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Optimization of Cellulase Production by *Trichoderma reesei* ATCC 26921 Using a Simplified Medium on Water Hyacinth Biomass

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Some environmental (pH and temperature) and biochemical (substrate concentration, nitrogen source and other nutrients) factors influencing cellulase production by *Trichoderma reesei* ATCC 26921 were studied during its growth in Mandels and Andreotti medium containing water hyacinth biomass as the major carbon source. Optimum pH, temperature and substrate concentration were found to be 4.8, 31 ± 1°C and 4 per cent (w/v) milled water hyacinth, respectively, for the maximum production of FPase, CMcase, and β-glucosidase. Among the different nitrogen sources tested, ammonium sulphate was found to be the best for cellulase production. In an attempt to simplify the composition of the basal medium, all other nutrients except $(NH_4)_2SO_4$, KH_2PO_4 , yeast-extract and Tween-80 were omitted from the medium. This did not reduce much the yield of cellulase. Addition of poultry manure further increased the cellulase production.

Introduction

Lignocellulosic plant waste has gained considerable interest because of its possible use in secondary fermentation process for the production of food[1,2], chemicals[3,4] or fuel[5-7]. In the process of its bioconversion to ethanol, the cost effectivity of cellulase is important as the enzyme hydrolyses the cellulose (a major component of lignocellulose) to simple sugar which is finally fermented to ethanol. In an assessment study on economics of cellulase process technology[8] it was estimated that the production cost of cellulase was ~$ 3.00/kg, cellulase protein having a specific activity of 0.6 IU/mg protein. However, this production cost may vary with carbon source used and can be lowered sufficiently by using abundantly growing weed biomass which is available without extra cost. Several workers have used weed biomass as substrate for cultivation of mushroom[9], cellulase production[10], enzymatic saccharification[11] or biogas production[12].

Water hyacinth, an aquatic weed of tropical and subtropical regions of the world, poses serious problem to water bodies through its high rate of propagation. Utilization of this biomass can be a part of water pollution control at one hand and production of food, fuel or chemical on the other hand, thus changing its status from a prolific pest into potential provider[13]. In terms of availability and cost, this lignocellulosic biomass may serve as a cheap substrate for the production of myco-protein[14,15], biogas[16], chemical[17], cellulase[18-20] as well as bioconversion to ethanol[21-24]. The present work is aimed at optimizing the cultural parameters and to simplify medium composition for maximum production of cellulases at minimum cost using water hyacinth biomass.

Materials and Methods

Organism — Stock culture of cellulase producing strain of *Trichoderma reesei* ATCC 26921, received courtesy USDA Laboratory, Peoria, Illinois and maintained on Potato-Dextrose-Agar (PDA) slants. Mycelial discs (7 mm diam), punched out from the edges of its 5 d old colonies in petridishes, were used as inocula.

Substrate — Water hyacinth [*Eichhornia crassipes* (Mart.) Solms.] were collected from local water bodies in and around Burdwan. After discarding the roots which were reported to contain heavy metals[25], the plants were washed overnight in water, oven dried at 65°C to constant weight and then milled to 40 mesh.

Cultural Studies — Mandels and Andreotti[26] medium, employed as basal medium for the enzyme production, contained the following components (g/l) : KH_2PO_4 , 2; $(NH_4)_2SO_4$, 1.4; Urea, 0.3; $MgSO_4$. $7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4$. $7H_2O$, 0.005; $MnSO_4$. H_2O, 0.0016; $ZnSO_4$, 0.0014; $CoCl_2$, 0.002; peptone, 1; yeast-extract, 0.05 and Tween-80, 2ml/l. Water hyacinth (40 mesh) was the major carbon source of the medium.

A range of initial pH (4-6.4), incubation at 25-40°C and substrate concentration (2-6 per cent w/v) were tested. In subsequent experiments, six different nitrogen (N) sources were added separately replacing all the N-sources of basal medium, to study their effects on cellulase production.

Cellulase Production — Erlenmeyer flasks (250 ml) containing the basal media (50 ml) and the substrate were autoclaved at 20 lb for 20 min and inoculated with 5 mycelial discs of *T. reesei* ATCC 26921. The flasks were then incubated at 30 ± 1°C on a rotary shaker (150 rpm). After 8d incubation, content of each flask was crushed with pinch of chilled neutral sand, filtered and the filtrate was centrifuged (10,000 g, 4°C). The supernatant was used for enzyme assay.

