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Research Article

Productionof Cellulolytic Enzymes by *Trichodermalignorum* immobilized in Polyurethane foam.

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ABSTRACT

Cellulase is an enzyme produced chiefly by cellulolytic fungi that are catalyzing the decomposition of cellulose to produce simple glucose units. Immobilized cells employed in various studies have several advantages over free cells.The production of cellulolytic enzymes by *Trichoderma lignorum*on carboxyl methyl cellulose nitrate medium and cotton agro waste medium by free and immobilized in polyurethane foam was studies. Effect of temperature and P^H concentration on the production of cellulase on both medium by free and immobilized cells were also study. The immobilized cells produced more enzymes as compared to free cells.

Key words: *Trichoderma lignorum*, cellulase enzyme, agro waste, immobilized cells, polyurethane foam.

INTRODUCTION

India is an agrarian country where agriculture generates abundant of agricultural waste which remains unutilized or improperly disposed caused environmental pollution. If this agricultural waste can convert to simple sugar,glucose[1] the problem of food energy can be overcame. The increased interest in the field of bioconversion of organic compound has lead to development of new method. This is in line with the findings of M.A.Milala[2]This has been profoundly influenced the studies in immobilization of microbial cells.[3] The bioconversion of higher carbohydrates like starch,cellulose etc.polymers of glucose can yield abundance of glucose from agro waste biomass with aim to efficiently manage and utilized the generated agro waste in the so called backward region of Maharashtra Marathwada, the problem of food and energycan be solved. Selecting most widely cultivated crop of this region generating large quantity of agro waste cotton. The work was undertaken to utilize the waste efficiently.Cellulase are synthesized by cellulolytic fungi.[4,5]Isolate the cellulolytic fungus *Trichoderma lignorum* from the local soil and employing them for the production of cellulose in free as well as immobilized state. This study was carried out on CMC as well as agro waste. Higher amount of enzyme was synthesized on agro waste medium by *Trichoderma lignorum.* Temperature and P^H affected synthesis of enzyme in both state.

MATERIALS AND METHODS

Isolation of fungi from soil

The soil samples were collected from fields under cultivation of Cotton.Soil samples from five

different locations in a field were collected to make a composite sample for isolation of cellulolytic fungi.

Dilution plate method was employed for isolation of fungi from soil. The most suitable dilution that gave measurable number of colonies per petriplate was found to be 1:1000. One ml of soil suspension from this last dilution was transferred aseptically to petriplates in triplicates and melted CMCN agar medium at 45˚c was poured over it. The suspension was thoroughly mixed with the above media by rotating the petriplates and then allowed to solidify. Petriplates were incubated at room temperature for 5 days in inverted position. Daily observations were recorded for appearance of colonies and number of colonies developed. When a new colony appeared, it was transferred to PDA slants for further studies. The fast growing and dominant fungus from each soil sample was selected for further studies in the production of cellulase.

Identification

After isolation from dilution plates pure cultures of individual fungi were obtained, each fungus was grown separately on CMCN medium and its growth and sporulation were recorded. Fungus was identified by colony characteristics as well as vegetative and reproductive structure as observed under microscope.

Media based on agricultural waste

When agricultural waste based media were used for production of cellulolytic enzymes, CMC in CMC Nitrate medium was replaced by wastes such as Cotton stalk and Pigeon pea stalk. The straws/stalk freshly collected from fields following harvest were ground to powder using 40 mesh pulverizer and sieving through specially designed sieves (120µ). The powder so obtained was used as carbon source. All these powders were treated with appropriate quantities (1:10v/v) 4% NaOH for 3 hours at 100˚c to delignify the material. The powders were used after freeing them from alkali by repeated washings. All above media were sterilized in autoclave at 15-lbs/sq. inch pressure for 20 minutes.

Immobilization in Polyurethane foams

For the natural attachment method using of polyurethane foam cubes as the carrier for fungal immobilization. Polyurethane foam had macropores larger than hundreds of micron. 5 mm of cubes of polyurethane foam were cut with sharp blade from the bulk. Cubes such obtained were transferred to flask containing the GN medium. The flask along with the cubes was autoclaved at 15lbs for 20 min. spore suspension was used to inoculate. The foam cubes and incubated at 27±2ºC. The cubes after regular time interval were observed for the growth of mycelium in pores these cubes were then thoroughly washed with sterilized distilled water and transferred to enzyme production medium.

