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Semi-Solid-State Fermentation of *Eicchornia crassipes* Biomass as Lignocellulosic Biopolymer for Cellulase and β-Glucosidase Production by Cocultivation of *Aspergillus niger* RK3 and *Trichoderma reesei* MTCC164

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Abstract

An aquatic weed biomass, *Eicchornia crassipes*, present in abundance and leading to a threatening level of water pollution was used as substrate for cellulase and β-glucosidase production using wild-type strain *Aspergillus niger* RK3 that was isolated from decomposing substrate. Alkali treatment of the biomass (10%) resulted in a 60–66% increase in endoglucanase, exoglucanase, and β-glucosidase production by the *A. niger* RK3 strain in semi-solid-state fermentation. Similarly, the alkali-treated biomass led to a 45–54% increase in endo- and exoglucanase and a higher (98%) increase in β-glucosidase production by *Trichoderma reesei* MTCC164 under similar conditions. However, the cocultivation of *A. niger* RK3 and *T. reesei* MTCC164 at a ratio of 3:1 showed a 20–24% increase in endo- and exoglucanase activities and about a 13% increase in the β-glucosidase activity over the maximum enzymatic activities observed under single culture conditions. Multistep physical (ultraviolet) and chemical (*N*-methyl-*N*'-nitrosoguanidine, sodium azide, colchicine) mutagenesis of the *A. niger* RK3 strain resulted in a highly cellulolytic mutant, UNSC-442, having an increase of 136, 138, and 96% in endoglucanase, exoglucanase, and β-glucosidase, activity, respectively. The cocultivation of mutant UNSC-442 along with *T. reesei* MTCC164 (at a ratio of 3:1) showed a further 10–11% increase in endo- and exoglucanase activities and a 29% increase in β-glucosidase activity in semi-solid-state fermentation.

Index Entries: Cellulase; β-glucosidase; *Eicchornia crassipes*; cocultivation; mutagenesis.

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Introduction

Cellulose, a major renewable resource available in nature, is an unbranched glucose polymer consisting of the anhydro-D-glucose units that are linked by the β-1,4-D-glucoside bonds. Hydrolysis of the cellulose requires the combined action of at least three enzymes: endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21).

Numerous efforts, with varying levels of success, have been made to utilize lignocellulosic materials mainly from agricultural wastes as substrate; however, the higher lignin content has been a deterrent toward bioconversion of agro-based lignocellulosic waste materials. Recently, attention has been diverted to the exploitation of aquatic weeds containing significant levels of cellulose and comparatively lower lignin content as the renewable energy source. These weeds occur abundantly and have fast growth rate, quick regenerative properties, easy propagation, and ecologic compatibility under diverse climatic conditions. In the present study, an aquatic weed, *Eicchornia crassipes*, which grows widespread and is abundant in polluted ponds, was selected as the biomass material for cellulase production and its subsequent bioconversion into simple sugars.

Most of the work regarding cellulase production during the last two decades has been done in submerged fermentation *(1–5)*, and little attention has been focused on solid-state fermentation *(6)*. Solid-state fermentation has many advantages over submerged fermentations, such as the mimicking nature, lower capital and smaller operational expenses and control, a simpler cultivational requirement, the production of concentrated enzyme, improved recovery, and smaller wastewater output *(7)*. The solid-state fermentation of *E. crassipes* biomass was attempted using a wild-type strain of *Aspergillus niger* RK3 isolated in our laboratory and by its mutants (obtained by physical and chemical mutagenesis). The impact of cocultivation studies on improving the economics of production were also studied. The isolated *A. niger* RK3 and its mutant along with the *Trichoderma reesei* MTCC164 were evaluated for cellulase levels under these conditions.

Materials and Methods

Microorganism

The strain we used was one of several strains isolated from decomposing materials. Isolated strains were analyzed for growth on carboxymethylcellulose (CMC)-agar and also for the zone diameter formed on Congo-red CMC-agar plate *(8)*. The strain showing notable growth and the maximum zone diameter on Congo-red CMC-agar was selected for the present study. Identification of this strain at the Indian Agricultural Research Institute, New Delhi, India, had indicated it to be *A. niger*, and the same was denoted as RK3 stain.

