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Effect of Inorganic Ions and pH on Cellulase Enzyme Activity in Crude Extracts Isolated from Solid Wastes Microbes

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Udupi district is a town located near Mangalore, Karnataka state. Temperature ranges from 30 to 35 degrees centigrade in daytime and is around 22 - 22° C ± 2°C during night. Humidity is normally high most of the time. The rainy season is from April to September.Solid waste samples weighing approximately 2 to 2.5 Kgs were randomly taken from Karkala and Udupi districts and then transferred into the nylon bags and brought to the laboratory for further evaluation for the incidence of bacteria.The objective of the project includes the collection of the Solid waste sample, isolation of the microflora and then the identification of these micoflora. Some of these methods are Blotter method. And to study wheather the organisums are having the capacity to produce cellulose enzyme., Cellulose is degraded by three major classes of hydrolases. These three classes have been isolated and purified from fungi. They range widely in temperature and pH optima, but the most common enzymes are most active at pH 5.5, 55°C. Three cellulases are of interest: 1) endoglucanase (carboxymethyl cellulase or CMCase), 2) cellobiohydrolase (CBH or filter paper activity) and 3) β-glucosidase. Endoglucanase (CMCase) attacks randomly in the interior of the cellulose structure. It is not very active against crystalline cellulose, but they are capable of hydrolyzing substituted cellulose such as carboxymethyl cellulose. It produces cellulodextrins also known as cellulooligosaccharides. Cellobiohydrolase (CBH) also known as exocellulase attacks crystalline cellulose from the non-reducing end and produces cellobiose. â-glucosidase hydrolyses cellobiose to glucose.

Key words: Blotter method, Cellulose, Cellobiohydrolase, Hydrolases.

"Solid waste" means any garbage, refuse, sludge from a waste treatment plant, water supply treatment plant, or air pollution control facility and other discarded material, including solid, liquid, semisolid, or contained gaseous material resulting from industrial, commercial, mining, and agricultural operations, and from community activities. Organic waste is a major component of municipal solid waste. Municipal Solid Waste (MSW) compost contains a significant amount of humic substances. (Wei.z *et al*., 2007).In India, the amount of waste per capita generated is estimated to increase at a rate of 1 – 1.33 % annually (Shekdar 1999), scenario assuming the daily per capita waste generation in 1995 as 0.456 kg , and the per capita increase in waste generation as 1.33%. The calculated value of daily per capita waste generation in 1997 is 0.468

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kg. It is evident that the total waste quantity generated in 2047 would be approximately above 260 million tones, more than five times the present level. Further a wide variety of pathogenic microorganisms have been reported to be present in these organic wastes. (Amalraj,S et al., 2006). Attempts were made to extract cellulase enzyme from solid waste microflora

MATERIAL AND METHODS

Inoculation of sample by spread plate method: The fungal samples were collected from various sources of solidwaste viz. straw, orange peel and wood source: Black color spores were seen on strawb) A grey-green growth was seen on orange peel. White colored fungal growth was seen on wood surface. The fungal samples were added to 10 ml sterile distilled water in 3 different test tubes labelled as black, grey and white for a, b, and c respectively. From each of the test tubes 0.5 ml volume was pipetted onto the PDA media on 3 separate Petri plates. The petri plates were labeled accordingly. Using L-shaped glass rod the sample was uniformly distributed. The Petri plates were incubated for 3 to 4 days in an inverted position at room temperature.

Isolation and subculture

After incubation appearance of the discrete, well separated colonies was examined, the next step was to subculture some of the cells from the colonies to separate agar plates with a sterile needle or loop for further tests and experiments. Each of these new cultures represents the growth of a single species and is called pure or stock culture.

Identification of fungi using cotton blue stains

Place a drop of distilled water on a clean slide. Transfer a small tuft of the fungus, preferably with spores and spore bearing structures into the drop using a flamed, cooled needle. Gently tease the material using the two mounting needles. Add a drop of lacto phenol cotton blue stain over the material. Gently mix the stain with the mold structures. Place a cover slip over the preparation taking care to avoid air bubbles in the slide. Examine slides under low and high power objectives of the microscope. The fungal species were identified based on their mold structures as viewed under the microscope.

