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Production, Optimization and Purification of Cellulase enzyme from Bacteria and Fungi

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Abstract:

In this study, cellulase producing bacteria and fungi were screened on the CMC agar plate. Cellulase producer produces a clear transparent zone into the CMC agar plate. Different parameters such as carbon source, nitrogen source, time and pH are used for maximum cellulase production in the liquid medium. Then Optimized condition was obtained for bacteria pH 9 after 72 hours of incubation at 37^0C and for fungi pH 8 after 120 hours of incubation at 28^0C.Then partially purified enzyme by ammonium sulphate precipitation method and Molecular weight determination by Native – PAGE.

Keywords — **cellulose, cellulase, screening, optimization, purification**

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I. INTRODUCTION

Cellulase is an industrially important enzyme produced chiefly by fungi, bacteria, and protozoan. Cellulase was first discovered in1983. Cellulase breaks down the cellulose into monosaccharide such as beta glucose (Crawford, 1981). Cellulose is an organic compound Polysaccharides consisting of a linear chain of several hundred to many thousands of beta (1-4) linked D-glucose units. Cellulose is the primary structure of the plant cell wall of green plants and many algae contain cellulose. Cellulose mainly obtained from cotton and wood pulp of many plants for industrial use. Cellulase enzyme is mainly used in food, textile, wine and brewing, detergent, pulp, and paper industries and in agriculture and also use in the bio refinery.

Cellulose degradation is a very complex process. In this process required three steps for cellulose degradation.

(1) Endoglucanases (CMCase) (EC3.2.1.4): It randomly cleaves internal bonds at sites that create new chain ends.

(2) Exoglucanases (EC 3.2.1.91): It cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in tetrascchacrides or disaccharides.

(3) Cellobiase (EC 3.2.1.21): It hydrolyses the exocellulase product into individual monosaccharide.

Cellulases have many industrial uses so, in this study focus on a maximum cellulase enzyme production from Bacteria and fungi, by using different cultural condition.

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II. MATERIALS AND METHODS

1. Screening of cellulase enzyme producing microbes:

Cellulase enzyme producing Bacteria and fungi were streak on CMCagar media plate containing 6 gm NaCl , 1 gm $(NH_4)_2SO_4$, 0.5 gm KH_2PO_4 , 0.5 gm K_2HPO_4 , 0.1gm $MgSO_4$, 0.1 gm $CaCl_2$, 1 gm CMC , 30 gm Agar in 1000 ml distilled water(Sonia Sethi et al. 2013).The plates were incubated at 37⁰C for 72 hours for bacteria and 28⁰C for 96 to 120 hours for fungi (Ismail Shareef et al. 2015) For visualize the hydrolysis zone plates were flooded with an aqueous solution of 0.1 % Congo red for 15 min. And remove it and further plates flooded with 1MNaCl remove it after 15 min and observe clear zone surrounded colonies.

2. Conformation test for cellulolytic activity of Microbes by cellulose Congo red agar:

On CMCagar plate zone observed colonies steak on CCRA medium plates for conformation. Colonies showing discoloration of Congo red were selected as positive colonies and measure the zone of diameters only these colonies were taken for further study. Store the strain on CCRA slant at 4⁰C (Mohammed Raw way et al. 2017).

3. Identification of Microbes:

Bacteria:

Morphological Characterization: Bacterial colony characteristics were observed like size, shape, arrangement, and colour. The bacterial strain was examined by Gram staining Methods. This method distinguishes the gram-positive and gram-negative bacteria.

Biochemical Characterization: The bacterial strain was identified by several biochemical tests such as Carbohydrate fermentation test, M-R test, V-P Test, Urea hydrolysis test, Gelatine hydrolysis test, TSI agar test Starch hydrolysis test, and Catalase test.

Fungi:

Morphological Characterization: The fungi were identified by mounting method by using lacto phenol.

4. Production of cellulase enzyme:

The bacterial strain was grown for cellulase enzyme production in a medium containing (g/l) 0.5 gm Glucose, 0.75 gm Peptone, 0.01gm $FeSO_4$, 0.5 gm KH_2PO_4, 0.5gm $MgSO_4$,0.1 % Congo red (Sonia Sethi et al. 2013). The fungi were placed in a medium containing (g/l) 0.002gm $FeSO_4$, 3 gm $NaNO_3$, 1gm Na_2HPO_4 , 0.1 gm $MgSO_4$, 0.5 gm KCl supplemented with 1% CMC as carbon source(Oyeleke , S.B. et al. 2012). The bacterial strain was grown in the medium for 3 days at 37ºC and fungal species were growing in the medium for 5 to 7 days at 28 ºC, at the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min. To obtain the crude extract, this is served as an enzyme source.