Sodium Hydroxide Pretreatment of Biomass

E. crassipes biomass, an aquatic natural waste, present in abundance in the northern region of India, was collected, chopped into small pieces of about 1 to 2 cm in length, washed thoroughly to remove surface dust, and dried at 65°C. The dried matter was added to 0.25 *M* NaOH (20 mL/g of substrate), autoclaved, and filtered through muslin cloth. The residue obtained was thoroughly washed and neutralized with 0.25 *N* HCl *(9)*. The substrate was then finally washed with distilled water and dried at 65°C.

X-ray Diffractographic Analysis of E. crassipes *Biomass*

The effect of alkali pretreatment on the crystalline structure of *E. crassipes* biomass was analyzed by X-ray diffraction *(10)*. Alkali-pretreated and untreated biomass was vacuum dried to remove the water content from the cellulosic substrate. Crystallinity was measured by X-ray diffraction using a Phillips X-ray diffractograph, having copper target with a nickel filter. The sample was mounted horizontally while the Geiger counter moved in a vertical arc. The samples were scanned in the range from 0 to 100° angle. Crystallinity index was calculated using the following expression *(11)*.

$$CrI = \frac{(I_0 - I_{am})}{I_0} \times 100$$

where, I_0 is the intensity of the peak at 0 angle, I_{am} is the intensity of peak at 2θ angle, and *CrI* is the crystallinity index.

Mutagenesis by Ultraviolet Radiation (Physical Treatment)

Ultraviolet (UV) mutagenesis was performed as previously described *(12)*. Briefly, a spore (2–4 d old) suspension (~10^6 spores/mL) was prepared in saline (0.85%) containing 0.01% Tween-80. Mutagenesis with UV irradiation was carried out using a UV tube (15 W) at a distance of 70 cm for 5–30 min (with 5-min intervals) in two repeated steps. Treated spores were wrapped with black paper and incubated for 48 h at 30°C in the dark. Spores were then plated on Congo-red CMC-agar plates for screening *(8,13)*.

Mutagenesis by Mixed Chemical Treatment

The mutant strains selected after UV mutagenesis were further treated with 10 µg/mL of *N*-methyl-*N*'-nitrosoguanidine (NTG) *(12)* for 45 min. The strains showing higher zone diameter on Congo-red CMC-agar plates as compared to UV-treated mutant strains were selected. The mutants obtained after UV-NTG mixed treatment were further subjected to sodium azide treatment at concentrations of 1, 2, 3, and 4 µg/plate *(14)*. The mutants showing a higher clear zone diameter were selected and further treated with colchicine at the concentrations of 0.01, 0.02, 0.5, 1.0, and 2.0 µg/mL *(15)*. The mutant showing a maximum zone diameter on Congo-red CMC-agar plates was finally selected.

Preparation of Inoculum

The culture on the potato dextrose agar (PDA) slant was incubated at 30°C for 24 h. The activated spores from *A. niger* RK3 and *T. reesei* MTCC164 at a ratio of 3:1 were collected and suspended in 5 mL of sterile solution of 2% Tween-80. Five milliliters of the spore suspension (10^8 spores/mL) was added to the fermentation flask *(16)* containing 100 mL of the production medium.

Preparation of Production Medium and Fermentation

The Mandel and Weber's modified medium *(17)* with suitable modifications was used. The modified medium contained the following: 2.0 g/L of KH_2PO_4, 2.1 g/L of $(NH_4)_2SO_4$, 0.004 g/L of NH_4Cl, 0.3 g/L of $MgSO_4 \cdot 7H_2O$, 0.3 g/L of $CaCl_2$, 0.00156 g/L of $MnSO_4 \cdot 5H_2O$, 0.0014 g/L of $ZnSO_4 \cdot 7H_2O$, 0.00266 g/L of $CoCl_2 \cdot 6H_2O$, 0.005 g/L of vanillin, 0.01 g/L of succinic acid, and 0.02 g/L of cellobiose. The pH of the medium was adjusted to 5.0 before sterilization. Medium (100 mL) containing 10% cellulosic substrate (*E. crassipes* biomass) was added to 5% of inoculum and incubated at 30°C for 18 d. The samples were collected at different time intervals after filtration of the semi-solid paste through muslin cloth and centrifuged (1000*g* for 15 min). The supernatant obtained was used for assaying endoglucanase, exoglucanase, and β-glucosidase and total soluble proteins. The total dry mycelial mass obtained after 18 d of incubation was also estimated.

