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Solid-State Fermentation of New Substrates for Production of Cellulase and Other Biopolymer-Hydrolyzing Enzymes

Scientific Note

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INTRODUCTION

With the realization that the use of leaded gasoline results in environmental pollution and can effect human health, interest in the use of ethanol either directly or as a gasohol is increasing. Production of ethanol from lignocellulosic biomass was commercially practiced during the world wars by acidic hydrolysis technique (*1,2*). However, these processes were found to be uneconomical after the war emergencies were over. An alternate route is the enzymatic hydrolysis of lignocellulosic biomass (*3*). However, the economics of the enzymatic hydrolysis process relies substantially on the cost associated with the production of cellulase enzyme (*4*). The liquid-state fermentation technique of cellulase enzyme production involves problems related to maintenance of cellulose concentration, inorganic nutrients, and pH (*5*). However, the authors feel that the solid-state fermentation (SF) technique may be more suitable for the large-scale

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production of cellulase enzyme (*6,7*). The cost of substrate and its potential for the induction and production of cellulase, along with other equally important enzymes, such as xylanase, β-1,3-glucanase, chitinase, and cellobiase enzymes, should be studied by selecting different cheap substrates in the SF process.

There are several latex- and resin-bearing plants that contain hydrocarbons and related compounds. These plants are called petrocrops, since these hydrocarbons and related compounds can be extracted from these plants by simple heptane extraction. In the present work, the spent residue (SR) obtained after heptane or hexane extraction was used as a substrate for the production of cellulase and related enzymes by the wheat litter decomposing fungi. The results are reported.

METHODS

The experimental techniques for the isolation of fungi (*6*), culture conditions (*6*), enzyme recovery (*6*), SF techniques (*6,8*), and enzyme assays (*6,8*) for estimation have been reported earlier (*6,8*). Petrocrops were soxhlet extracted (*9*) with heptane or hexane for 8 h. The spent residue obtained was used as a substrate for SF using different fungi (*6*). The enzyme assay was performed by using standard procedures (*6–8*) and the enzyme activities were reported in terms of mg reducing sugar produced/mL (*8*). The SF experiments were performed using SR (2 g) in a sterilized watch glass kept in a covered Petri dish maintaining sufficient humidity (*6,8*) at different temperatures. The enzyme was recovered using citrate buffer (*6,8*).

RESULTS

About 37 wheat litter decomosing fungi were isolated and examined for their cellulolytic activities. These were tested for their cellulase enzyme activity. Seven fungi, i.e., *Alternaria tennissima, Aspergillus flavus, A. luchuensis, Fusarium moriliforme, Penicillium chrysogenum, P. expansum* and *Trichoderma viride,* showed more than 1000 Filter Paper Decomposition (FPD) units/mg activity (*6*). Cellulase enzyme activity of *P. chrysogenum* was found to be the highest (2530 $\pm$ 230), and this was followed by *T. viride* (2370 $\pm$ 240) in terms of FPD units. *P. chrysogenum* was therefore selected for the present studies.

Atrocarpus integrifolia (stem) and *Plumeria rubra* (stem) were found to be suitable plants for the production of biocrude and ethanol (*8*). The SRs of these plants were also used as substrates for the production of cellulase enzyme by *P. chrysogenum* in SF techniques. Table 1 shows the FPD activities of the cellulase enzymes produced by using the SRs of petrocrops and

Table 1
Cellulase Production by *Penicillium Chrysogenum* in Solid Culture Using Spent Residues (SR)

Type of lignocellulosic residue	FPD activity, U/mg
A. integrifolia (leaf)—SR	3500 ± 250
A. interfolia (leaf)	1840 ± 350
Nerium indicum (stem)—SR	2145 ± 230
Plumeria rubra (stem)—SR	2858 ± 270
Carisa carandus (stem)—SR	2650 ± 358
Calotropis procera (stem)—SR	1850 ± 425
Ipomoea purpurea (stem)—SR	2150 ± 450
Plumeria acutifolia (stem)—SR	1760 ± 325
Helianthus annus (stem)	3187 ± 159
Bagasse	1257 ± 230
Bagasse—SR	2380 ± 195
Wheat straw	1457 ± 225
A. integrifolia (stem)	1780 ± 225
A. integrifolia (stem)—SR	3486 ± 530

SR—Spent residue obtained after heptane or hexane exraction.
FPD activity—filter paper decomposition activity.

by using agroresidues, such as bagasse and wheat straw. The FPD activity of the cellulase enzyme produced by using *A. integrifolia* (3486 ± 530 and 3500 ± 250) was found to be the highest (Table 1). In general, petrocrop SRs were found to be better substrates for the production of cellulase enzyme.