Bioconversion studies

For production of cellulase 75 ml of CMC or agrowaste based medium was poured to 50ml flask. The flask was sterilized 15lbs for 20 min. The flasks were inoculated with spore suspension containing 1 X 10^5 spores per ml (for free cell studies) or with the stated amount of immobilized units in matrix (for immobilized cells studies) the flask were incubated at a room temperature and (the flask were shaken intermittently) after regular time interval samples were drawn and assessed for enzyme activity.

Effect of pH on enzyme synthesis

The pH of substrate and the enzyme preparation were adjusted using acetate or phosphate buffers to the desired pH. The enzyme activity was assessed according as stated.

Effect of temperature on enzymes synthesis.

The reaction mixture containing substrate enzyme preparation and buffer were incubated at various temperatures as desired in serological

water bath. The enzyme activity was assessed according as stated.

Enzyme assays

The fungi which were isolated in the course of this study were assayed for FPase, CMCase and β-glucosidase. Assay for Cellulases was done following method of Berghem and Petterson (1973) with slight modifications.

***For FPase enzyme*:** 100 mg of Filter paper dust was suspended in 1 ml of 0.01 M Sodium acetate buffer of pH 4.8 was incubated with 2 ml of crude enzyme solution.

***For CMCase enzyme*:** 2ml of crude enzyme solution was mixed with 4 ml of CMC and 1ml of the 0.01 M Sodium acetate buffer of pH 4.8 and were incubated at 27±2 °C. Aliquots were drawn from the mixture at regular time interval and the release of glucose due to the enzyme activity was assayed by 3 5 Dinitrosalicylic acid method (Miller, 1959) using D glucose as standard.

***For β glucosidase*:** Activity of β glycosidase was assayed by the method of Eberhart *et al* (1963) using p-nitro β glucoside as substrate. The reaction mixture consist of 50 mg of p-nitro β glucoside in 2 ml of 0.01 M acetate buffer at 4.8 pH and 1 ml crude enzyme preparation incubated at 27±2 °C. At regular time intervals aliquot were drawn and added with 0.1 N NaOH and the release of p-nitro phenol was from substrate was estimated for absorbance at 420 nm in a spectrophotometer.

Soluble reducing sugars (equivalent to glucose) were released from filter paper was estimated and the Cellulase activity was expressed in enzyme units. Enzyme activity is expressed in Units (U). 1 enzyme unit is equivalent to amount of enzyme required to release 1 µ mole of D glucose (for FPase and CMCase) or P nitro phenol (For β-glucosidase) per minute from respective substrate.

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RESULT AND DISCUSSION

The enzymes produced by free and immobilized cells of *Trichoderma lignorum* in polyurethane foam varied. The enzymes produced by immobilized cells were higher than free cells in CMC medium. The maximum enzymes were produced on 8th day where immobilized enzymes prouced 0.10 U/ml Fpase 0.29 U/ml. CMCase and 0.22 U/ml β-glucosidase where free cells prouduced 0.12 U/ml Fpase, 0.28 U/ml CMCase and 0.15 U/ml β-glucosidase (Table no.1).

Similarly the enzymes produced by immobilized cells were higher than free cells in cotton agro waste medium. The maximum enzymes were produce on 9th day where immobilized were produce 0.19 U/ml Fpase, 0.35 U/ml CMCase and 0.12 U/ml β-glucosidase, where free cell produced 0.16 U/ml Fpase, 0.33 U/ml CMCase and 0.13 U/ml β-glucosidase (Table no.2).

The effect of pH and temperature on production of cellulases was studied. It was found that *Trichoderma lignorum* produced maximum enzymes at 5.5 pH in free as well as immobilized cells (Table no.3).

Similarly the effect of temperature on production of cellulases and was also studied. It was found that maximum enzymes was produced at 40°C in free as well as in immobilized cells (Table no. 4).

