Journal of Industrial Microbiology, 2 (1987) 9–13
Elsevier
SIM 00056

Production of mycelial protein and hydrolytic enzymes from paper mill sludges by cellulolytic fungi

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Received 28 July 1986
Revised 22 December 1986
Accepted 6 January 1987

Key words: Lignocellulosic waste; *Trichoderma*; Single cell protein production; Cellulase; Xylanase

SUMMARY

Characterization of lignocellulosic wastes from three paper mills in New York State indicated that a kraft mill sludge contained substantial quantities of utilizable cellulose and hemicellulose. This residue was tested as a carbon source for seven cellulolytic fungi. *Trichoderma reesei* DAOM 167654 accumulated a product of over 22% crude protein, and caused a conversion of sludge to protein of almost 15% in 3 days growth in shake flasks. *T. reesei* also produced the highest levels of cellulase, while *T. longibrachiatum* produced more xylanase (35 units/ml) than other fungi examined.

INTRODUCTION

A serious disadvantage to the use of most lignocellulosic material as substrate(s) for single cell protein (SCP), hydrolytic enzyme production, or saccharification to component carbohydrates is the need for costly pre-treatment [14].

Waste sludges from pulp and paper mills represent a potential source of lignocellulosic material which has been rendered accessible to enzymatic attack by the pulping process [10]. Sludges, which are generally dewatered to 20–40% solids before disposal in landfills, are composed of wood fibers of varying length and paper additives such as clay, starch, gelatin, TiO_2 and lime. The quantity and composition of the lignocellulosic fraction, and its susceptibility to enzymatic attack, are largely dependent on the type of pulping process utilized. A common pulping process, known as kraft pulping, makes use of sodium sulfide and sodium hydroxide to delignify wood. Approximately 41 kg of sludge are generated in the production of 1000 kg of paper at a typical paper mill in the United States [4].

In this study, sludges from three pulp and paper mills in northern New York State were characterized for potential use as substrates for SCP and hydrolytic enzyme production. Primary sludge from one of these mills was tested as a growth substrate for production of SCP, cellulase and xylanase by seven cellulolytic fungi.

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MATERIALS AND METHODS

Materials

Sludge A was a primary sludge obtained from a mill producing and utilizing kraft pulp from hardwood and softwood. Sludge B was obtained from a mill using a mixture (3:2) of sulfite pulp from hardwood and kraft pulp from softwood. Sludge C was a mixture of primary and secondary wastes obtained from a mill utilizing both mechanical pulp from softwood and kraft pulp from hardwood. The sludges were obtained in a partially dewatered state, and were manually shredded, dried overnight at 65°C, and ground in a Wiley mill to pass a 20-mesh screen. The sludges were then mixed to insure uniformity before analysis or use as fungal growth substrates.

Organisms, media and culture methods

Trichoderma reesei DAOM 167654, *Trichoderma longibrachiatum* and *Scytalidium lignicola* were provided by Dr. Chun-Juan Wang (SUNY College of Environmental Science and Forestry). *Sporotrichum pulverulentum* was obtained from Dr. T. Jeffries (Forest Products Laboratory, Madison, Wisconsin), while *Talaromyces vermiculatus*, PS No. 3 and PS No. 4 were isolated from paper sludge by the authors. Fungi were maintained on potato dextrose agar (Difco, Detroit, MI) plates. The salts solution was that of Park [11] modified to contain, in g/L: $(NH_4)_2SO_4$ (1.4), yeast extract (0.5), and the following trace elements in μg/L: $FeSO_4$ (0.5), H_3BO_3 (0.6), $CoSO_4.7H_2O$ (0.24), $CuSO_4.5H_2O$ (0.02), $MnCl_2.4H_2O$ (0.02), $NaMoO_4.2H_2O$ (0.05), and $ZnSO_4.7H_2O$ (0.24). Culturing of fungi on sludge is described in the legend for Table 2.

Methods of analyses

Water- and ethanol/benzene (1:2)-extractable materials were determined by measuring the loss in weight after extraction for 24 h in a Soxhlet apparatus [18]. Ash content was estimated as the material remaining after heating at 500°C in a muffle furnace for 5 h. Cellulose was determined using the procedure of Updegraff [22]. Alkali-soluble material was measured according to TAPPI [19]. The residue remaining after hydrolysis with 72% H_2SO_4 [20] was subjected to the ashing procedure described above, and the material lost was measured as lignin.

