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PRODUCTION OF CELLULASES BY A NEW ISOLATE OF *TRICHODERMA PSEUDOKONINGII* ON SLUDGE

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The production of cellulase by *Trichoderma pseudokoningii* Rifai on sludge was studied. The optimum conditions of pH and temperature were found to be 5.5 and 35°C respectively. The maximum enzyme yield (0.66 mg/ml) was obtained when 3% concentration (w/v) of sludge was used.

Lignocellulosic substrates provide energy in the form of glucose through their hydrolysis by microbial cellulolytic enzymes. A serious disadvantage to the use of most lignocellulosic material as substrates for enzyme production or saccharification to component carbohydrates is the need for costly pretreatment (Royer & Nakas, 1983). Waste sludges from pulp and paper mills represent a potential source of lignocellulosic material which has been rendered accessible to enzymatic attack by pulping process (Pamment *et al.*, 1979). The study of cellulase enzyme complex, which essentially comprised of three main components, has attracted much attention because of the economic feasibility of a hydrolysis process which can provide the optimal use of the photosynthetic products i.e. cellulose (Bagga & Sandhu, 1987).

In the present investigations, sludge from straw product factory, Bhopal was used as a potential substrate for cellulase enzyme production by *Trichoderma pseudokoningii*, a new isolate from sludge. As cellulase enzymes being extracellular in nature and directly affected by physical factors it is therefore aimed to investigate the effect of temperature and pH on the cellulase production.

MATERIALS AND METHODS

The waste sludge was collected from straw product factory, Bhopal. The strain of *Trichoderma pseudokoningii* Rifai was isolated from sludge using dilution plate technique (Warcup, 1950) and identified from CMI, England as *Trichoderma pseudokoningii* IMI 320933. The stock culture was maintained on Potato Dextrose Agar (PDA) slants and the enrichment culturing was done in basal medium devised by Mandels & Sternberg (1976) which contained 1% (w/v) sludge or Microcrystalline Cellulose Powder (MCCP) as cellulosic substrate.

Effect of pH and temperature on cellulase production: The basal medium was buffered by citrate phosphate buffer (0.05 M) and was supplemented with 1% (w/v) MCCP or sludge. To see the effect of pH the medium was adjusted at different pH levels ranging from 3.5 to 8.5. The inoculated media were then incubated at 35±1°C in stationary phase for 8 days. Similarly to see the effect of temperature on cellulase production the basal medium was adjusted at predetermined optimum pH (pH 5.5) and after inoculation the medium was incubated at 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C temperatures for the period of 8 days.

Effect of substrate concentrations: To assess the effect of substrate concentrations, the basal medium was supplemented with different concentrations of sludge ranging from 0.5% to 3% and was adjusted at pH 5.5 and incubated at 35°C. The cellulase activity was measured in the culture filterated obtained after 8 days of incubation.

Enzyme assays: Endo-B-glucanase (EC 3.2.1.4) and Exo-B-glucanase (EC 3.2.1.91) were assayed according to the method of Mandels *et al.* (1976). The reducing sugar was estimated by DNS method (Miller, 1959), the B-glucosidase (EC 3.2.1.91) was assayed by the method described by Herr (1979). The extr-
soluble protein was estimate-
method of Lowry *et al.* (1951)

All enzyme activities w(
reducing sugar released ml^{-1} o.

RESULTS

Effect of pH and Temperature: Trichoderma pseudokoningii produced high activity cellulase on sludge than that of MCCP. The exo-ß-glucanase and endo-ß-glucanase activities were maximum at pH 5.5

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Table 1: Effect of pH of the medium on cellulase activity of (reducing sugar released, mg ml^{-1}) *Trichoderma pseudokoningii* . x

Substrate	Enzyme	pH					
		3.5	4.5	5.5	6.5	7.5	8.5
Sludge	Exo-β-glucanase	0.11	0.26	0.42	0.34	0.25	0.23
	Endo-β-glucanase	0.06	0.08	0.18	0.12	0.09	0.07
	β-glucosidase	0.03	0.035	0.04	0.052	0.024	0.014
MCCP	Exo-β-glucanase	0.06	0.14	0.22	0.16	0.11	0.08
	Endo-β-glucanase	0.03	0.055	0.13	0.07	0.055	0.04
	β-glucosidase	0.015	0.025	0.03	0.04	0.035	0.01

Table 2: Effect of temperature on cellulase activity (reducing sugar released, mg ml^{-1}) of *Trichoderma pseudokoningii*.