Analytical Methods

Endoglucanase Assay

Carboxymethylcellulase (CMCase) activity was determined as described previously *(18)*. Briefly, the assay mixture (2.0 mL) contained 0.5 m*M* substrate (CMC), 50 m*M* sodium citrate buffer (pH 5.0), and 0.5 mL of the supernatant obtained from fermentation broth as the source of enzyme. After incubation at 50°C for 30 min, the reducing sugar released was estimated using the dinitrosalicylic (DNS) method as described previously *(19)*.

Exoglucanase Assay

Filter paper (FPase) activity was determined using Whatmann no. 1 strips (1 × 6 cm, 10 mg) by the DNS method as described previously *(18)*.

β-Glucosidase

β-Glucosidase activity was determined using *p*-nitrophenol β-D-glucopyranoside as a substrate *(20)*. Briefly, the assay mixture in a total volume of 1.0 mL contained 1 m*M* substrate in 50 m*M* sodium citrate buffer (pH 5.0) and 0.1 mL of supernatant from the fermentation broth, following incubation at 50°C for 30 min. Two milliliters of 4% sodium carbonate was added and the *p*-nitrophenol released was measured at 410 nm. The enzyme activity was expressed as micromoles of *p*-nitrophenol liber-

ated/(minute·milliliter) of enzyme. Total soluble protein was estimated according to the method of Lowry et al. *(21)*.

Scanning Electron Microscopic Studies

The morphologic features of the wild-type *A. niger* RK3 and the mutant strain were compared using scanning electron microscope model LEO-435 VP.

Results and Discussion

High endoglucanase (CMCase), exoglucanase (FPase), and β-glucosidase (25.18, 16.33, and 9.23 U/g of substrate, respectively) activities were observed with alkali-treated *E. crassipes* biomass as the substrate (10%) in semi-solid-state fermentation after 18 d of incubation with *A. niger* RK3. The activities observed were 60–66% higher compared with those of CMCase, FPase, and β-glucosidase as observed with untreated *E. crassipes* biomass. Similar results were obtained when *T. reesei* MTCC164 was used as the inoculum with alkali-treated *E. crassipes* biomass. Under these conditions a 42–54% increase in endo- and exoglucanase activities and a 98% increase in β-glucosidase activity was observed as compared with the untreated biomass (Table 1).

However, the maximum levels of endoglucanase, exoglucanase, and β-glucosidase activities were observed when alkali-treated *E. crassipes* biomass was cocultured with *A. niger* RK3 and *T. reesei* MTCC164 at a ratio of 3:1. Incubation under cocultured conditions resulted in a 20–24% increase in endoglucanase and exoglucanase activities over the respective maximum activities observed under single culture conditions (Table 1). However, the increase in β-glucosidase activity was 13% higher compared with the maximum activity observed when *A. niger* RK3 was used as the single culture under similar conditions.

Many other chemicals, particularly perchloric acid, paraacetic acid, sulfuric acid, formic acid, and ammonia freeze explosion, and many other organic solvents, such as *n*-propylamine, ethylenediamine, and *n*-butylamine, have also been used for pretreatment of lignocellulosics *(22)*. However, alkali pretreatment with sodium hydroxide is widely accepted as the most effective and presently used by many researchers *(23)*. Acid treatment of lignocellulosics solubilized the cellulose counterpart with lignin. On the other hand, alkali pretreatment solubilized only lignin contents of lignocellulosics and is economically feasible as compared to acid hydrolysis *(16)*. X-ray diffractograph of *E. crassipes* showed a peak at 2θ angle of 22°, indicating the degree of crystallinity (CrI = –166%) in the untreated *E. cressipes* biomass. A significant increase in the size of this peak (intensity 80–200) was recorded in the alkali-treated *E. crassipes* biomass at the same 2θ angle of 22° (Fig. 1). It therefore indicated that the crystalline structure of the cellulose was damaged (CrI = –566%) because of the lignin removal owing to the alkali pretreatment. This treatment would therefore lead to

