PRODUCTION OF CELLULASE USING AGRO-WASTES AS SUBSTRATE

PRE-TREATMENT OF SUBSTRATE

The physically pre-treated substrates were further subjected to pre-treatment by alkali pre-treatment, and acid pre-treatment. Each of the substrates was assessed using the combined treatment of acid, alkali and, autoclaving techniques. 1 g of each substrate in 10 mL of 1 % H_2SO_4 was autoclaved at 121 °C for 15 min. After cooling, the supernatant was decanted, and the residues were washed with 1% NaOH and then with distilled water till the pH becomes neutral, then the residues were dried in an oven at 70 °C for 24h.

SOLID STATE FERMENTATION

Experiments were carried out in 250 mL Erlenmeyer flasks, each containing 5 g of pre-treated agricultural waste (rice husk, wheat bran, coconut husk, banana fibre, sorghum straw husk) and 50 mL mineral solution.

Sterilized by autoclaving it and there after inoculated with 10 mL inoculum of the isolate. The flasks were incubated for 7 days at room temperature at 125 rpm. Samples were withdrawn after 7 days. Citrate buffer (0.05M, pH 5.0) was added to the fermented substrate to a total volume of 100 mL and mixed for 1 h on rotary shaker. The suspension was filtered, centrifuged, and the supernatant was used as the crude enzyme for assay of enzyme activity.

RESULT AND DISCUSSION

ISOLATION FROM COLLECTED SAMPLE

Total 10 bacterial, 6 fungus and 4 actinomycetes isolates were obtained from collected soil sample on nutrient agar medium, potato dextrose agar medium and actinomycetes isolate agar medium. These cultures were purified by streaking on respective method. Cultural and morphological characteristics of these isolates were observed (Table 1). Into that different gram positive and gram-negative organisms are observed having a diverse cultural characteristic. Typical actinomycetes colonies are also observed as cellulase producers.

Table 1 Cultural and morphological characteristics of isolated microorganisms.

Sr. No.	Isolate	Gram's Reaction	Morphological characteristics	Cultural characteristics	CI value
1	B1	+	Rods, motile, narrow, thin	medium, round, undulate, smooth, raised, opaque, moist, off-white	3.6
2	B2	+	Rods, non-motile, large, thick	medium, round, entire, smooth, raised, opaque, moist, nil	2.1
3	B3	+	Rods, motile, large, thin	medium, round, curled, smooth, convex, moist, off-white	0
4	B4	-	Rods, non-motile, narrow, thin	medium, round, entire, flat, moist, yellow	0
5	B5	+	Rods, non-motile, narrow, thin	medium, round, entire, smooth, convex, opaque, viscous, white	5.3
6	B6	-	Short rods, motile, narrow, thin	small, round, entire, smooth, raised, transparent, moist, nil	4.5
7	B7	-	Short rods, non-motile, thin	small, round, entire, smooth, raised, transparent, moist, nil	0
8	B8	+	Round, non-motile, small	small, round, entire, smooth, raised, transparent, moist, yellow	3.11
9	B9	+	Round, non-motile, small	small, round, entire, smooth, raised, opaque, moist, white	2.6
10	B10	-	Short rods, non-motile, narrow, thin	small, round, undulate, smooth, flat, opaque, moist, nil	1.1
11	F1	NA	Branched mycelia, conidiospore	large, irregular, raised, filamentous, white colony, white spores	5.2
12	F2	NA	Branched mycelia	large, irregular, flat, filamentous, pink colony, pink spores, pink color diffused in media	3.9
13	F3	NA	Branched mycelia	Medium, round, raised, filamentous, dark green colony, black spores, black color diffused in media	0
14	F4	NA	Branched mycelia	large, irregular, raised, filamentous, white colony, green spores	2.8
15	F5	NA	Branched mycelia	large, irregular, raised, filamentous, white colony, black spores	0
16	F6	NA	Branched mycelia	medium, irregular, raised, filamentous, white colony, black spores, yellow color diffused in media	0
17	A1	+	Branched filaments, spores	small, round, filamentous, black diffused in media, raised, opaque	0
18	A2	+	Branched filaments, spores	small, round, filamentous, grey diffused in media, raised, opaque	3.6
19	A3	+	Branched filaments, spores	small, round, filamentous, white diffused in media, convex, opaque	5.1
20	A4	+	Branched filaments, spores	small, round, filamentous, shiny white, flat, opaque	0
B*- Bacteria F*- Fungi A*- Actinomycetes *NA- Not applicable					

QUALITATIVE STUDY FOR CELLULASE ENZYME

Paudel *et al.* [25] studied different bacterial isolates and screened out 26 bacteria for cellulase enzyme production. Also Sive *et al.* [26] studied for fungi and found different cellulolytic fungi and observed 4 positive fungi. Daquioag [27] investigate for actinomycetes from soil sample and obtained different maximum zone of clearance 20 mm. In present study from isolated cultures 7 bacteria are able to hydrolyse cellulose (figure 1(b)). while 3 fungi and 2 actinomycetes isolates shown positive result. Into that as shown in figure 1 (a) maximum CI value observed by isolate B5 which is 5.3, F1 which is 5.2 and A3 which is 5.1. rest of the isolate shows positive result but having lower CI value. Higher zone producer isolates are carry forwarded for further analysis.