Substrate	Enzyme	20	25	30	35	40	45	50	55
Sludge	Exo-β-Glucanase	0.16	0.28	0.35	0.45	0.30	0.22	0.11	0.07
	Endo-β-glucanase	0.09	0.13	0.16	0.20	0.12	0.08	0.06	0.045
	β-glucosidase	0.009	0.018	0.025	0.055	0.03	0.025	0.02	0.015
MCCP	Exo-β-Glucanase	0.07	0.12	0.22	0.16	0.13	0.09	0.06	0.03
	Endo-β-glucanase	0.04	0.075	0.12	0.14	0.11	0.08	0.05	0.02
	β-glucosidase	0.015	0.025	0.03	0.05	0.04	0.035	0.025	0.02

and temperature 35°C on both the substrates. On sludge, the exo-ß-glucanase and endo-ß-glucanase were 2 fold and 1.4 fold more in comparison to MCCP. The ß-glucosidase activity was maximum at pH 6.5 and its activity on sludge was 1.3 times high in comparison to that of MCCP (Table 1). The extracellular protein was recorded maximum at pH 5.5 on sludge (0.45 mg/ml) and at pH 4.5 on MCCP (0.24 mg/ml).

The effect of temperature on cellulase production varied. The maximum exo-ß-glucanase activity was obtained at 35°C on sludge (0.45 mg/ml) and at 30°C on MCCP (0.22 mg/ml) but the endo-ß-glucanase and ß-glucosidase activities were maximum on both the substrates at 35°C (Table 2). The extracellular soluble protein production was maximum at 35°C on MCCP and sludge but the amount of protein produced on sludge was 2-2 times more than that on MCCP (Table 3 and 4).

Effect of different concentrations of sludge : The results of the effect of different concentrations of sludge on cellulase production showed that the cellulase activity was very low at 0.5% concentration but with the increase in sludge concentration up to 3% the cellulase activity gradually increased. The exo-ß-glucanase activity (0.45 mg/ml) and endo-ß-glucanase) activity (0.22 mg/ml) obtained at 1% concentration of sludge were found to increase by 1.4 and 2 folds respectively at 3% concentration of sludge. The maximum β-glucosidase activity was recorded at 2.5% concentration of sludge which was nearly 3 fold higher in comparison to that obtained

Table 3: Effect of pH of the medium on protein production of *T. pseudodoningii* using sludge and MCCP.

pH	Extracellular Protein (mg/ml)	
	Substrate-Sludge	Substrate-MCCP
3.5	0.08	0.19
4.5	0.21	0.24
5.5	0.45	0.21
6.5	0.40	0.17
7.5	0.19	0.11
8.5	0.15	0.8

at 1% concentration. The maximum amount of soluble protein (0.69 mg/ml) was recorded at 3% concentration which showed a decline phase with further increase in the concentration of sludge.

Table 4: Effect of different temperatures on protein production of *T. pseudokoningii* using sludge and MCCP.

Temperature °C	Extracellular Protein (mg/ml) Substrate-Sludge	Substrate-MCCP
20	0.24	0.10
25	0.33	0.12
30	0.40	0.18
35	0.48	0.22
40	0.35	0.16
45	0.26	0.12
50	0.19	0.09
55	0.11	0.07

The relationship between enzyme and substrate concentration followed Michelis-Menten type of enzyme kinetics with a saturating concentration of 30 mg/ml for exo-ß-glucanase and endo-ß-glucanase activities and 25 mg/ml for ß-glucosidase activity. A Lineweaver-Burk plot of data revealed apparent Km values of 22 mg/ml for exo-ß-glucanase and endo-ß-glucanase activities and 15 mg/ml for ß-glucosidase activity. The Vmax values were 1 mg/ml, 0.58 mg/ml and 0.52 mg/ml for exo-ß-glucanase, endo-ß-glucanase and ß-glucosidase activities respectively (Fig.1).

DISCUSSION

Trichoderma pseudokoningii Rifai isolated from sludge produced higher cellulase on sludge in comparison to MCCP which was mainly due to structural difference between the two substrates. The higher crystallinity of MCCP (76%) as evidenced by theX-ray diffraction method affected the cellulase production due to its resistance while on the other hand the lesser crystallinity index of sludge (69%) provided more amorphous regions accessible for enzyme production by the test fungus.