Table 1
Analysis of Cellulase Levels from Alkali-Treated and Untreated *E. crassipes* Biomass Under Single and Coculture Conditions[a]

	Endoglucanase activity (U/g)		Exoglucanase activity (U/g)		β-Glucosidase activity (U/g)	
Organism	Untreated	Treated	Untreated	Treated	Untreated	Treated
A. niger RK3	15.15 ± 1.2	25.18 ± 1.5	10.14 ± 0.8	16.33 ± 1.2	5.71 ± 0.3	9.23 ± 0.7
T. reesei MTCC 164	18.13 ± 1.4	27.99 ± 1.9	12.88 ± 0.9	18.48 ± 1.2	3.00 ± 0.2	5.95 ± 0.4
A. niger RK3 + *T. reesei* MTCC164	29.54 ± 1.7	33.95 ± 2.1	14.43 ± 1.1	22.89 ± 2.3	7.73 ± 0.5	10.44 ± 0.9

[a]Values represent means of three replications ± SD. Ten percent alkali-treated and untreated substrate (*E. crassipes* biomass) was used. *A. niger* RK3 + *T. reesei* MTCC164 were used at a ratio of 3:1.

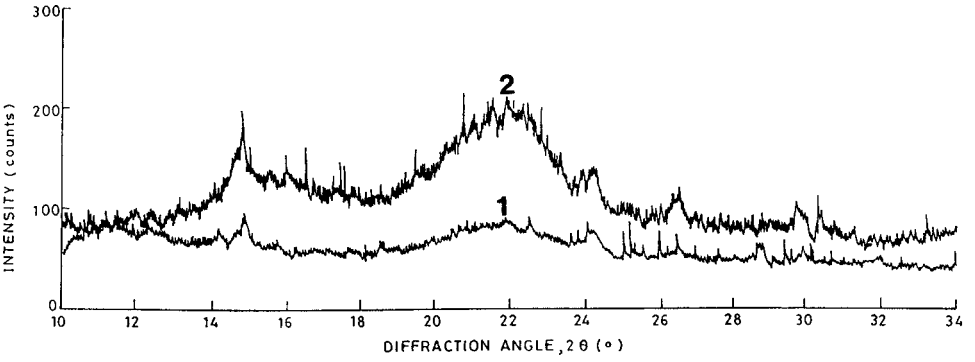

Fig. 1. X-ray diffractographic analysis of alkali-treated *E. crassipes* biomass. Peak 1 represents the crystalline complexity in untreated condition, and peak 2 represents the reduced crystalline complexity in alkali-treated condition.

the enhanced digestibility of the cellulose fiber. Similar observations were recorded with *Mangifera indica* sawdust used as the lignocellulosic substrate for cellulase production *(11)*.

Substrate concentration is a major factor for semi-solid-state fermentation that can significantly influence the moisture content and optimal microbial growth for bioconversion. Maximum levels of endoglucanase, exoglucanase, and β-glucosidase were observed at 10–12% substrate concentration, whereas higher substrate concentrations were inhibitory (data not shown). Similar observations were recorded with respect to dry mycelial weight and total soluble protein concentration (Fig. 2A,B). However, with respect to agricultural wastes, e.g., paddy straw, wheat straw, sawdust, and sugarcane bagasse, higher substrate concentrations (20%) were observed *(24)* to yield maximal enzymatic activities. Higher cellulosic content and the moisture retention ability of *E. crassipes* biomass may be the major factors leading to increased enzymatic activities with comparatively lower substrate concentration as observed in our experiments. The higher cellulose, lower lignin, and moisture content favor microbial growth, subsequent enzymatic adsorption, and degradation of the substrate, thus leading to increased levels of enzyme production *(7,24)*.