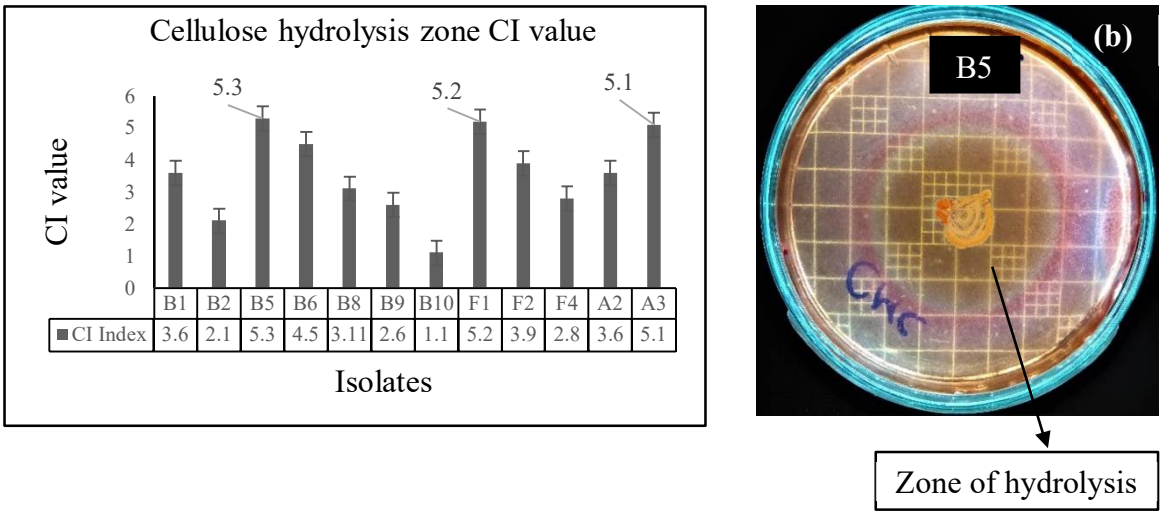


Figure 1 (a) Zone of hydrolysis after treatment of Congo red and NaCl. (b) Zone of hydrolysis on CMC agar plate.

QUANTITATIVE STUDY OF CELLULASE ENZYME

Paudel *et al.* [25], Sive *et al.* [26] and Daquioag [27] have studied CMCase activity and FPase activity of crude enzyme produced by isolates and they have found maximum CMCase activity of 2.31 U/mL for bacteria, 2.46 U/mL for fungi and 1.98 U/mL for actinomycetes. In present study during the incubation of 5 days maximum CMCase activity of B5 1.45 U/mL, F1 2.89 U/mL, A3 3.68 have been found. Maximum FPase activity is 3.12 U/mL observed after 84 hours incubation. Further B5 have higher activity of CMCase but not FPase (Figure 2 (a),(b)) but F1 have higher FPase activity with compare to CMCase activity. A3 have moderate activity of CMCase and FPase enzyme.

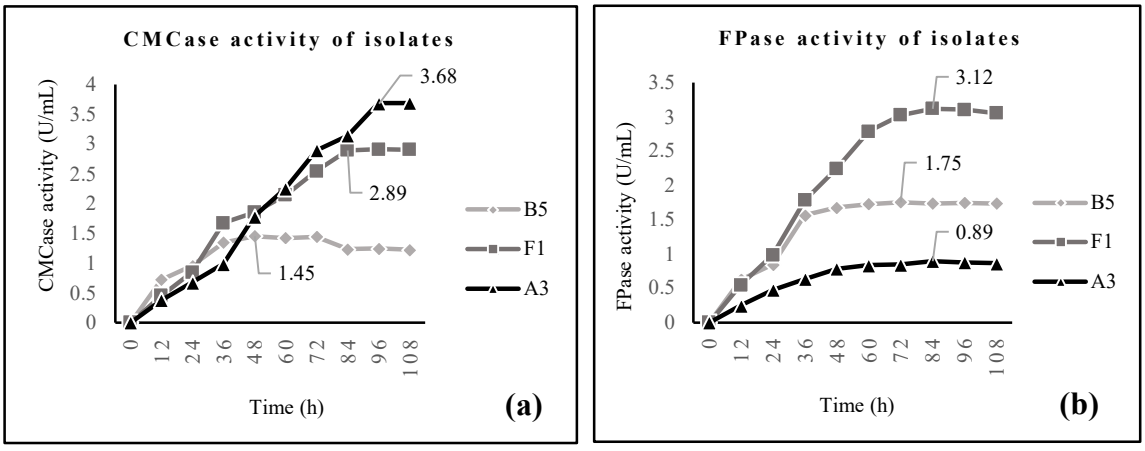


Figure 2 Quantitative study of screened out microorganisms for enzyme activity (a) CMCase activity of isolates (b) FPase activity of isolates