Table 1: Production of cellulaseson carboxy methyl cellulose nitrate medium free and immobilized cell

Age of culture filtrate (Days)	Cellulase activity (U / ml)					
	Free cell			Immobilized cell		
	Fpase	CMCase	β-Glycosidase	Fpase	CMCase	β-Glycosidase
01	0.00	0.00	0.00	0.00	0.00	0.00
02	0.00	0.01	0.00	0.01	0.01	0.00
03	0.00	0.03	0.01	0.01	0.04	0.02
04	0.01	0.07	0.08	0.03	0.08	0.10
05	0.03	0.09	0.10	0.05	0.11	0.12
06	0.04	0.17	0.11	0.07	0.20	0.16
07	0.09	0.21	0.12	0.07	0.24	0.19
08	0.12	0.28	0.15	0.10	0.29	0.22
09	0.11	0.26	0.09	0.13	0.28	0.21
10	0.08	0.24	0.10	0.08	0.26	0.12

Table 2: Production of cellulases on Cotton agro waste medium by free and immobilized cells

Age of culture filtrate (Days)	Cellulase activity (U / ml)					
	Free cell			Immobilized cell		
	Fpase	CMCase	β-Glycosidase	Fpase	CMCase	β-Glycosidase
01	0.00	0.00	0.00	0.00	0.00	0.00
02	0.00	0.00	0.01	0.00	0.00	0.01
03	0.01	0.02	0.02	0.02	0.02	0.02
04	0.03	0.05	0.03	0.04	0.06	0.05
05	0.06	0.09	0.08	0.07	0.11	0.10
06	0.08	0.14	0.11	0.09	0.17	0.14
07	0.13	0.20	0.15	0.14	0.22	0.20
08	0.15	0.27	0.16	0.18	0.29	0.25
09	0.16	0.33	0.13	0.19	0.35	0.12
10	0.15	0.32	0.11	0.17	0.15	0.18

Table 3: Effect of pH on production cellulases enzyme on cotton agro waste based medium

pH of culture filtrate	Cellulase activity (U / ml)					
	Free cell			Immobilized cell		
	Fpase	CMCase	β-Glycosidase	Fpase	CMCase	β-Glycosidase
3.0	0.09	0.20	0.07	0.09	0.21	0.08
3.5	0.11	0.27	0.11	0.13	0.29	0.33
4.0	0.13	0.30	0.12	0.15	0.33	0.36
4.5	0.13	0.31	0.13	0.16	0.35	0.40
5.0	0.14	0.36	0.13	0.18	0.40	0.44
5.5	0.18	0.39	0.15	0.21	0.44	0.49
6.0	0.16	0.40	0.13	0.20	0.49	0.54
6.5	0.13	0.37	0.14	0.17	0.42	0.46
7.0	0.11	0.22	0.11	0.14	0.24	0.28
7.5	0.07	0.18	0.07	0.09	0.18	0.20
8.0	0.05	0.10	0.04	0.07	0.12	0.14
8.5	0.04	0.03	0.02	0.03	0.05	0.08
9.0	0.02	0.01	0.01	0.02	0.02	0.04
9.5	0.00	0.00	0.00	0.01	0.00	0.01
10.0	0.00	0.00	0.00	0.00	0.00	0.00
10.5	0.00	0.00	0.00	0.00	0.00	0.00

Table 4: Effect of temperature on production cellulases enzymes on cotton agro waste based medium

Temp. (°C)	Cellulase activity (U / ml)					
	Free cell			Immobilized cell		
	Fpase	CMCase	β-Glycosidase	Fpase	CMCase	β-Glycosidase
20	0.12	0.19	0.10	0.15	0.21	0.12
25	0.13	0.26	0.11	0.18	0.29	0.13
30	0.17	0.29	0.13	0.20	0.33	0.15
35	0.22	0.39	0.15	0.24	0.42	0.17
40	0.25	0.43	0.17	0.28	0.48	0.18
45	0.19	0.32	0.14	0.21	0.38	0.16
50	0.16	0.22	0.10	0.18	0.27	0.12
55	0.12	0.10	0.07	0.15	0.19	0.10
60	0.10	0.08	0.05	0.13	0.16	0.07
65	0.05	0.03	0.02	0.07	0.13	0.05

A little difference in enzyme production in CMC as well as cotton agro waste based medium by free cell but marked and enhanced production was observed with immobilized cells. The effect of PH and temperature cellulose production was recorded. The synthesis was favored by PH 6-6.5 in free and immobilized state. The maximum cellulose was released at 40-45^{0}C.

CONCLUSION:

Agro waste in the form of cellulose which is the most abundant renewable biomass in the biosphere has shownto be used in the production of valuable products by fungi.

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