The wild-type *A. niger* RK3 was subjected to multistep mutagenesis by UV irradiation; mutants with high zone diameter were selected and subsequently treated in a stepwise manner with chemical mutagens as described in Materials and Methods. Maximum zone diameters obtained after respective mutagenesis are given in Table 2, and mutant UNSC-442 was selected for further studies. The growth of the wild-type strain was slower, and the mycelia observed were thin, long, and sporadically branched, whereas the mutant UNSC-442 was comparatively fast growing with extended mycelia that were highly branched and swollen, leading to increased biomass production (Fig. 3A,B). However, the biochemical and molecular basis for morphologic differentiation and increased enzyme production remains to be investigated. The enzymatic activities observed

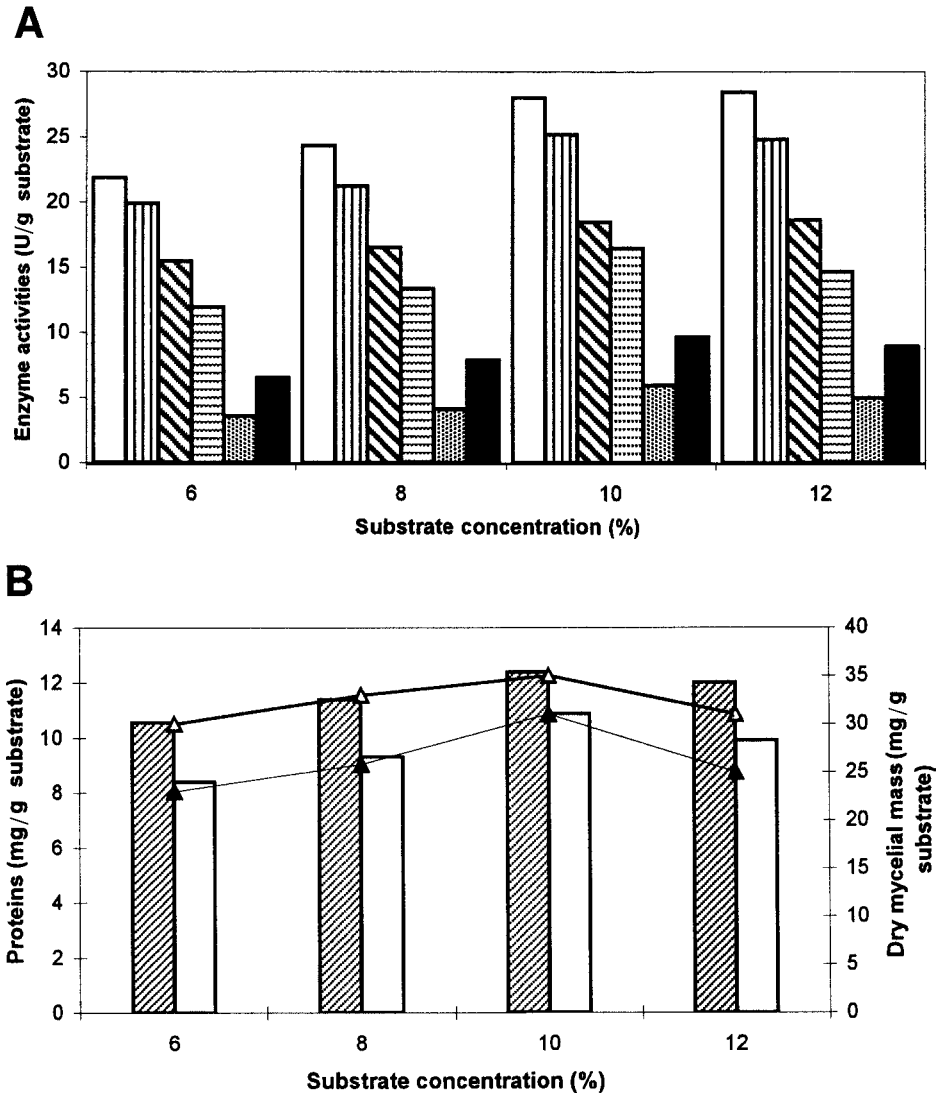

Fig. 2. **(A)** Effect of substrate concentrations on cellulase production in semi-solid-state fermentation. □, *T. reesei* MTCC164 (endoglucanase); ⊞, *A. niger* RK3 (endoglucanase); ▨, *T. reesei* MTCC164 (exoglucanase); ▤, *A. niger* RK3 (exoglucanase); ▩, *T. reesei* MTCC164 (β-glucosidase); ■, *A. niger* RK3 (β-glucosidase). **(B)** Effect of substrate concentrations on total soluble proteins and dry mycelial mass in semi-solid-state fermentation. ▨, *T. reesei* MTCC164 (proteins); □, *A. niger* RK3 (proteins); —△—, *T. reesei* MTCC164 (dry mycelial mass); —▲—, *A. niger* RK3 (dry mycelial mass).

in mutant UNSC-442 were significantly higher as compared to the wild-type *A. niger* RK3 strain. The mutant UNSC442 showed an increase of 136, 138, and 96% in endoglucanase, exoglucanase, and β-glucosidase activities, respectively (Fig. 4A). The cocultivation of mutant UNSC-442 with *T. reesei* MTCC164 showed a further increase in endoglucanase (65.21 U/g)

Table 2
Zone Diameter of Mutants After Physical and Chemical Mutagenesis of *A. niger* RK3 on Congo-Red CMC-Agar Plates

Sodium azide no.	Treatment	Mutants obtained	Zone diameter (mm)[a]
1	Wild-type *A. niger* RK3	—	2.0
2	UV	UV-40	4.0
3	UV + NTG (UN)	UN-44	4.6
4	UN + sodium azide (UNS)	UNS-442	5.3
5	UNS + colchicine (UNSC)	UNSC-442	8.3

[a]Values represent the maximum zone diameter observed after subsequent mutagenesis.