Extraction of crude enzyme from fungi

We had previously weighed the empty Whatman filter papers. Subject the filter paper with its contents (fungal mycelium) for drying in a hot air oven at 105 ° C for 48 hours. Weigh the dried filter papers with its contents and note down the weight. Keep the filter paper with mycelium in the oven once again for 2 more hours and weigh it once again. If there is no difference in weight, it indicates that the mycelium is completely dry. Subtract the empty filter paper weight from the weight of the filter paper with mycelia. Express the weight of the mycelium in gm/liter of culture.

Quantitative estimation of protein by Bradford's method in the culture

This is a rapid, simple and sensitive method for estimation of proteins in sample extract. The color development is virtually complete within 2 minutes and the color is stable for about 1 hour. Unlike Lowry's method, ions such as NH_4^+, Na^+, K^+, phenols and carbohydrates such as sucrose do not interfere in this assay. This procedure is based on interaction of a dye, Coomassie Brilliant Blue, with proteins. The unbound dye has absorbance maxima at 495 nm. However, on interaction with proteins, the dye turns blue and its absorbance maxima are displaced to 595 nm. Thus from the absorbance at 595 nm the amount of protein in a sample solution can be quantitatively estimated. However, as in Lowry's procedure, detergents such as SDS, Triton X-100 etc., interfere in estimation of proteins by this method.

Filter paper activity(FPA) for total cellulose activity in crude extracts

Take 3 ml of crude cellulase in 3 test tubes Whatman No.1 filter paper strip (1×6 cm; 50 mg) was immersed in the enzyme in each of the test tubes.1 ml of buffer was added to each of the test tubes. Blank was prepared by taking 3 ml of distilled water instead of crude enzyme in a test tube and adding filter paper and 1 ml of buffer to it. Incubate the test tubes at 50 ° C for 1 hour in water bath. Remove the test tubes and add 2 ml of DNSA to each of the test tubes. Place the test tubes in water bath at 70 ° C for 10 minutes. Cool the test tubes, Set the colorimeter to zero using blank. Measure the OD at 540nm.one unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1µmole of reducing sugar from filter paper per ml per minute

RESULTS AND DISCUSSION

Cellulose is the most abundant organic source of food, fuel and chemicals. However, its usefulness is dependent on its depolymerisation to fermentable sugar using microbial system. Many fungi secrete cellulase that exhibit the ability to degrade cellulose. When the fungi *Aspergillus*, *Fusarium* and *Penicillium* were grown on a medium containing cellulose (E.g. CMC, filter paper, etc.) , they were capable of producing cellulase enzyme. The crude cellulase extract was isolated from these fungi and a study on the biochemical characteristics of the enzyme was conducted. Cellulase has large scale industrial and environmental applications and thus the fungal sources can be tapped for large scale production of the enzyme. The production of fungal cellulase was found to be dependent on a number of factors viz. substrate, substrate concentration, temperature, pH, incubation period, inorganic ions etc. and varies from specie to specie. Large scale production of cellulase for commercial purposes requires for identification of high yielding fungal sources and optimization of process conditions. In recent years, high cellulase yielding mutant and recombinant varieties have also been developed.

