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STUDY ON THE CELLULASE ENZYME PRODUCING ACTIVITY OF BACTERIA ISOLATED FROM MANURE WASTE AND DEGRADING SOIL

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***Abstract*— Cellulolytic bacteria were isolated from manure wastes (cow dung) and degrading soil (municipal solid waste). Nine bacterial strains were screened the cellulolytic activities. Six strains showed clear zone formation on Berg's medium. CMC (carboxyl methyl cellulose) and cellulose were used as substrates for cellulase activities. Among six strains, cd3 and mw7 were observed in quantitative measurement determined by dinitrosalicylic acid (DNS) method. Maximum enzyme producing activity showed 1.702mg/ml and 1.677mg/ml from cd3 and mw7 for 1% CMC substrate. On the other hand, it was expressed 0.563mg/ml and 0.415mg/ml for 1% cellulose substrate respectively. It was also studied for cellulase enzyme producing activity optimizing with kinetic growth parameters such as different carbon source including various concentration of cellulose, incubation time, temperature, and pH. Starch substrate showed 0.909mg/ml and 0.851mg/ml in enzyme producing activity. The optimum substrate concentration of cellulose was 0.25% for cd3 but 1% for mw7 showing the amount of reducing sugar formation 0.628mg/ml and 0.669mg/ml. The optimum incubation parameters for cd3 were 84 hours, 40°C and pH 6. Mw7 also had optimum parameters 60 hours, 40° C and pH6.**

***Index Terms*— Bacteria, Cellulase, DNS, optimum, reducing sugar**

I. INTRODUCTION

Enzymes are biological catalysts which are the most remarkable, highly specialized and energized protein molecules found in every cell and are necessary for life [1]. In addition, they are usually proteins of three-dimensional structures. They drive or accelerate chemical reactions in which they remain unchanged [2]. During the past two decades, usage of enzyme in industrial process has significantly increased[3]. Cellulase is one of the most useful enzymes in industry and a class of enzyme that catalyzes the cellulolysis i.e., hydrolysis of cellulose [4]. This is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units; this is a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides. However, this process can be significantly intensified in a proper solvent, e.g. in an ionic liquid [5]. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system [6]. Three general types of enzymes make up the cellulase enzyme complex. Endocellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulase polysaccharide chains. Exocellulase cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. Cellobiase or beta-glucosidase hydrolyses the endocellulase product into individual monosaccharides [7]. These enzymes carry out hydrolysis by the synergistic action [8]. These complex cellulases are used in preparation of the minute rice by macerozyme, pharmaceutical formulations, detergent preparation, and fashion design in textile industry/fabric modification, food processing, brewing and paper and pulp industry [9].

Cellulolytic enzymes are synthesized by a number of microorganisms. Among the microbes, bacteria are more reliable due to their adoptability in any environmental conditions, relatively very faster growth than any other microbes and extremely have the capacity to produce highly stable enzyme complement and they serve as highly potent sources of individually important enzymes [10].

For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts [11]. The optimization of the development of any fermentation process, particularly physical and chemical

parameters are of primary importance, owning to their impact on the economy and practicability of the process. Various process parameters like incubation time, temperature, pH, agitation, etc., seem to influence microbial growth and production of cellulase; thus a judicious selection of these parameters can dramatically improve the enzyme yield [12]. In my research, the cellulase producing bacteria were isolated, screened and measured in quantity by optimizing with kinetic growth parameters such as different carbon sources and various concentrations, incubation period, temperature and pH.

II. MATERIALS AND METHODS

A. *Culture Source Collection and Bacterial Isolation*

Manure wastes (cow dung) and degrading soil (municipal solid waste) samples were collected from Mandalay Technological University Campus and Mandalay Urban Area for the isolation of cellulolytic bacteria. The medium used for the isolation of cellulolytic bacteria contains CMC 1%, NaNO3 0.2%, MgSO4 0.05%, K2HPO4 0.005%, FeSO4 0.001%, CaCl2 0.002% and MnSO4 0.002%, distilled water 1L and agar 10g. The plates were incubated for 72hours at 37° C. Bacterial colonies were isolated and subcultured to obtain single strain. The purified cultures were maintained at 4° C for further analysis.

B. *Screening of Cellulase Producing Activity*

Qualitative assay using Congo red was carried out. The Cellulase Producing Activity of bacterial isolates were screened on Berg's media, where zone of clearance was observed visually by staining plates with 0.1% Congo red, taken for 15minutes, and destained with 1M NaCl. Diameter of clear zone was measured in millimeter. It was occurred in cellulase activity.