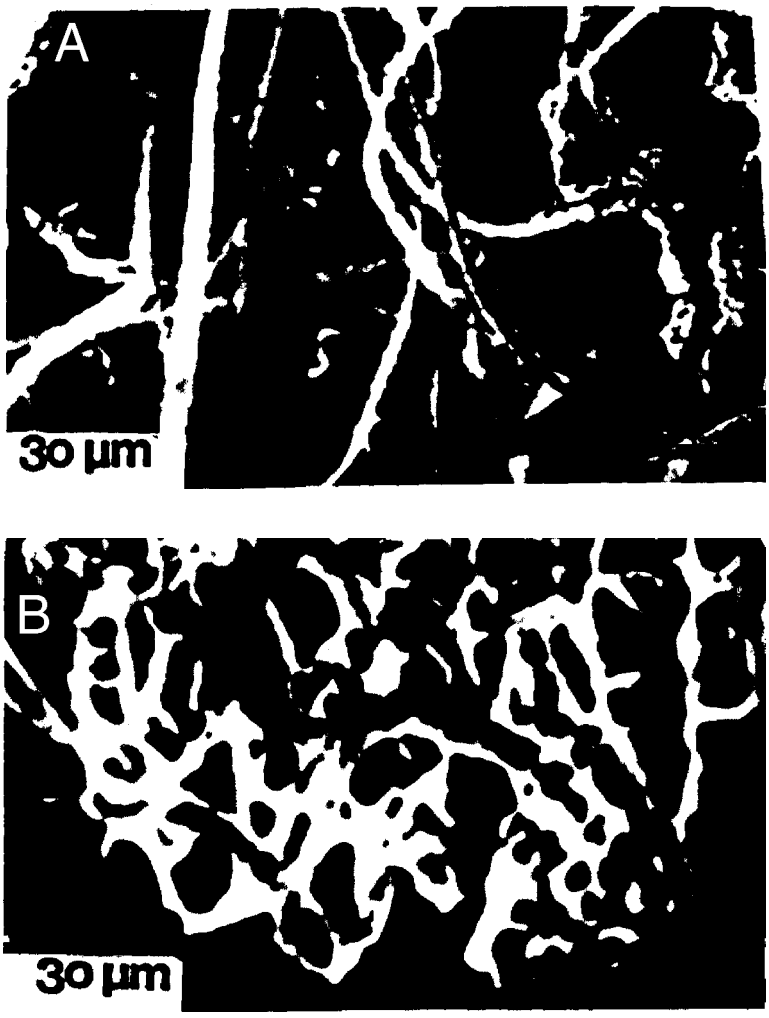

Fig. 3. Scanning electron micrograph of 72-h-old mycelia of **(A)** the wild-type *A. niger* RK3 and **(B)** the mutant *A. niger* UNSC-442.