Heptane extraction was a beneficial pretreatment for the production of cellulase enzyme (Table 1). The optimum pH for the maximum activity and extracellular protein production was 5.5, and fermentation time was 5 d. The effect of temperature on growth and enzyme production of *P. chrysogenum* through the SF process is shown in Fig. 1 in terms of FPD activity and extracellular protein produced. The loss in SR increased up to 5th d of fermentation, and then it decreased. Maximum loss in SR was observed when the SF was performed at 35 and 45°C (Fig. 1). Temperatures above 45°C showed inhibitory effects (Fig. 1). During the SF process, *P. chrysogenum* also excreted xylanase and chitinase enzymes. However, activities of these enzymes were maximum at the late lag phase of *P. chrysogenum*. The cellobiase and β-1,3-glucanase enzymes showed maximum activities during the 5th d of the SF process.

Table 2 shows the effect of addition of various nutrients i.e., carbon compounds, such as, glucose, maltose, corn starch, sucrose, and distillery effluents, and $MgCl_2$, $CaCl_2$, and NaCl on activities of xylanase, β-1,3-glucanase, chitinase, and cellobiase enzymes produced by *P. chrysogenum*

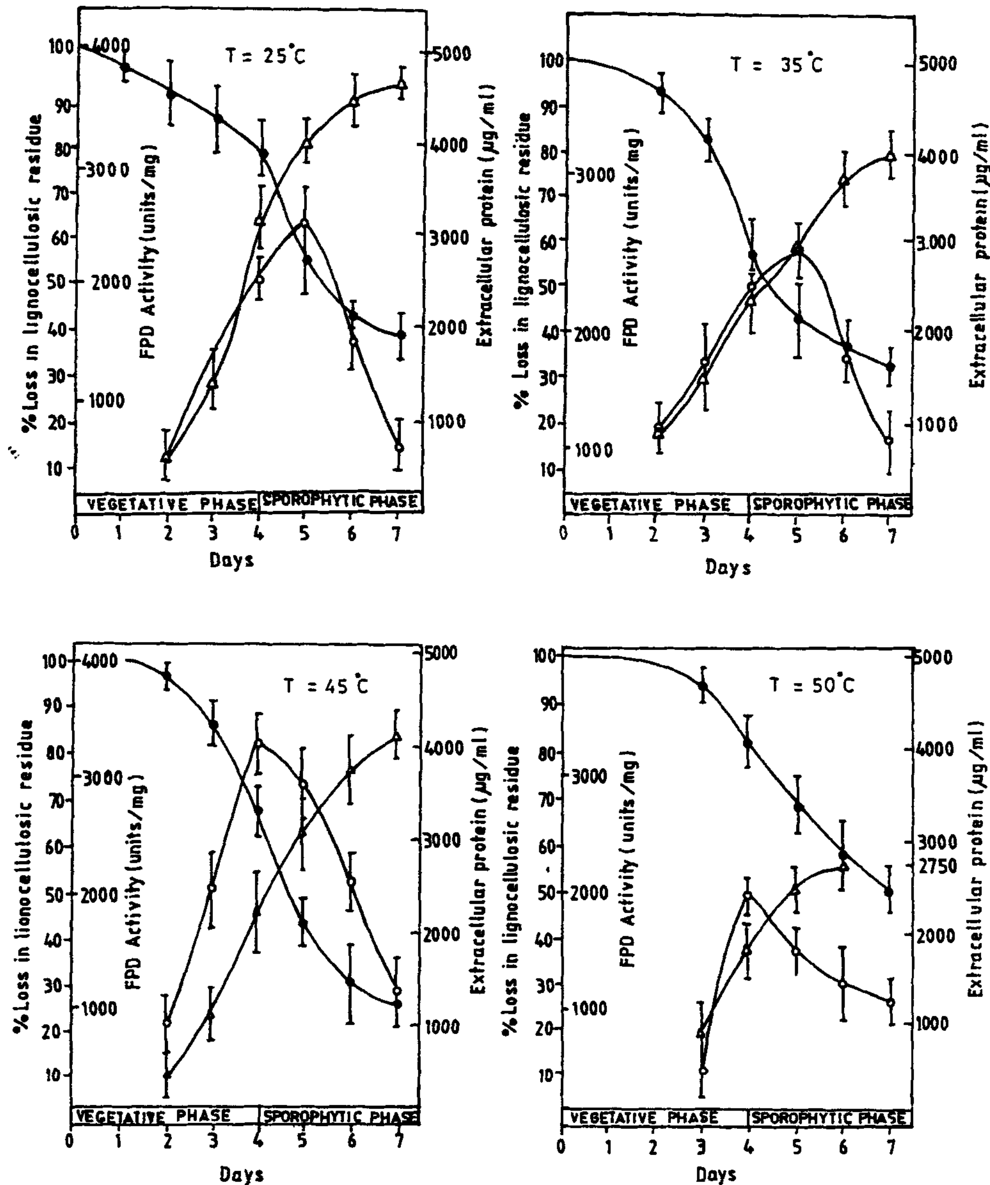

Fig. 1. Relationship of growth and cellulase enzyme production by *P. chrysogenum*. ● % Loss in lignocellulosic residue, ○ specific enzyme production rate, and △ extracellular protein.