Crude protein was estimated by multiplying Kjeldahl nitrogen [2] by 6.25. The filter paper cellulase assay was performed according to the procedure of Mandels et al. [5] except that enzyme solutions were diluted with sodium citrate buffer (0.05 M, pH 4.8) to produce between 0.7 and 1.2 μmol of reducing sugar as glucose in the assay. The xylanase assay was identical to the carboxymethyl cellulase assay of Mandels et al. [6] except that oat spelt xylan (Sigma) was used as substrate, and enzyme solutions were diluted with buffer to produce between 0.7 and 1.2 μmol of reducing sugar as xylose in the assay. Activity in the assays was expressed as micromoles of reducing sugar as glucose or xylose, measured by the dinitrosalicylic assay procedure of Miller [7], released per min per ml of undiluted enzyme.

Duncans multiple range test (performed using the computer package program provided by the SAS Institute, Inc., Cary, North Carolina, release 82.3B at Syracuse University) was used to determine the significance of differences in enzyme and crude protein yield among the fungi examined.

RESULTS

Substrate characterization

Table 1 shows the compositional analyses of the three paper sludges. The ash content was high in all of the sludges, particularly sludge C. This material was a mixture of primary and secondary sludges, and therefore contained more nitrogen than the other two residues. Initial pH levels of autoclaved solutions (1.0% (w/v) sludge in salts) were within a satisfactory range for fungal growth. Major differences in cellulose and lignin content were found among the three sludges. Sludge A contained more cellulose and less lignin than the other two residues, while sludge C contained the highest amount of lignin and lowest amounts of cellulose. Sludge B was intermediate in the amounts of both components. The concentration of hemicellulose and degraded

Table 1

Paper waste characterization

	Sludge A	Sludge B	Sludge C
Wood used and pulping process	Hardwood (kraft) Softwood (kraft)	Hardwood (sulfite) Softwood (kraft)	Softwood (mechanical)
Type of sludge	Primary	Primary	Primary plus secondary
% Ash[a]	24.4 ± 0.1	23.0 ± 0.0	38.0 ± 0.1
% Total Kjeldahl nitrogen	0.14 ± 0.01	0.14 ± 0.00	0.87 ± 0.01
% Crude protein[b]	0.90 ± 0.04	0.86 ± 0.02	5.43 ± 0.05
pH (1% w/v with salts)	6.6	4.9	5.2
% Hot water extractable[c]	2.0 ± 0.0	7.0 ± 0.3	6.9 ± 0.1
% Ethanol/benzene[d]	3.1	2.7	5.7
1% Alkali-soluble[e]	16.8	14.5	20.6
% Cellulose	52.0 ± 0.6	33.7 ± 1.3	16.7 ± 0.8
% Acid-insoluble lignin	3.5 ± 0.0	11.3 ± 0.3	19.75 ± 0.4

[a] Ash is mainly clay filler and boiler ash.
[b] % Crude protein is total Kjeldahl nitrogen × 6.25.
[c] Hot water extractables include: tannins, resins, gums, starches, sugars and coloring matter.
[d] Ethanol/benzene (1:2) extractables include: waxes, lipids and some resins and gums.
[e] Alkali-soluble material consists of hemicellulose and degraded cellulose.

cellulose (as estimated by percent alkali-soluble material) was between 14 and 21% in all three sludges.

Because of its high cellulose and low lignin content, sludge A was selected for testing as a substrate for mycelial protein and hydrolytic enzyme production (Table 2). All fungal products contained higher percentages of crude protein after 7 days' growth than after 3. However, the percent conversion of sludge to protein (a reflection of quantity of crude protein) decreased for all fungi, except PS-4, during this time period. *T. reesei* mycelium contained the highest percentage of crude protein after both incubation periods. Highest quantities of protein were found in the mycelia of *T. reesei* and *S. lignicola* after 3 days' growth. *T. reesei* generated the highest levels of cellulase activity, while *T. longibrachiatum* produced significantly higher amounts of xylanase than the other fungi examined.