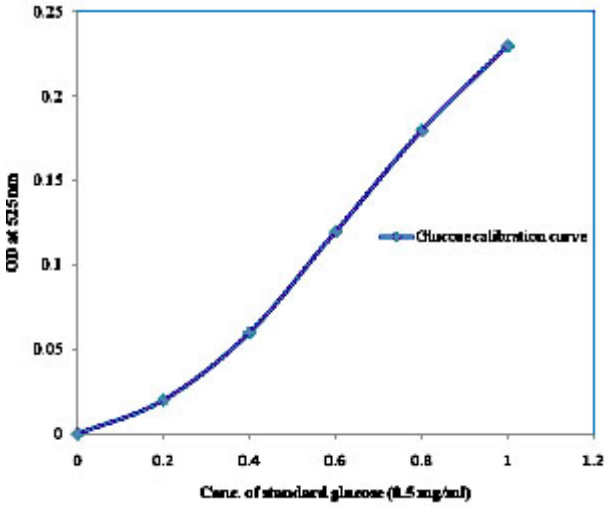

Fig. 1. Glucose calibration curve

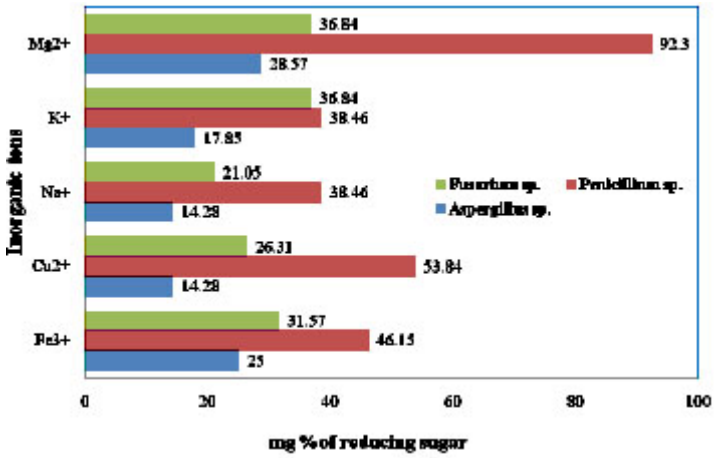

Fig. 2. Effect of inorganic ions at 5mM concentration on cellulase activity in crude extract

Present study was aimed at identifying the highest yielding genus among the three test organisms used and also determining the optimum conditions required for maximum cellulase activity.

Reducing sugar (glucose) in the culture broth was determined by dinitrosalicylic acid (DNS) method with glucose as the standard. It is simple, sensitive and adaptable during handling of a large number of samples at a time. The highest amount of glucose was produced in the culture broth of *Aspergillus* while least concentration of glucose was present in the culture broth of *Penicillium*. *Aspergillus* showed maximum cellulase activity, followed by *Fusarium* and *Penicillium* respectively. Filter paper activity (FPA) for total cellulose activity in the cultural filtrate was determined according to the method of Mandels *et al.* Filter paper activity is a combined assay for Endoglucanase and Exoglucanase cellulase. Filter paper was chosen as a typical substrate for cellulase activity determination since it has both crystalline and amorphous fractions, moderate susceptibility and availability as a convenient and reproducible substrate. Here again the reducing sugar released was estimated by dinitrosalicylic method. Again, cellulase activity was determined for each of the fungal species and the same trend was observed as indicated previously by glucose estimation. Dahot and Noomrio. Determined the reducing sugars in culture broth of *Aspergillus fumigatus* by DNSA method and CM- cellulase activity was found to be 0.027µmol/ml broth for 24 hr incubation period.

The protein contents of the three culture broths were determined by Bradford's method with bovine serum albumin (BSA) as standard. Highest protein .concentration (0.1217 mg/ml) was seen in the culture broth of *Aspergillus*, followed by *Fusarium* (0.413 mg/ml) and *Penicillium* (0.0347 mg/ml) respectively. Dahot and Noomrio [13] determined the protein content in culture broth of *A. Fumigatus* by Lowry's method as 0.50mg/ml for

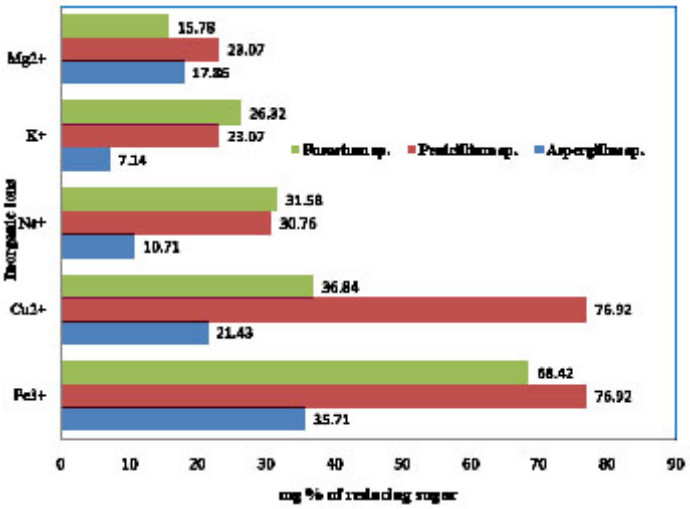

Fig. 3. Effect of inorganic ions at 10 mM concentration on cellulase activity in crude extract

24 hr incubation period.In case of pH, it was observed that the enzyme activity has a broad pH range between 3.0 and 9.0.Here, in the present study, the pH activity profile for the cellulase obtained from *Aspergillus, Fusarium and Penicillium* showed a sharp optimum in the range pH 3-4.The optimum pH for the cellulase from *A.niger* was found out to be 3.6 while the cellulase obtained from *Fusarium* and *Penicillium* showed maximum activity at pH 3.4. But pH requirement was determined at pH 5.0 in fungal species *Trichoderma harizianum, Fusarium avenaceum*. The cellulase from *A. Niger* reported by Ikeda et al. is characterized by a pH optimum at pH 2.5.

