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# Cellulase production by *Trichoderma reesei* on pretreated water hyacinth : effect of nutrients

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Cellulase production by *Trichoderma reesei* AICC 26921 was studied in submerged culture using Mandels and Andreotti medium containing pretreated water hyacinth biomass as the sole carbon source and ammonium sulphate as the nitrogen source. In an attempt to develop a simple, cost-effective medium all other nutrients except $(NH_4)_2 SO_4$ were omi tted from the medium which resulted in significant reduction of enzyme production. Successive addition of $KH_2 PO_4$ and yeast extract to the medium enhanced enzyme production to the same level or even higher level than that obtained with Mandels and Andreotti medium.

**Key words :** Cellulase production, *Trichoderma reesei*, water hyacinth, simple medium

## INTRODUCTION

Cellulose, the major constituent of plant biomass, is the most abundant and replenishable organic material on earth. It has considerable potential as a source of chemical, energy or microbial protein. Before it is used, it must be hydrolyzed into monomeric sugar which in turn can then be used as carbon source in various fermentation processes. Enzymatic hydrolysis of cellulose, though a slower process, produces purer sugar under mild conditions. This process requires participation of at least three types of enzymes viz., endo–1, 4–β–glucansase, exo–1, 4–β–glucanase and β–glucosidase which are collectively reffered to as the cellulase system (Awafo *et al.*, 1996). For large scale hydrolysis of cellulose, an economical production of large amount of cellulase is of utmost importance. As such selection and improvemtent of suitable cellulase producing strain, development of cheap and simple fermentation media and methods are essential (Enari and Markkanen, 1977).

Among a wide range of cellulolytic organisms *Trichoderma reesei* and its mutants are still considered the most promising one for commercial production of cellulase (Singh and Garg, 1996). The basal mediun used for cellulase production with *T. reesei* was developed by Mandels and Reese (1957) which contained carbon source in the form of different cellulase preparations, nitrogen sources as ammonium sulphate, urea, peptone and many other essential minerals viz., Ca, Mg, Fe, Mn, Zn, Co. Studies on cellulase production by *Trichoderma* spp. have shown that aquatic weed water hyacinth [ *Eichhornia creassipes* (Mart.) Solms.] can be used as cheap carbon source (Ahmed *et al.*, 1995; Mukhopadhyay and Nandi, 1997, 1999). It has been also found that cellulase production by *T. reesei* can be achieved on untreated water hyacinth biomass using a simple medium (Mukhopadhyay and Nandi, 1999). In the present work cellulase production has been studied on pretreated water hyacinth biomass. The work also aims to study effects of nutrients other than carbon and nitrogen on cellulase production by *T. reesei*.

## MATERIALS AND METHODS

### *Organism*

The cellulase producing strain of *Trichoderma reesei* ACTC 26 921 used in the present study was received by courtesy of U. S. Departmet of Agiculture, Peoria, Illinois. Stock culture was maintained on

Potato-Dextrose–Ager (PDA ) slants. For cellulase production mycelial discs (7 mm diameter), punched out from the edged of its 5 days old colonies grown in pertridishes, were used as inocula.

***Substrate and its pretreatement***

Water hyacinth [*Eichhornia crassipes* (Mart.) Solms.] was collected from local water bodies in and around Burdwan and sun dried after discarding the roots which were reported to contain heavy metals (Zhu *et al.*, 1999). Dried plants were chopped into small pieces and soaked overnight in 0.1 N $H_2SO_4$, washed thoroughly with excess water to neutrality and subsequently in 1% (w/v) NaOH. It was then autoclaved at 121°C for 1 h and subsequently washed under running tap water until neutrality. Finally, the wet biomass was dried at 65° C to constant weight and ground to 40 mesh to use as carbon source in cellulase production medium.

***Cellulase production***

Initially, Mandels and Andreotti (1978) medium was employed as basal medium for cellulase production (Mukhopadhyay and Nandi, 1999). Pretreated water hyacinth powder (4% w/v) and ammonium sulphate (4.2 g/L) was used as major carbon and nitrogen source in the medium. Erlenmeyer flask (250 ml) containing 50 ml basal medium (pH 4.8) and the carbon source were autoclaved at 121°C for 20 min and inoculated with five mycelial discs of *T. reesei*. The flasks were incubated at 31 ± 1°C on rotary shaker (150 rpm). After 8 days incubatiom, content of each flask was crushed with a pinch of chilled neutral sand, filtered and the filtrate was centrifuged. (10,000 g, 4° C ). The supernatant was used for enzyme assay.

To study the effect different nutrient composition of medium on cellulase production, all other nutrients except ammonium sulphate [$(NH_4)_2 SO_4$] and Tween-80 were omitted from the basal medium. In further experiments, enzyme production was recorded by successive addition of potassium dihydrogen orthophosphate (2 g/L) and yeast extract (0.5 g/L) to the medium. A control set with only the carbon source (pretreated water hyacinth) and Tween–80 was run parallely for comparison.