5. Enzyme assay:

The culture medium was centrifuged at 5000 rpm for 15 min. To obtain the crude extract , take supernant 200µl then add 1800 µl of 0.5 % CMC in 50 Mm sodium phosphate buffer (pH: 7) , incubate it at 37 ºC for 30 min. After add 3000 µl of DNS reagent and then incubate it in boiling water bath for 5 min. Take O.D. at 575 nm (G.L. Miller et al. 1959).

6. Optimization for maximum cellulase production:

Effect of time:

Production medium incubates with positive colonies and after every 24 take-ups- culture medium and determines the enzyme activity by the above methods. Different times for bacteria 24 to 120 hr and for fungi 24 to 192 hr.

Effect of carbon sources:

The effect of various carbon sources such as dextrose and fructose at the concentration of 1% to 5% was examined in the medium.

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Effect of nitrogen sources:

The effect of various nitrogen sources such as peptone and yeast extract at the concentration of 1% to 4% was examined in the medium.

Effect of pH:

Flasks with a broth containing the optimum concentration of carbon and nitrogen sources are taken and the pH of the medium adjusted to 3.0, 4.0, 6.0, 7.0, 8.0 and 9.0 in the different flask using 1 N NaOH and 1N HCl and sterilized medium. The strain was inoculated and incubates at particular suitable temperature and time. At the end of incubation, the culture was taken and measure cellulase activity.

7. Partial Purification of cellulase enzyme:

Ammonium sulphate precipitation method:

The optimized cultural medium prepared and add positive colonies incubate it at 37ºC for 72 hours For bacteria and 28ºC for 96 to 120 hours for fungi. Then the prepared crude enzyme was brought to 90% saturation with solid ammonium sulfate. The mixture was kept overnight at 4 ºC. Then the culture medium was centrifuged at 3000 rpm for 30 min. at 4 ºC. After taking pallet and add into 2 ml of 0.02M Sodium phosphate buffer containing 1mM EDTA and 1mM 2 – mercaptoethanol. After the partially purified enzyme was added into a semi-permeable membrane and put it overnight into a phosphate buffer in a magnetic stirrer for remove salt and obtained protein. Store it in refrigerators for further use (Farjana Islam et al. 2018).

Protein estimation and molecular weight determination:

Protein estimation in the crude enzyme was estimated by using folic lowery's method with BSA as a standard and SDS –PAGE was used for molecular determination (Farjana Isalam et al. 2018).

III. RESULT AND DISCUSSION

1. Screening

After 72 hours of incubation CMC agar plate were flooded with an aqueous solution of 0.1% Congo red for 15 min. And after further plate flooded with 1N NaCl and observes clear zone surrounded colonies and take those colonies and further steak on CCRA plate for conformation and obtain four-strain hydrolyze the cellulose and produce clear zone, and also observe 1 fungal strain which produces clear zone. Zone of Diameter shown in the table: 1

Fig. 1: (A) before dye addition

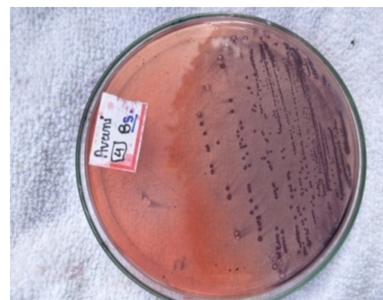

Fig. 1: (B) After dye addition.

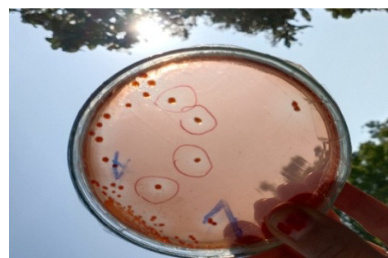

Fig. 2: Clear zone on CCRA plate.