Enzyme Assay — Cellulase (FPase) and endoglucanase (CMcase) activities were assayed by the method reported by Mandels *et al.*[27] using Whatman No. 1 filter paper (50 mg) and carboxymethyl cellulose (0.5per cent w/v in 0.05 *M* acetate buffer, pH 5) as substrates, respectively. The reducing sugars during the enzymatic reaction were measured by dinitrosalicylic acid (DNS) method[28] using *D*-glucose as a standard. The β- glucosidase activity was determined following Macris[29] method using *p*-nitrophenyl β-D-glucopyranoside (4 m*M*) as a substrate.

Enzyme activity was expressed in units (U) which was the amount of enzyme needed to liberate 1 μmol of glucose equivalent (in case of FPase and CMcase) or 1 μmol of *p*-nitrophenol (for β-glucosidase) per minute from the respective substrate under the conditions of assay. Total nitrogen present in poultry manure was estimated following Vogel[30].

Results and Discussion

Effect of pH

For determining the optimum pH, a range of initial pH (4-6.4) of the medium was tested, keeping the incubation temperature constant at 30 ± 1°C. An acidic pH favoured cellulase production while the yield was markedly reduced above pH 6 (Table 1). Since the highest yields of cellulases were obtained at pH 4.8, it was considered optimum for the test strain of *Trichoderma* on water hyacinth. Ryu and Mandels[31] have reported significant impact of the initial pH of a medium on cellulase production with an optimum value lying between 3 and 4 in the case of pure cellulose as substrate and at a higher pH in the case of natural cellulosics as substrates. Maximum cellulase production by strains of *T. reesei* was also reported to occur between 3 and 5 (refs 32-35).

Effect of Incubation Temperature

The medium was adjusted to an initial pH 4.8 during optimization of incubation temperature. It was observed that cellulase yield did not change significantly at 31-34°C, although the maximum yield was obtained at 31 ± 1°C (Table 2). As such the necessity of maintaining a constant temperature was not essential, thus facilitating operation. It was also observed in a parallel study that

Table 1 — Production of cellulases by *T. reesei* ATCC 26921 at different initial pH of Mandels and Andreotti medium containing water hyacinth biomass (3 per cent w/v) after 8d incubation at 30 ± 1°C

Initial pH	Cellulase activity (U/ml)		
	FPase	CMcase	β-glucosidase
4	0.03	0.12	0.01
4.4	0.04	0.27	0.03
4.6	0.09	0.36	0.08
4.8	0.12	0.44	0.11
5	0.13	0.41	0.11
5.2	0.1	0.32	0.1
5.4	0.09	0.27	0.09
5.6	0.06	0.24	0.08
6	0.02	0.11	0.07
6.4	0.06	0.07	0.05
LSD (P=0.05)	0.04	0.02	0.02

Table 2 — Production of cellulases by *T. reesei* ATCC 26921 in Mandels and Andreotti medium (pH 4.8) containing water hyacinth biomass after 8d incubation at different temperatures

Incubation temperature (°C)	Cellulase activity		
	FPase	CMcase	β-glucosidase
25	0.07	0.19	0.08
28	0.12	0.38	0.10
31	0.12	0.44	0.11
34	0.14	0.40	0.10
37	0.10	0.32	0.06
40	0.04	0.15	0.01
LSD (P=0.05)	0.02	0.06	0.03

an initial exposure at 34 ± 1°C for 2 d followed by incubation at 30 ± 1°C further increased the yield over that recorded at constant temperature. This was in accordance with the observation by Tangnu *et al.*[34] in *T. reesei* Rut C-30.

Effect of Substrate Concentration

In the earlier experiments 3 per cent (w/v) water hyacinth was used as the major carbon source. On the basis of cellulose content of water hyacinth (38.5 per cent of dry weight) this was approximately equivalent to 1 per cent pure cellulose. With different concentrations (w/v) of the substrate (2-6 per cent) there was corresponding increase in cellulase yield up to 4 per cent (Table 3). However, the ratio of β-glucosidase to FPase increased up to 6 per cent. This might be due to the slurry consistency of the culture at higher substrate concentration which created almost still condition favouring relatively more β-glucosidase activity than FPase activity as earlier pointed out by Kalra and Sandhu[36].

Effect of Nitrogen Sources

Maintaining the optimum pH, incubation temperature, substrate concentration at 4.8, 31 ± 1°C and 4 per cent (w/v) respectively, six different nitrogen sources (Table 4) were tested separately to find the best one for cellulase production. Each source was added in amount equivalent to the total nitrogen present in basal medium (587 mg nitrogen/l of medium) keeping the available nitrogen constant. Amongst the sources $(NH_4)_2SO_4$ induced the best production. Linko *et al.*[37] have reported ammonium salt to be an excellent source of nitrogen for *Trichoderma*.