DISCUSSION

The large differences in cellulose, lignin, and ash content of the sludges are consistent with the variability found by Pamment et al. [10] and Harkin et al. [3] and are explained by the different pulping methods and pulps utilized. High ash content lowers the value of sludge as a carbon source, but may have additional effects on growth by providing trace elements and buffering, by chelating metals and fungal metabolic products, and adsorbing cellulase enzymes [1].

The high yield of protein from sludge A relative to alpha cellulose [13] demonstrates the increased susceptibility of the chemically pulped fibers present in sludges to enzymatic attack. Despite the high ash content of sludge A, several of the fungi (particularly *T. reesei*) accumulated biomass at levels comparable wth other microorganisms grown on

Table 2

Mycelial protein and extracellular enzyme production by fungi grown on sludge

Flasks (0.80 g sludge A plus 80 ml salts solution) were incubated on a reciprocating shaker (80 oscillations/min, 4 inch oscillation) at 28°C. *S. pulverulentum* was inoculated with a 1 mm disc from an 8 day PDA plate colony. Other fungi were inoculated with 4 ml of mycelium ground for three 10-s bursts at high speed in an Omnimixer unit (Sorvall, Norwalk, CT) from an 8 day disc inoculated culture grown on the identical medium. Flask contents were filtered through glass-fiber filter discs (Whatman 934-AH) and the filtrate used for the enzyme assays. Mycelium was washed and dried (65°C overnight) before analysis. Values shown are the mean of three replicates. Means within the same column with the same letter are not significantly different (DF = 14, α = 0.05) as determined by Duncan's multiple range test.

Fungus	Percent protein[a]		Percent conversion of sludge to protein[b]		Filter paper cellulase (units/ml)		Xylanase (units/ml)	
	3 day	7 day	3 day	7 day	3 day	7 day	3 day	7 day
T. reesei	22.4A	25.2A	14.9A	12.8AB	0.08A	0.22A	8.4B	8.7BC
T. longibrachiatum	18.6C	22.0B	13.0B	12.6B	0.03C	0.10C	34.1A	35.1A
S. pulverulentum	17.3D	20.8BC	11.8C	9.4D	0.05B	0.18B	3.0CD	9.6B
S. lignicola	19.8B	21.6B	14.7A	13.5A	0.03C	0.04D	4.0C	7.8BC
T. vermiculatus	17.7CD	20.2BC	13.1B	12.1B	0.02CD	0.04D	1.9CD	2.9CD
PS No. 3	14.2E	19.1C	10.6D	9.3D	0.02CD	0.17B	3.0CD	9.0BC
PS No. 4	1.0F	15.0D	0.9E	10.6C	0.01D	0.02D	0.3D	0.8D

[a] Percent protein = (wt. crude protein/wt. mycelium) × 100.

[b] Percent conversion = (wt. crude protein/initial wt. of substrate) × 100.

low ash substrates [3,9,10,17]. Crude protein yields were higher than those obtained when *Chaetomium cellulolyticum* was cultured in shake flasks on a paper sludge of similar composition [10]. It should be remembered, however, that crude protein is merely a convenient estimate for protein, and measures indigestible chitin, and nucleic acids, which have negative value as a food source. *C. cellulolyticum* is an acceptable food supplement for rats, and contains a favorable amino acid balance [9], aspects not examined in this study. Mycelia of *T. viride* [12], *S. pulverulentum* [21] and *T longibrachiatum* [17] have been shown to be suitable food sources in animal feeding trials.

T. reesei produced the highest levels of cellulase of the fungi examined, though yields were far lower than those of hypercellulolytic mutants of *T. reesei* [8]. Sandhu and Kalra [16] also noted significant xylanase generation by *T. longibrachiatum*, and recent research in our laboratory [15] has shown that production of xylanase by *T. longibrachiatum* can be improved significantly through manipulation of culture conditions.

A logical follow-up to the described experiments would be scale-up studies to extend our laboratory information to future applications. Also, the nutritional value of fungal mycelium generated from paper sludges must be determined. The presence of impurities in paper sludge may be less important in enzyme generation for the purpose of achieving fermentable sugars from polysaccharides. This concept may have particular application for paper sludges containing high concentrations of ash, paper additives, and other impurities.

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