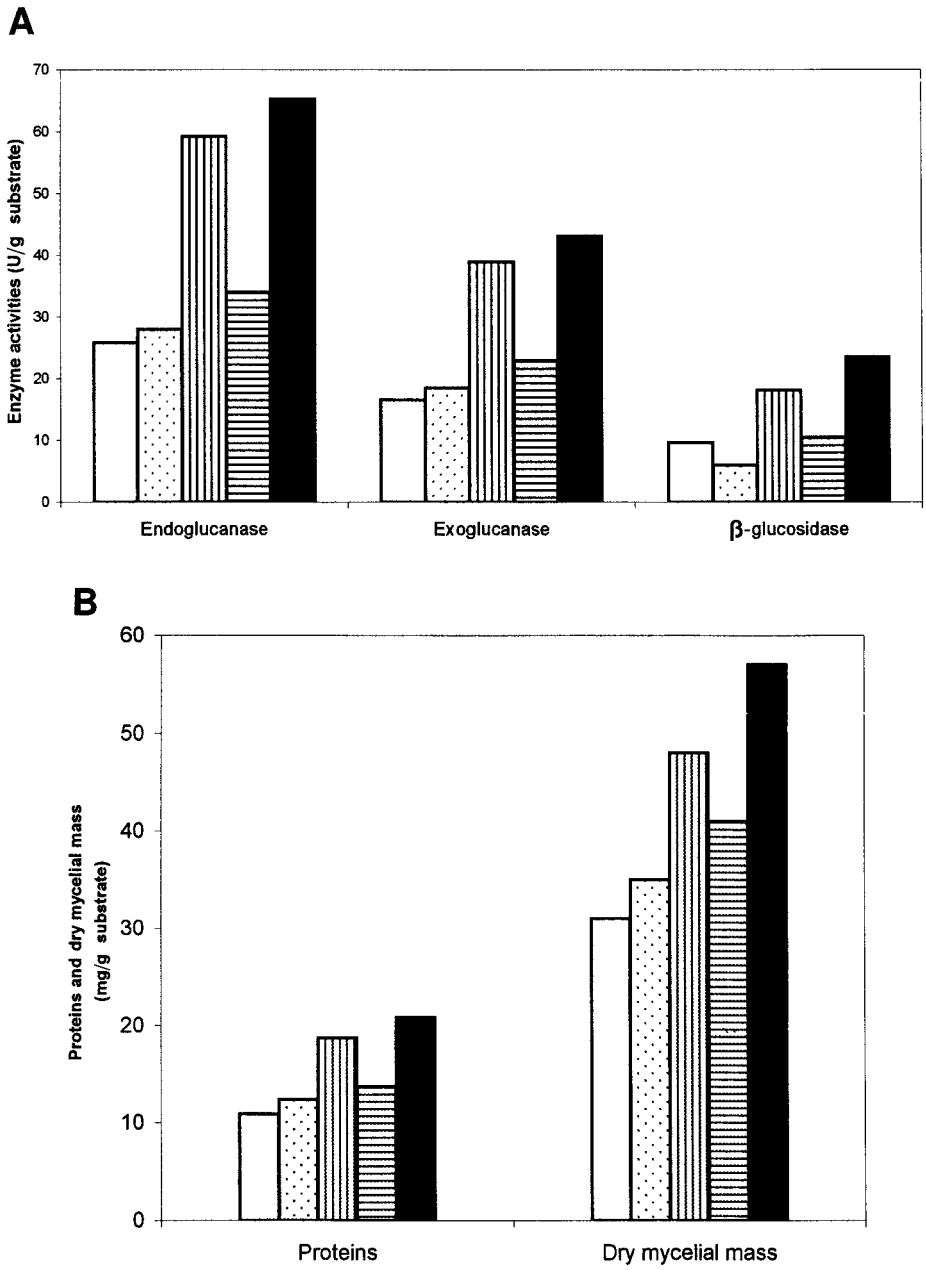

Fig. 4. **(A)** Production of endoglucanase, exoglucanase, and β-glucosidase by single and cocultivation conditions of *A. niger* RK3 mutant and *T. reesei* MTCC164 in semi-solid-state fermentation. □, *A. niger* RK3; ▒, *T. reesei* MTCC164; ▥, UNSC-442; ▤, *A. niger* RK3 + *T. reesei* MTCC164; ■, UNSC-442 + *T. reesei* MTCC164. **(B)** Production of total soluble proteins and dry mycelial mass by single and cocultivation conditions of *A. niger* RK3 mutant and *T. reesei* MTCC-164 in semi-solid-state fermentation. □, *A. niger* RK3; ▒,*T. reesei* MTCC164; ▥, UNSC-442; ▤, *A. niger* RK3 + *T. reesei* MTCC164; ■, UNSC-442 + *T. reesei* MTCC164.

exoglucanase (43.10 U/g), and β-glucosidase (23.47 U/g) activities that were ~10, 11, and 29% higher compared with the enzymatic activities obtained with mutant UNSC-442 strain (Fig. 4A).

The higher enzyme activities were related to the increased total soluble protein concentration and the dry mycelial weight under cocultivation with mutant UNSC-442 strain and *T. reesei* MTCC164 as compared to the total soluble protein concentration or the dry mycelial weight obtained either from mutant UNSC-442 or with that of *T. reesei* MTCC164 under single cultivation condition (Fig. 4B).

Very little residual free liquid remains in the medium during fermentation, and, therefore, a concentrated form of enzyme may be obtained by semi-solid-state fermentation which is advantageous, whereas the enzyme becomes diluted in the submerged fermentation. The concentrated form of the enzyme obtained during semi-solid-state fermentation can be easier to process during purification than a diluted one.