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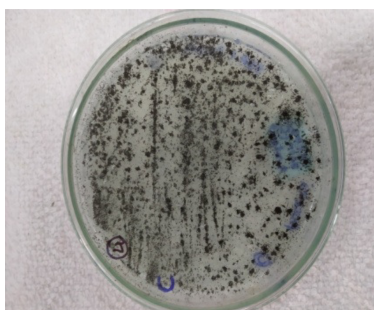
Fig. 3: (A) Fungi on CMC agar plate

Fig. 3: plate (B) Fungi on CCRA plate.

Table: 1: Zone of diameter:

Code	Mean clear zone diameter (ZD)mm	Mean colony diameter (CD)mm	H-C Value (ZD/CD) mm
P1	13	1	13
P2	3	1	3
B1	3	1	3
B2	10	1	10
F	36	22	1.63

2. Morphological characterization:

Bacterial colony characteristics were shown in table:2. Some Bacteria are gram-positive and some are gram-negative observed.

Table 2: Colony Characteristics:

	P1	P2	B1	B2
Size	Small	Small	Small	Small
Colour	White	White	White	White
Shape	Round	Round	Round	Round
Margin	Entire	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth	Smooth
Consistency	Moist	Moist	Moist	Moist
Optical characters	Opaque	Opaque	Opaque	Opaque

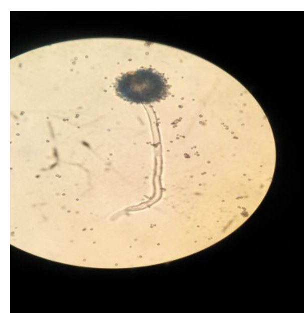
Fig. 4: Microscopic observation of fungi

3. Biochemical Characteristics:

Table: 3: Biochemical Characteristics

Sr. No.	Test	P1	P2	B1	B2
1	Catalase test	+	+	+	+
2	Indole test	-	-	-	-
3	Citrate utilization test	-	+	-	-
4	Urea hydrolysis test	-	-	-	-
5	Methyl red (M-R) test	-	+	-	-
6	Voges- Proskauer (V-P) test	-	-	-	-
7	Starch hydrolysis test	-	-	+	+
8	Gelatin hydrolysis test	+	+	+	+
9	Carbohydrate				

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	fermentation test Lactose Maltose Sucrose	+ - -	+ + +	+ - -	+ + +
10	Triple sugar iron (TSI)agar test Slant Butt H_2S Gas	Ak Ak - -	Ak Ak - -	Ak Ac - -	Ak Ac - -

4. Optimization:

Effect of time:

All the four bacterial strain and one fungal strain inoculate into a fermentation medium and after every 24 hours withdrew the sample and determine the enzyme activity was observed maximum production for bacteria after 72 hours at 37 C and for fungi after 120 hours at 28 C. Shown in fig.5 and 6 (A).

Effect of carbon source:

Various Carbon source concentrations such as 1% to 5% use and obtain a maximum enzyme activity at 3% of Dextrose and Fructose for bacteria and 4% Dextrose and 3% fructose for fungi were observed Shown in fig. 5 and fig. 6 (C) and (D).

Effect of Nitrogen source:

Various Nitrogen source concentration such as 1% to 5% use and obtain a maximum enzyme activity at 3% of Peptone and 3% Yeast extract for bacteria and 2% Peptone and 4% Yeast extract for fungi was observed. Shown in fig. 5 and 6 (E) and (F).

Effect of pH:

The optimum pH was observed 9.0 for bacteria and 8.0 for fungi shown in fig. 5 and 6 (B).