Glucose and protein estimation IN crude extracts
Calculations

Concentration of unknown = (OD of the unknown/OD of the standard) × concentration of standard

For,

Aspergillus sp.

Concentration of unknown sugar	= (0.28/0.23)×0.5
	= 0.6086 mg/ ml glucose
	= 608.6 µg/ml glucose
	= 121.72 µg/ml/min
Activity (mol. wt of glucose)	= (121.72 µg/ml/min)/
	= 0.676µmol/ml/min
Concentration of unknown protein	= (0.28/0.23)×0.1
	= 0.1217 mg/ml
Specific activity of	= (0.676l/ml/min) / (0.1217 mg/ml)
Aspergillus sp	= 5.55 µmol/mg of protein

Similarly,

Scale

X-axis: 1 cm= 0.2 mg/ml

Y- axis: 1 cm =0.05 OD

Effect of 5mM solutions of inorganic ions on enzyme activity

It is seen that the activity is enhanced by the addition of inorganic ions for *Penicillium sp.* and the best enhancer being Mg^{2+} ion. There is moderate increase in cellulase activity of *Fusarium sp*. on the addition of Mg^{2+} and K^{+} ions. The activity of *Aspergillus niger* is inhibited on the addition of inorganic ions and its activity is inhibited the most by Na^{+} and Cu^{2+}.

Effect of 10mM solutions of inorganic ions on enzyme activity

The cellulase activity is enhanced for *Penicillium sp.*and Cu^{2+} and Fe^{3+} ions are the best enhancers. There is moderate increase in cellulase activity of Fusarium sp. and the best enhancer is Fe^{3+} ion. The cellulase activity is inhibited for *Aspergillus sp.* The best inhibitor is K^{+} ion.

Effect of inorganic ions at 10 mM concentration on cellulase activity in crude extract

The effect of inorganic ions (Fe^{3+}, Cu^{2+}, Na^{+}, K^{+} and Mg^{2+}) on fungal cellulase activity was studied. Cellulase assays performed using 10mM concentration of salts of these ions showed that inorganic ions inhibit cellulase produced by *A.niger*, the maximum inhibition being caused by K^{+} ions. Though Fe^{3+} ions also inhibit the cellulase produced by *A.niger*, it was found to be a poor inhibitor compared to the other ions. On the other hand, these inorganic ions were found to enhance the activity of cellulases obtained from *Fusarium* and *Penicillium.*

The enhancement in cellulase activity was more in case of *Penicillium* than *Fusarium.* Of all the ions, Fe^{3+} ions are the best enhancers of fungal cellulase from both the species. Cu^{2+} ions are equally good enhancers of cellulase obtained from *Penicillium*, but show less enhancement in enzymatic activity for cellulase obtained from *Fusarium* as compared to Fe^{3+} ions. K^{+} and Mg^{2+} ions also enhance the cellulase activity for these two fungal species but they are poor enhancers.

When 5mM concentration of inorganic ions were used in cellulase assay it was observed that though all these ions inhibit cellulase from *A.niger*, $Cu^{2+}$and Na^{+} ions are the best inhibitors while Mg^{2+} ions are poor inhibitors of cellulase from this fungus. K^{+}, Fe^{3+} and Mg^{2+} ions enhance the cellulase activity in *Fusarium*, but only moderately. The activity of cellulase derived from *Penicillium* is highly enhanced by these metal ions at 5mM concentration, Mg^{2+} ions being the best enhancers followed by Fe^{3+} and Cu^{2+} ions respectively.

In case of pH, it was observed that the enzyme activity has a broad pH range between 3.0 and 9.0Here, in the present study, the pH activity profile for the cellulase obtained from *Aspergillus, Fusarium and Penicillium* showed a sharp optimum in the range pH 3-4.The optimum pH for the cellulase from *A.niger* was found out to be 3.6 while the cellulase obtained from *Fusarium* and *Penicillium* showed maximum activity at pH 3.4. But pH requirement was determined at pH 5.0 in fungal species *Trichoderma harizianum* , *Fusarium avenaceum* . The cellulase from *A. Niger* reported by Ikeda *et al.*,[24] is characterized by a pH optimum at pH 2.5.

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