C. *Inoculum Development*

Pure culture of selected bacterial isolates were inoculated in broth medium containing CMC 1%, NaNO3 0.2%, MgSO4 0.05%, K2HPO4 0.005%, FeSO4 0.001%, CaCl2 0.002% and MnSO4 0.002% for 48 hour of fermentation period. After fermentation, 10% bacterial broth were used as inoculum source for cellulase assay.

D. *Cellulase Enzyme Production*

100ml of autoclaved producing medium CMC 1%, NaNO3 0.2%, MgSO4 0.05%, K2HPO4 0.005%, FeSO4 0.001%, CaCl2 0.002% and MnSO4 0.002% with 10% inoculum culture broth was incubated on water bath shaker at 120 rpm at 37 C for 5 day for celluase enzyme production. Bacterial cultures were harvested with 12 hours incubation time interval by centrifugation at 5000rpm for 20mins. The culture supernatants were used for the assay of extracellular enzyme.

E. *Cellulase Enzyme Assay*

The activity of cellulase was assayed using DNS method. 0.5ml of culture supernatant was mixed with 1ml of 0.05M citrate buffer (pH 4.8) solution in test tubes containing 1% cellulose substrate. The resulting reaction mixture was incubated at 50 C for 60min in a water bath shaker at 80 – 85 rpm. After reaction time, 3ml of DNS reagent were added to the reaction mixture and this mixture was boiled for exactly 5minutes to terminate the reaction in a vigorously boiling water bath. After that cool in a cold water bath, then record the absorbance which was measured by spectrophotometer at 540nm against the blank without enzyme filtrate.

F. *Effect of Incubation Time*

Different incubation times (12, 24, 36, 48, 60, 72, 84, 96, 108 hours) were employed to study their effect on the cellulase production. The culture filtrates were collected at respective time interval and assayed cellulase activity.

G. *Effect of Carbon Sources*

The Berg's medium containing 1% CMC was replaced with 1% carbon sources such as cellulose and starch. The flasks were inoculated with 10% inoculum and incubated at 37 C in water bath shaker for 5 days. The assay was carried out at 12 hours incubation time interval.

H. *Effect of pH*

To determine the optimum pH for cellulase production, the pH of the Berg's medium containing 1% cellulose was adjusted to 5, 6, 7, 8, and 9 with 1N NaOH and 1N HCl. The assay was carried out at 60 hours and 84 hours incubation time for respective isolates.

I. *Effect of Temperature*

The production of Berg's medium containing 1% cellulose was carried out at different temperature such as 30 C, 35 C and 40 C at fixed pH to study optimum temperature. The assay was carried out at 60 hours and 84 hours incubation time for respective isolates.

J. *Effect of Cellulose Concentration*

To determine the optimum cellulose concentration for cellulase production, Berg's medium with different concentration of cellulose (0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%) were prepared and incubated with inoculum culture broths at fixed pH and temperature for each culture, the sample were withdrawn at 60 hours and 84 hours for respective isolate.

III. RESULTS AND DISCUSSION

The present study is aimed to develop the bacterial strains which can produce cellulase enzyme. A totally 9 bacterial isolates were selected by the use of Berg's medium and screened for their cellulolytic activity on various

concentrations of CMC and cellulose agar plates by using Congo red indicator. It was changed the brown-red color to colorless forming clear zone around the colonies that was indicated as cellulase activity. Among 9 bacterial isolates, only six strains were able to produce cellulase producing activity. According to screening, cd3 isolate had the highest activity with a diameter of 30mm at every concentration of cellulose and CMC agar plates. It possesses the stable activity on Berg's medium testing in experimental replication. On the other hand, bacterial isolate mw7 also gave good result in clear zone (22mm). According to microscopic morphology, the two strains were different in species. So cd3 and mw7 isolates were selected for further study. In quantitative determination, Cellulase activity can be measured by determination of reducing sugar formation by DNS colorimetric method using 1% cellulose as substrate. The reducing sugar concentrations were calculated according to glucose standard curve Figure (4).