***Enzyme assay***

Total cellulase (FPase) and endoglucanase activities were assayed following the method of Mandels *et al.* (1976) and β–glucosidase activity was determined following Macris (1999). Reducing sugars released during the enzymatic reaction were measured by dinitrosalicylic acid (DNS) method (Miller, 1959) using D–glucose as the standard.

Cellulase activity was expressed in international units (IU) which was the amount of enzyme needed to liberate 1 μ mol of glucose equivalent (in case of FPase and CMCase) or 1 μ mol of ρ–nitrophenol (for β–glucosidase) per minute from the respective substrate under the assay conditions.

## RESULTS

Effect of various nutrient compositions on cellulase production, by including and excluding different macro–and micro-elements have been summarized in Table 1. From the results it is evident that FPase and CMCase yields were reduced significantly when all the nutrients of basal medium except $(NH_4)_2 SO_4$ were omitted, suggesting the necessity for nutrient enrichment of the medium. Omission also resulted in a decrease in final pH (3.5) of the medium. However, the pH value could be controlled at the favourable range by adding 2 g/L of potassium dihydrogen orthophosphate ($KH_2PO_4$) to the medium. This in turn helped to maintain satisfactory level of FPase and CMCase acvitities. In the next step, addition of complex organic additive like yeast extract (YE) at a concentration of 0.5 g/L along with $KH_2PO_4$ resulted in further enhancement of cellulase activities to a level equal to or even higher than that obtained by using all the nutrients. Tween–80 was used in all combinations as surface-active agent which was known to stimulate cellulase secretion.

## DISCUSSION

Cellulase production by *T. reesei* and other microbes is influenced by several biochemical factors (Gomes *et al.*, 1989) such as type and concentration of cellulosic substrate, organic and inorganic nitrogen sources, macro and trace elements etc. Cellulosic substrate is used as carbon source for inductive production of cellulase while nitrogen sources play important role in biosynthesis of protein by different microorganisms (Nigam *et al.*, 1988). Mandels and Reese (1957) reported that calcium plus trace levels of Fe, Mn,Zn and Co were required for enzyme production of *T. reesei*. But from the present

**Table 1.** Cellulase activities of *Trichoderma reesei* ATCC 26 921 in media (pH 4.8) with different nutrient compositions at 31 ± 1°C after 8 days in shake cultures

| Composition of medium | Cellulase activity (IU/ml) | | |
|---|---|---|---|
| | FPase | CMCase | β-glucosidase |
| (a) Carbon source (4% w/v of pretreated water hyacinth) + Tween–80 (2 ml/L) | 0.001 | 0.023 | 0.05 |
| (b) Carbon source + other mutrients of MA* medium except nitrogen sources + $(NH_4)_2SO_4$ (4.2 g/L) + Tween–80 (2 ml/L) | 0.15 | 0.42 | 0.13 |
| (c) Carbon source + $(NH_4)_2SO_4$ (4.2 g/L) + Tween–80 (2 ml/L) | 0.1 | 0.25 | 0.1 |
| (d) Carbon source + $(NH_4)_2SO_4$ (4.2 g/L) + Tween–80 (2 ml/L) + $KH_2PO_4$ (2 g/L) | 0.12 | 0.3 | 0.1 |
| (e) Carbon source + $(NH_4)_2SO_4$ (4.2 g/L) + Tween–80 (2 ml/L) + $KH_2PO_4$ (2 g/L) + Yeast extract (0.5 g/L) | 0.14 | 0.45 | 0.12 |
| LSD (P = 0.05) | 0.02 | 0.04 | 0.01 |

* Mandels and Andreotti (1978) medium.

investigation it is evident that carbon and nitrogen source along with phosphate salt and organic nitrogen could replace all other nutrients in the Mandels and Andreotti (1978) medium, a modified medium of Mandels and Reese (1957). In the present work, supplied $KH_2PO_4$ possibly not only controlled pH of the medium (Wayman and Chen, 1992) at the favourable range (4.8 – 5.0) but also supplied the required phosphate salt (Yamanobe *et al.*, 1987) for better cellulase production (Ismail *et al.*, 1995). Yeast extract provided organic nitrogen which was needed for maximum enzyme production. Addition of YE (0.5 g/L) increased the CMCase activity significantly probably by providing organic nitrogen.

Minimization in the number of nutrients resulted in considerable simplification of the medium as well as in the reduction of its cost for cellulase production by *T. reesei*. Moreover, cellulase produced in this medium contained FPase to β–glucosidase in the ratio of 1 : 0 : 9 which was close to 1 : 1. A cellulase system having the above ratio close to 1.0 was considered a 'complete' system since it established a threshold enzyme ratio for elimination of accumulated cellobiose during hydrolysis of cellulose (Awafo *et al.*, 1996). Thus, it can be suggested from the present study that a complete cellulase system can be produced by a cost effective method using biomass of the aquatic weed, water hyacinth as the sole carbon source in a simple medium containing only $(NH_4)_2SO_4$, $KH_2PO_4$, YE and Tween–80.

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