in the SF process. Glucose was found to be the most effective nutrient for promoting activity of the glucanase enzyme. Interestingly, distillery effluent was found to be an even more effective nutrient than glucose for promoting activities of cellulase, xylanase, chitinase, and cellobiase enzymes. Behera and Misra (*10*) had reported that various types of cations, ammonia, and organic nitrogenous compounds, such as soluble proteins and amino acids, are present in the distillery effluents, which can promote the production of active enyzmes in the SF process.

Table 2
Influence of Cellulosic Substrate, Sugar Mill Effluent, and Inorganic Nutrient on Enzyme Activities Produced by *Penicillium Chrysogenum* in (Spent Residue) (*P. Rubra*) Solid Culture Medium

Growth substrate	Cellulase, U/mg	Xylanase, U/mL	β-1,3-Glucanase, U/mL	Chitinase, U/mL	Cellobiase U/mL
SR	3460	45.7	6.3	1235	0.15
SR + glucose	4515.3	47.35	27.1	1265.87	0.1504
SR + maltose	4169	22.14	14.35	1352.32	0.16
SR + cornstarch	Nil	Nil	Nil	Nil	Nil
SR + sucrose	3766.3	13.84	11.9	1327.62	0.1505
SR + effluent (1:5, v/v)	4671	59	7.71	1772.22	0.180
SR + $MgCl_2$	3539.58	5.82	1.594	1290.57	0.1508
SR + KCl	Nil	Nil	Nil	Nil	Nil
SR + $CaCl_2$	4055.12	22.29	13.28	1401.72	0.165
SR + NaCl	3511.9	Nil	Nil	Nil	Nil

SR = *P. rubra* spent residue.
Standard deviation = ±5%.

DISCUSSION

Extraction of petrocrops with heptane can remove hydrocarbons, terpenoids, steroids, chlorophylls, waxes, resins, and so forth, which are compounds present in the lignocellulosic biomass. This would increase the porosity of biomass, and create more space and surface area for improved mass transfer and microbial penetration. The concentration of cellulose increases in the SR as a result of removal of extractives. The cementing materials between lignocellulosic biomass will be removed and loosened. The folds of cellulosic fibers are unfolded and loosened for increasing their accessibility by *P. chrysogenum*. The continuous increase in the extracellular protein (2500 μg/mL) on the 5th d of SF with increase in temperature was the result of the release of cell-separating enzymes, avicelase, carboxymethyl cellulase, xylanase, cellobiase, chitinase, and so forth, during SF (Fig. 1). Recently, submerged fed-batch cultivation of cellulase producing fungi on lactose or whey-containing media has created interesting results (*4*) that are comparable to the present SF technique (*6,7*).

CONCLUSIONS

Based on above studies, the following conclusions may be drawn.

1. In comparison to raw petrocrops and agroresidues, petrocrop SRs are better substrates for the production of cellulase enzymes;
2. Removal of nonpolar extractives, such as hydrocarbons, terpenes, latex, steroids, waxes, resins, and so on, renders the SRs better substrates for the production of active cellulase enzyme;

3. The addition of distillery effluents to the petrocrop SRs results in enhancing the activity of cellulase, xylanase; β-1,3-glucanase, chitinase, and cellobiase enzymes produced in the SF process;
4. Solid-state fermentation of petrocrop SRs (*6,7*) is a simple technique (*6,7*) for the production of active cellulase, xylanase, β-1,3-glucanase, chitinase, and cellobiase enzymes by *P. chrysogenum*.
5. Future petrocrop industries may have an integrated process, where biocrude will be extracted from potential petrocrops and the SRs will be processed in two different units, i.e., for the production of cellulase enzyme by the SF process and for the enzymatic hydrolysis of (delignified) SRs to obtain fermentable sugars and ethanol (*1,4,7,8*) and;
6. The maximum FPD activity (3500 U/mg) was obtained by using *A. integrifolia* SRs in the SF process at 45°C for 5 d. During the SF process, *P. chrysogenum* also excreted xylanase, β-1,3-glucanase, cellobiase, and chitinase enzymes. These enzymes are also important for food, fuel, and carbohydrate industries.

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