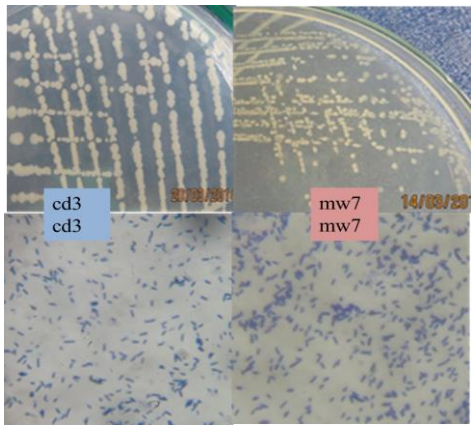

Fig 1. Colonial and Microscopic morphologies of cd3 and mw7 strains

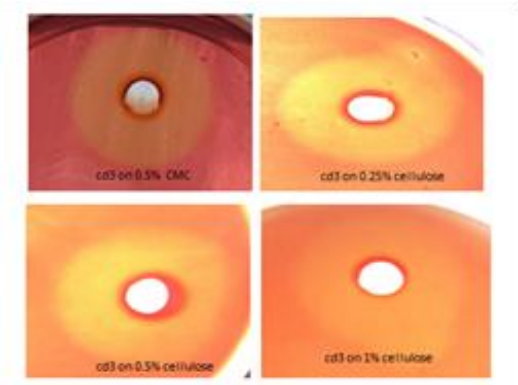

Fig 2. Screening of Cellulase enzyme activity of cd3 strain with difference carbon sources

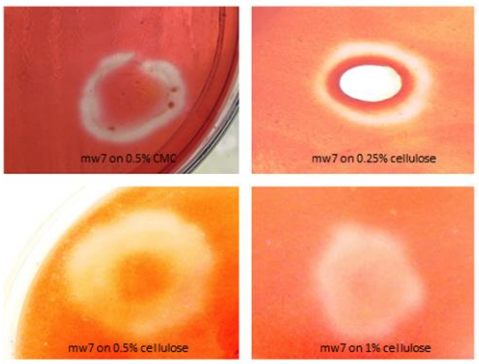

Fig 3. Screening of Cellulase enzyme activity of mw7 strain with difference carbon sources

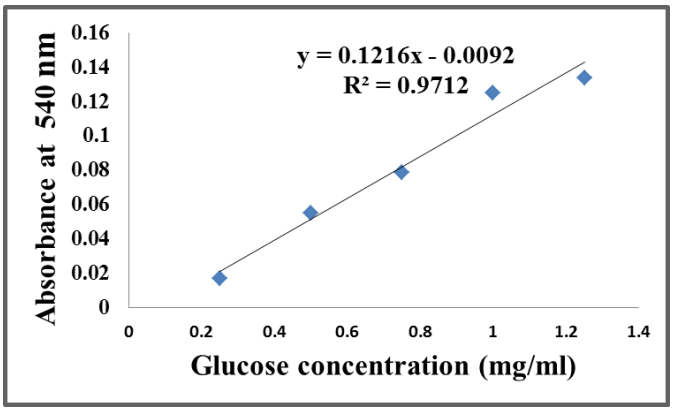

Fig 4. Standard curve for glucose at 540nm

Nutrient sources were found to be the important factor for the cellulase production. Since carbon is considered as the primary nutrient for the bacteria, carbon sources of CMC, cellulose and starch were utilized for the cellulase production.

The effect of different carbon sources (Carboxymethyl cellulose, Cellulose, Bacteriological starch) was studied for the cellulase enzyme activity. cd3 and mw7 strains were more favour CMC substrate. The cellulose substrate which was difficult to break down was selected for the studying on cellulase producing. The effect of cellulose concentration was also studied for the enzyme producing activity shown in figure 7. According to figure, the optimum cellulose concentration was 1% for both strains.

The effect of incubation time was tested by inoculation in the Berg's medium supplemented with 1% CMC, cellulose, and bacteriological starch respectively. The incubation period was 4.5 days. The optimum incubation periods for cd3 were 36 hrs, 84 hrs and 108 hrs showing in enzyme activity 1.702 mg/ml, 0.562 mg/ml and 0.909 mg/ml. On the other hands, the optimum incubation periods for mw7 were 60 hrs, 60 hrs and 108 hrs showing in enzyme activity 1.677mg/ml, 0.415 mg/ml and 0.851 mg/ml.

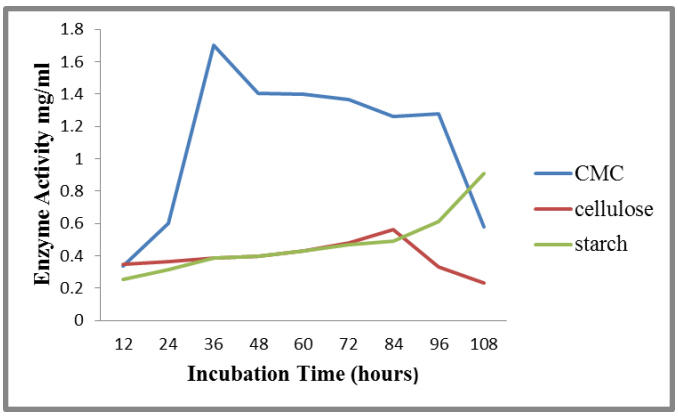

Fig 5. Effect of incubation time and carbon sources on cellulase production by cd3