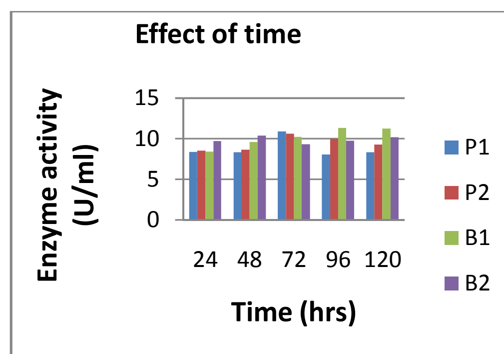

Fig. 5: Bacteria (A) Effect of time

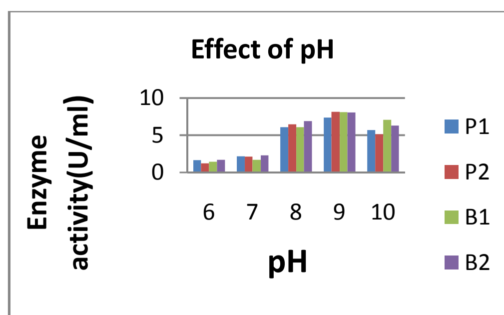

Fig. 5: Bacteria (B) Effect of pH

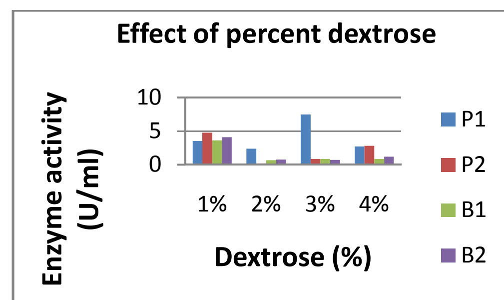

Fig. 5: Bacteria (C) Effect of Dextrose

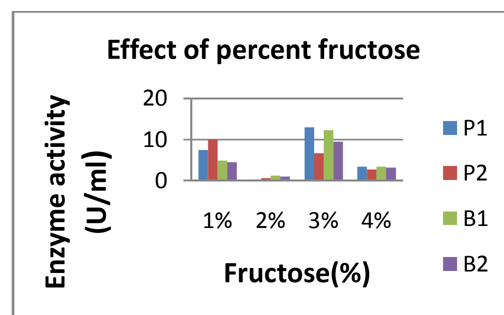

Fig. 5: Bacteria (D) Effect of fructose

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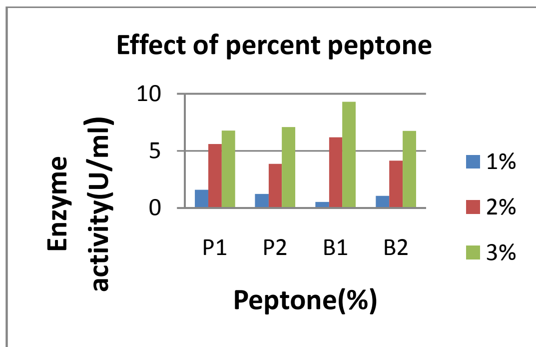

Fig. 5: Bacteria (E) Effect of peptone

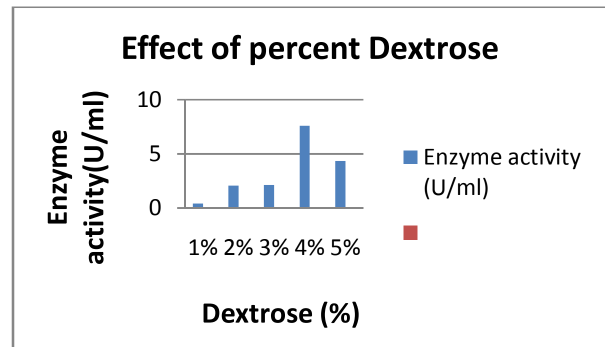

Fig. 6: Fungi: (C) Effect of Dextrose

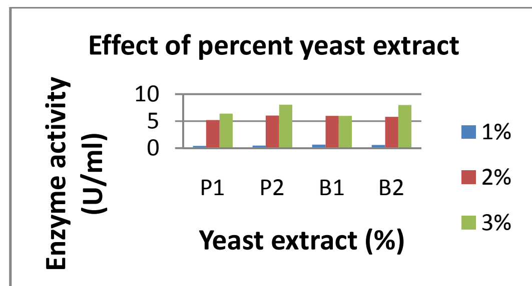

Fig. 5: Bacteria (F) Effect of yeast extract.