On comparing the results obtained at different levels of pH and temparature, it was observed that pH 5.5 and temperature 35°C were optimum conditions for maximum cellulase production by *Trichoderma pseudokoningii* the alkaline pH levels (pH 7.5 and pH 8.5) did not favour cellulase zproduction which may be due to the reason that the induction of saccharifying cellulase (exo-ß-glucanase and endo-β-glucanase) occurs at the stage of high metabolic activity when the pH of medium lies in acidic range of pH. The pH 5.5 has already reported optimum for cellulase production (Mandels *et al.*, 1975) because very less enzyme is required to produce sugar at this pH. The preference of narrow pH range for cellulase production have also been reported by earlier workers in cellulolytic microorganisms (Mandels *et al.*, 1976; Bisen *et al.*, 1982). In the present investigations, the ß-glucosidase activity was found to favour pH 6.5 for its maximum activity as this enzyme gets suppressed in the acidic condition of the medium where the accumulation of glucose and cellobiose due to glucanase activities inhibit the enzyme activity (Bagga & Sandhu, 1987) the poor β-glucosidase activity in the acidic range of pH has been reported by several workers (Sternberg, 1976; Ghose, 1977; Woodward & Arnold, 1981).

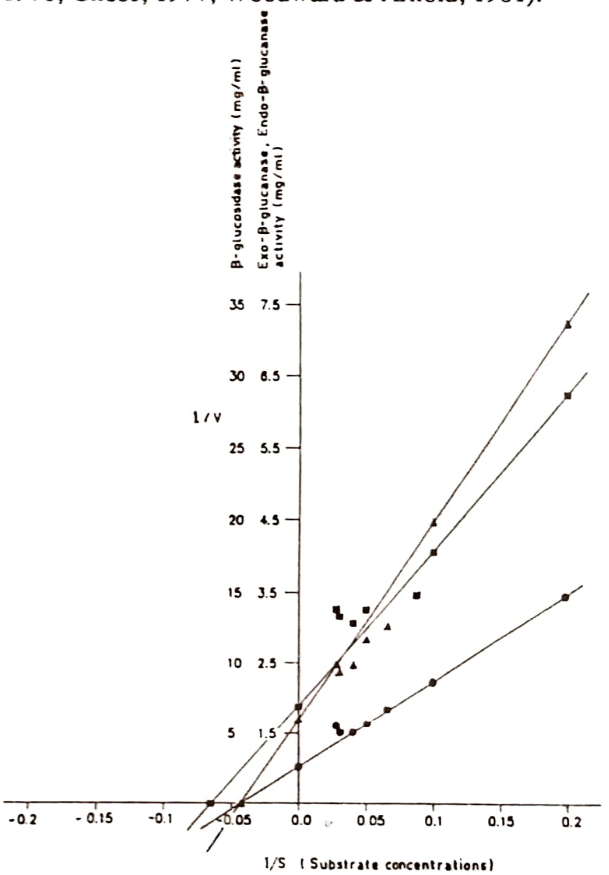

Fig. 1. Lineweaver - Burk plot for endoglucanase ( ) exoglucanase ( ) and ß-glucosidase ( ) activity at different concentrations of sludge.

The results showed that all the components of cellulase complex were found to be directly affected by the incubation temperatures which may be due to the reason that temperature highly exerts selective effects on metabolic pathways by repression of

particular protein synthesis (Pirt, 1975). The protein production was maximum at pH range of 4.5 to 5.5 and temperature 35°C. With further increase in temperature and pH the protein value gradually declined because at high temperatures it gets denatured, while alkaline conditions were not found favourable for its production.

When this test fungus was grown on different concentrations of sludge the data revealed that there was a gradual increase in cellulase activity with the increase in substrate concentrations up to 3%. This is in agreement with the earlier findings of Markkanen (1975) that the increased concentrations of substrate give rise to high sugar yields due to the increase in the amount of easily hydrolyzable cellulose. In the present study, maximum cellulase activity was recorded up to 3% concentrations of sludge because as soon as the saturation point was accomplished between enzyme and substrate, further increase in substrate concentration does not enhance the cellulase production. This leds to the conclusion that up to 3% concentration, the nitrogen level in the medium was adequate to suport cellulase production but above this concentration nitrogen level in the medium acts as limiting factor resulting repression in cellulase production.

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