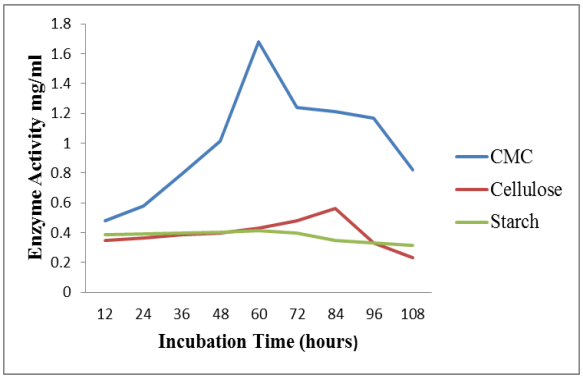

Fig 6. Effect of incubation time and carbon sources on cellulase production by mw7

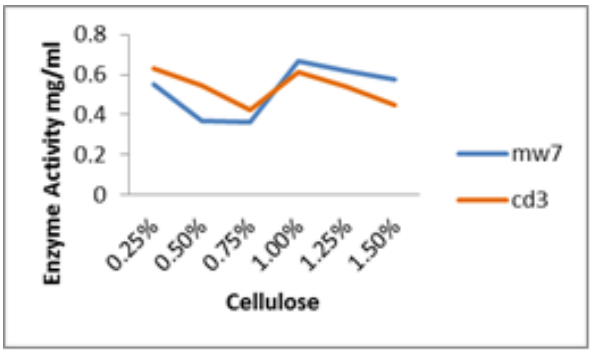

Fig 7. Effect of cellulose concentration on cellulase producing activity of cd3 and mw7 strains

The main parameters like pH and temperature are very essential parameters of the cellulase production. To optimize the optimum pH for the better cellulase production, productions was made in various pH. The higher cellulase activity was found as 0.661mg/ml at pH6 for the cellulase production of mw7 isolate. Similarly, cd3 has maximum cellulase activity as 0.719mg/ml at pH6 (figure 8). As the temperature is found to be also important environmental parameter, various temperature such as 30□ C, 35□ C and 40□C were analyzed on cellulase producing at pH6. Both isolates cd3 and mw7 showed maximum enzyme yield at 40□ as 0.694 mg/ml for mw7 and 0.851 mg/ml for cd3 (figure9).

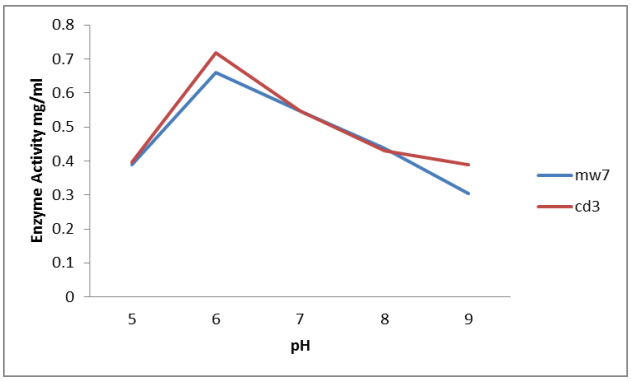

Fig 8. Effect of pH on cellulase producing activity of cd3 and mw7 strains

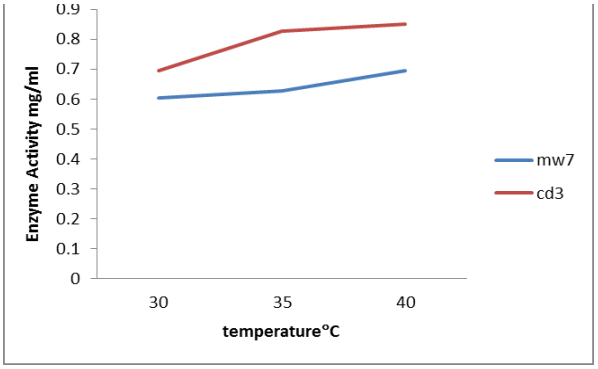

Fig 9. Effect of temperature on cellulase producing activity of cd3 and mw7 strains

IV. CONCLUSION

Four bacterial strains from manure wastes (cow dung) and five strains from degrading soil (municipal solid waste) were isolated and studied the cellulase producing activity. Six strains gave positive result to clear zone formation. Among them, cd3 and mw7 were selected for quantitative assay by DNS method. CMC was the best carbon source but cellulose substrate was used for culture optimization. The two strains showed similar pattern of optimization parameters such as 1% cellulose, pH6, temperature 40 but differed in incubation period.

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