Enzyme production can be improved further by coculturing of two or more microbial strain and has been used increasingly for enhanced enzyme production *(16,25,26)*. Improved cellulose hydrolytic activities have been observed by the cocultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus* in the semi-solid-state fermentation systems *(16)*. The present investigation has also indicated that *T. reesei* MTCC164 and *A. niger* RK3 together are more productive for cellulase and β-glucosidase production, and the levels of the enzymatic activities observed are higher as compared to the *T. reesei* and *Aspergillus phoemics* combination *(27)*.

A. niger RK3 used in our study is a natural isolate. This strain was subjected to UV and chemical mutagenesis, thus resulting in the mutant with significantly higher levels of the cellulase and β-glucosidase production. *T. reesei* MTCC164 when used alone accumulates cellobiose because of the lower β-glucosidase levels in the enzyme complex, and this may lead to the inhibition of the endo- and exoglucanase enzymes, thus retarding the overall rate of hydrolysis owing to catabolite repression. The inhibition may be eliminated to some extent by incorporating the β-glucosidase through *A. niger* under coculture conditions. *A. niger* RK3 and *T. reesei* MTCC164 can be cocultured productively with a significant increase in the levels of the endoglucanase, exoglucanase, and β-glucosidase activities, as observed in the present study. Thus, *T. reesei* MTCC164 and *A. niger* RK3 possess a high degree of synergism, and, in addition, the other related molecular factors and the metabolic regulation may lead to improved enzyme production. Further study in this significant area will help widen the biotechnologic approaches in utilizing lignocellulosic material for the generation of fuels and feedstock.

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