EFFECT OF PH

Figure 3 (a) Effect of pH on enzyme production (b) Effect of pH on enzyme activity

After production of enzyme effect of pH, temperature and concentration of CMC have been studied for the better production and to check the stability of enzyme. The strain B1, F1 and A3 showed maximum activity at pH 7 after 4 days incubation. And maximum enzyme activity observed at pH 7. At pH 9 activity observed higher in F1 but minimum in A3 and B5. Further pH 3 shows minimum activity.

Figure 3 (a) Effect of pH on enzyme production (b) Effect of pH on enzyme activity

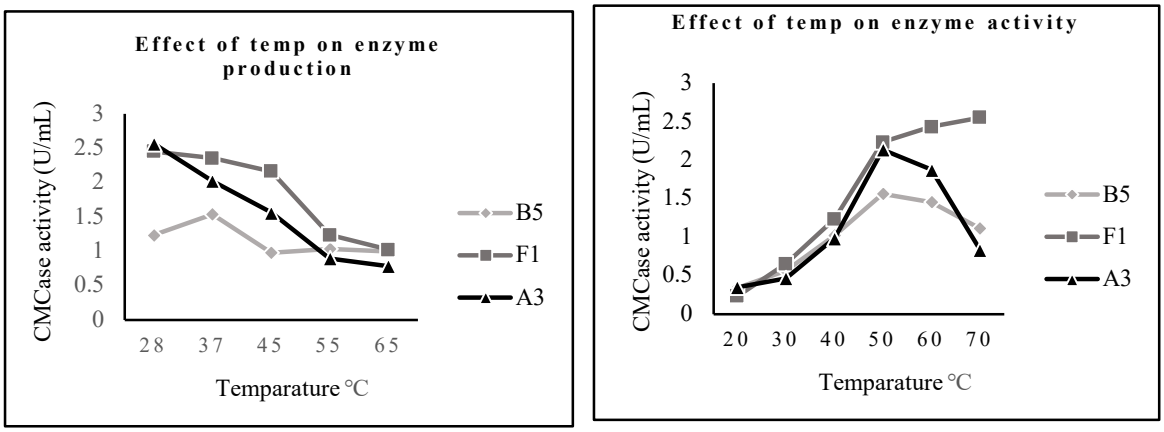
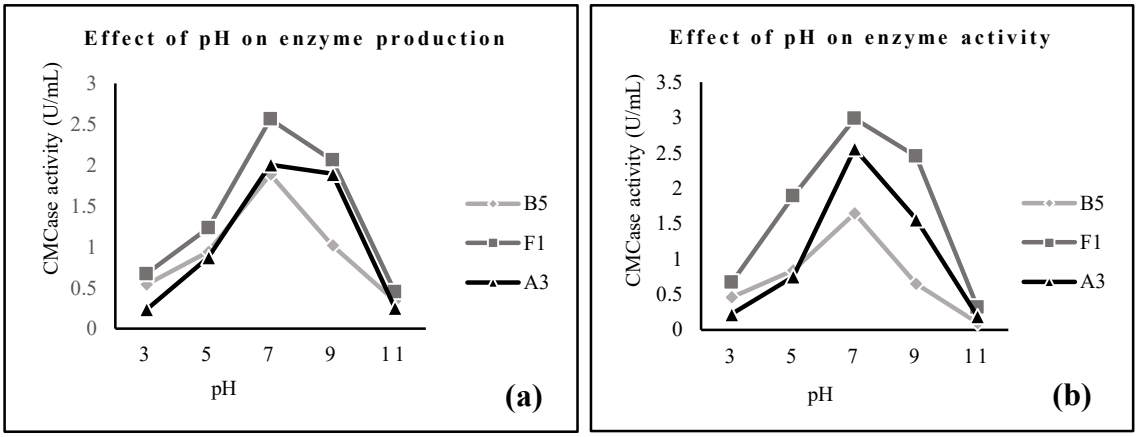


Figure 4 (a) Effect of temperature on enzyme production (b) Effect of temperature on enzyme activity



EFFECT OF TEMPERATURE

Figure 4 (a) Effect of temperature on enzyme production (b) Effect of temperature on enzyme activity

Different temperature studies for the better production by Paudel *et al.* [25] and found best activity at 50 °C temperature. In present research maximum production found at 28 °C of isolate F1 and A3 but B5 shows maximum production at 37 °C temperature. Maximum enzyme activity observed at 70 °C for F1; A3 and B5 shows maximum activity at 50 °C (Figure 4 (a), (b)).