Table 3 — Production of cellulases by *T. reesei* ATCC 26921 on different concentrations of powdered water hyacinth in Mandels and Andreotti medium (pH 4.8) after 8d incubation at 31 ± 1°C

Water hyacinth (w/v), per cent	Cellulase activity (U/ml)		
	FPase	CMcase	β-glucosidase
2	0.1	0.3	0.05
3	0.14	0.44	0.1
4	0.17	0.48	0.13
5	0.12	0.32	0.1
6	0.07	0.2	0.06
LSD (P=0.05)	0.03	0.02	0.03

Experiments using different concentrations (g/l) of $(NH_4)_2SO_4$ showed (Table 5) that 3.5 g/l was optimum for cellulase production by *T. reesei* ATCC 26921.

Effect of Other Nutrients

In an attempt to simplify composition of the basal medium, all nutrients except $(NH_4)_2SO_4$ (3.5 g/l) and Tween-80 (2 ml/l) were omitted from Mandels and Andreotti[26] medium. This omission resulted in higher final pH (6.4) and reduced cellulase yield to a certain

Table 4 — Production of cellulases by *T. reesei* ATCC 26921 in Mandels and Andreotti medium (pH 4.8) containing different nitrogen source* and 4 per cent (w/v) water hyacinth

Nitrogen source	Cellulase activity (U/ml)		
	FPase	CMcase	β-glucosidase
Ammonium sulphate	0.15	0.4	0.12
Ammonium chloride	0.1	0.34	0.07
Ammonium nitrate	0.11	0.27	0.09
Potassium nitrate	0.08	0.29	0.04
Urea	0.11	0.37	0.07
Peptone	0.09	0.3	0.08
LSD (P=0.05)	0.02	0.02	0.02

*Each nitrogen source was present in amount equivalent to 587 mg N/L of medium

Table 5 — Production of cellulases by *T. reesei* ATCC 26921 in Mandels and Andreotti medium (pH 4.8) containing concentration (g/l) of $(NH_4)_2SO_4$

Ammonium sulphate (g/l)	Cellulase activity (U/ml)		
	FPase	CMcase	β-glucosidase
0.7	0.07	0.21	0.05
1.4	0.1	0.27	0.07
2.1	0.12	0.3	0.09
2.8	0.14	0.38	0.12
3.5	0.18	0.49	0.11
4.2	0.17	0.46	0.11
4.9	0.13	0.4	0.05
LSD (P=0.05)	0.02	0.03	0.02

Table 6 — Effect of poultry manure on the production of cellulase by *T. reesei* ATCC 26921 in a medium containing $(NH_4)_2SO_4$ (3.5 g/l), KH_2PO_4 (2g/l), yeast-extract (0.05 g/l), Tween-80 (2ml/l) and water hyacinth (4 per cent), pH 4.8 after 8d incubation at 31 ± 1°C

Poultry manure, per cent (w/w)	Cellulase activity (U/ml)		
	FPase	CMcase	β-glucosidase
1	0.17	0.44	0.14
2	0.19	0.52	0.17
4	0.15	0.38	0.12
6	0.12	0.32	0.06
LSD (P=0.05)	0.03	0.04	0.01

extent. Addition of KH_2PO_4 (2 g/l) and yeast-extract (0.05 g/l) however, overcame the reduction and favoured better yield of the cellulase. Wayman and Chen[34] used KH_2PO_4 (0.1 *M*) to maintain the pH of the medium at lower level.

Effect of Cheap Nitrogen Additive

To study any further promotion in cellulase production, poultry manure containing 1.6 per cent (of dry wt) total nitrogen, was used as cheap, complex nitrogen additive. Of the different concentrations (w/w) tried (1-6 per cent), further increase in the yield was obtained at 2 per cent (Table 6). Such cheap nitrogen additive was earlier reported to help in high cellulase activity[38,29].

From the results, it can be concluded that production of cellulase complex by *T. reesei* ATCC 26921 can be achieved on cheap substrate like water hyacinth using a simple medium containing $(NH_4)_2SO_4$, KH_2PO_4, yeast-extract, Tween-80 and 4 per cent (w/v) substrate, maintained at pH 4.8 and 31 ± 1°C. Addition of cheap poultry manure further improved the yield. Ali and Akhand[18] used only NH_4NO_3 and Tween-80 for cellulase production by strain of *Trichoderma* on water hyacinth. Thus, a plant biomass, in a simplified medium supplemented with poultry manure proved to be a cost-effective substrate for cellulase production as well as cellulolytic hydrolysis. This may be of practical importance in large-scale enzymatic hydrolysis of lignocellulosic plant waste during bioethanol production.

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