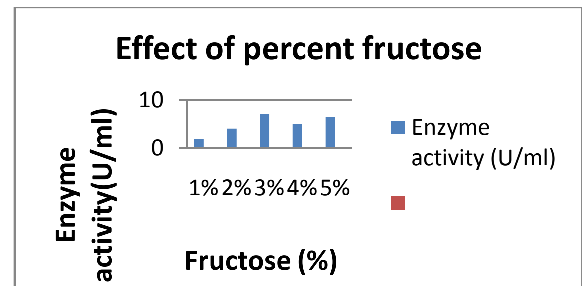

Fig. 6: Fungi: (D) Effect of fructose

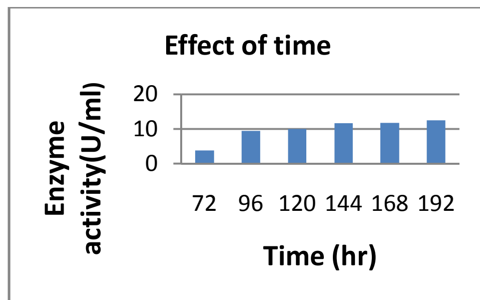

Fig. 6: Fungi: (A) Effect of time

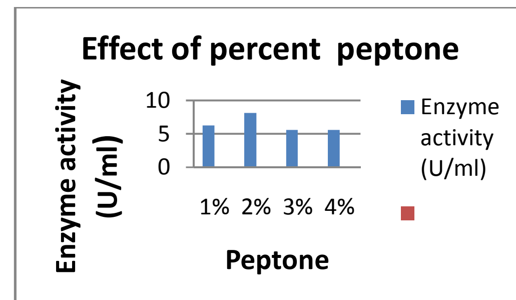

Fig. 6: Fungi: (E) Effect of peptone

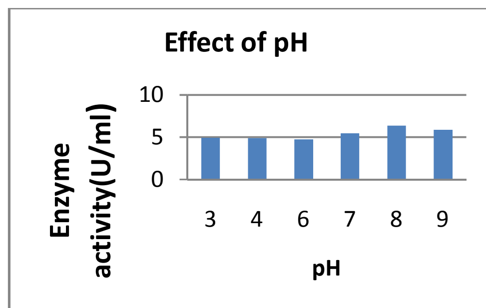

Fig. 6: Fungi: (B) Effect of pH

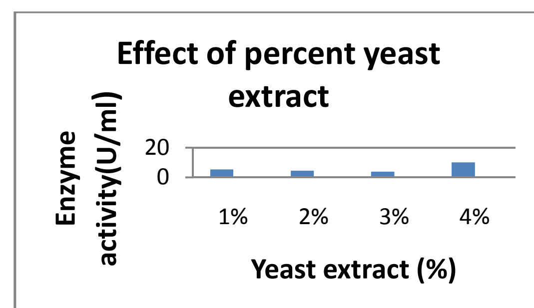

Fig. 6: Fungi: (F) Effect of yeast extract.

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5. Partial Purification:

Ammonium sulphate precipitation method:

Sown the two separate layer in fig no.7.

Protein estimation and molecular weight determination:

Band was observed by Native –PAGE analysis. Shown in fig. 8.

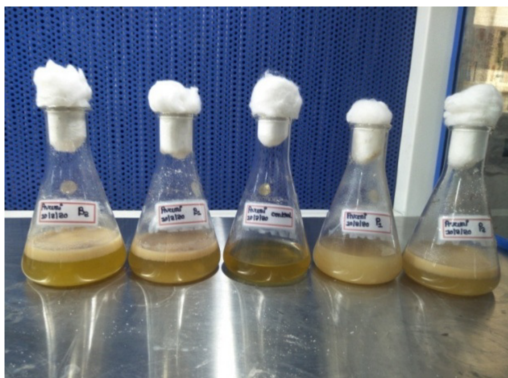

Fig. 7: Ammonium sulphate precipitation

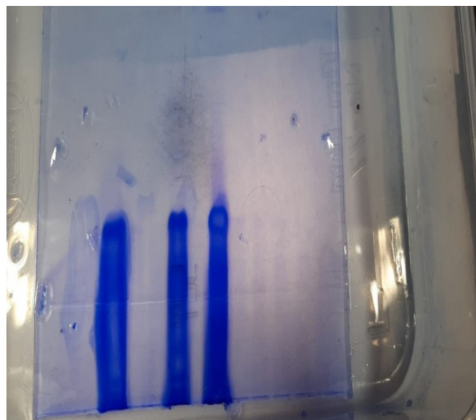

Fig. 8 Native PAGE Band of protein

IV. CONCLUSIONS

Results of this study indicate that cellulase producing bacterial and fungal strains can be grown at different optimized conditions. This isolated bacterial strain showed maximum cellulase activities pH 9, 37^0 C temperature with 3% fructose as a carbon source and 3 % peptone as a nitrogen source at 72 hours, incubation period and the fungal strain showed maximum cellulase active pH 8, 28^0 C temperature with 3% fructose as a carbon source and 4 % peptone as a nitrogen source at 120 hours incubation period. The purified cellulase enzyme is useful for several industrial applications.

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