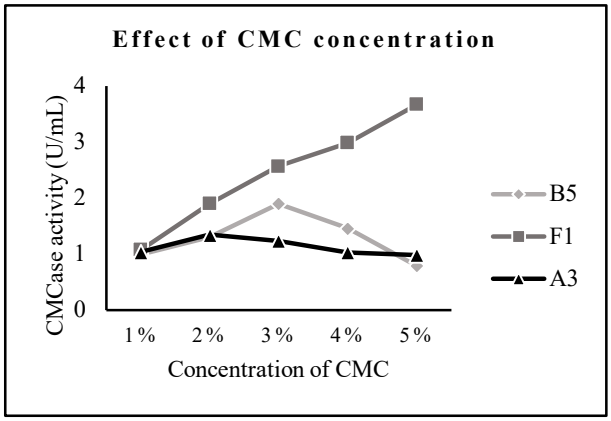


Figure 5 Effect of CMC concentration on enzyme production

EFFECT OF CMC CONCENTRATION

Figure 5 Effect of CMC concentration on enzyme production

Effect of substrate concentration is important parameter for production of enzyme. In (Figure 5) F1 shows maximum enzyme activity 3.67 U/mL in 5% CMC concentration and isolate B5 shows highest activity in media supplemented with 3% CMC concentration further A3 isolate shows higher activity in 2% CMC concentration. B5 and A3 are able to grow in higher concentration of CMC but shows minimum enzyme activity.

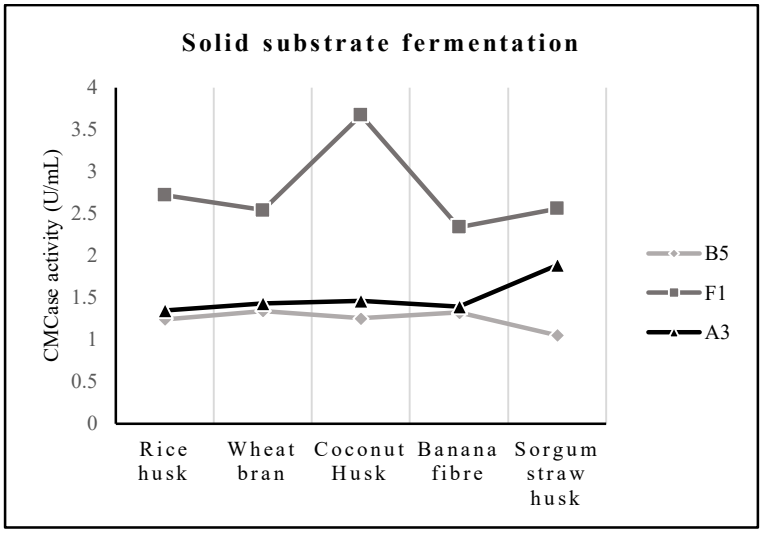


Figure 6 Solid substrate fermentation

SOLID SUBSTRATE FERMENTATION

After the pre-treatment of substrate it is supplemented with salt solution and culture was inoculated into it. Sive *et al.* [26] observed maximum activity of enzyme in wheat straw after incubation of 7 days. In present study after incubation of 7 days crude enzyme

Figure 6 Solid substrate fermentation

extracted from the flask in different substrate diverse activity have been found. With compare to B5 and A3 isolate F1 have higher capacity to utilize agricultural waste as a substrate (Figure 6)). By utilizing coconut husk it produces enzyme having highest activity 3.67 U/mL further isolate A3 observed higher activity by utilizing sorgum straw husk which is ~ 2.0 U/mL. B3 shows minimum utilization of agricultural waste as substrate.

CONCLUSION

7 cellulase-producing bacterial, 3 fungal and 2 actinomycetes isolates were obtained from collected soil samples. The isolates B5, F1 and A3 produced higher cellulase in plate assay than other isolates. Further enzyme production has been carried out and into that strain F1 produced maximum CMCase (3.67 ± 0.21 U/mL) at pH 6 and 50 °C after 84 h of incubation. Maximum FPase (3.12 ± 0.15 U/mL)

produced by F1. Further F1 also observed for better production by utilizing solid substrates. Strain F1 can be used for further studies of cellulase enzyme and their application in various industries.

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