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Lignocellulolytic Enzymes Produced by *Volvariella volvacea*, the Edible Straw Mushroom

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Volvariella volvacea, commonly known as the straw or paddy mushroom, had the following growth characteristics: minimum temperature, 25°C; optimal temperature, 37°C; maximal temperature, 40°C; pH optimum 6.0. Optimal pH for cellulase production was 5.5. The optimal initial pH for cellulase production and mycelial growth was found to be 6.0. The pH and temperature optima for cellulolytic activity were 5.0 and 50°C, respectively. Maximal cellulolytic activity was obtained within 5 days in shake-flask culture. The cellulases were found to be partly cell free and partly cell bound during growth on microcrystalline cellulose. The endoglucanase activity was primarily extracellular, and β-glucosidase activity was found exclusively extracellularly. Weak cellulase activity was detected when cells were grown on cellobiose and lactose. *V. volvacea* could not digest the lignin portion of newspaper in shake-flask cultivation. Phenol oxidase, an important enzyme in lignin biodegradation, also was lacking in the cell-free filtrate. However, the organism oxidized phenolic compounds when it was cultured on agar plates containing commercial lignin.

Cellulose occurs as the structural element of plants and is thus present as a major component in agricultural and municipal waste and is the earth's most abundant renewable resource. Unfortunately, humans and most other animals except for the ruminants cannot digest it. Before the use of this resource, it is necessary to convert it to usable forms, such as glucose, methane, ethanol, single-cell protein, or other feedstock that will fit into the economy. However, there are always two main obstacles hindering the efficient transformation of cellulose: (i) the highly ordered crystalline cellulose structure and (ii) a lignin seal usually surrounding cellulose fibers.

Volvariella volvacea, known from ancient times, is commonly called paddy mushroom, straw mushroom, banana mushroom, or Chinese mushroom. It has been cultivated successfully on cotton waste, a relatively crystalline cellulose (3). This implies that the organism may have the potential to produce high exoglucanase activity to degrade crystalline cellulose. If "lignase" activity could be clearly demonstrated, both crystallinity and lignification problems could be overcome simultaneously. This study was undertaken to provide essential information on quantitative production of cellulase and lignase by *V. volvacea*.

For simplicity and economy, it is practical, especially in the developing countries, to grow microbial cells on lignocellulose waste for human or animal food and produce enzymes and glucose in a unified and continuous process. Producing mushrooms on lignocellulose waste is a good approach to achieve this goal. By this method, the fruiting body can be harvested as a food commodity, and the extracellular enzymes produced on the cellulose material can be used later for the production of sugar through saccharification in the reactor. Since cellulase is strongly absorbed to cellulose at high concentration of substrate (9, 14), the unhydrolyzed residual cellulosic mushroom bed can be put into the reactor without removing the enzyme from the cellulose. Newsprint, because of its high cellulose content, consistent composition, and ready availability was chosen as one source of lignocellulose in this study.

MATERIALS AND METHODS

Culture. *V. volvacea* (NRRL 3778) was supplied by Northern Regional Research Center, Peoria, Ill. Stock cultures of the cells were grown and maintained on Difco mycological media slants in 70-ml screw-capped tubes and transferred biweekly.

Preparation of inoculum and cultivation. Stock cultures from slants were inoculated into 100 ml of modified Mandels and Reese medium (20) in 500-ml Erlenmeyer flasks described below. They were incubated in a New Brunswick rotary shaker at optimal growth temperature, which was determined to be 37°C at a speed of 180 rpm. After 4 days of incubation, the submerged culture was transferred to sterile 400-ml Omnimixer cylinders and comminuted 2 min at a

moderate speed with the cylinder immersed in an ice bath. Two milliliters of the inoculum was uniformly applied to a fresh medium with a 10-ml pipette. The cells were activated again in the same way and were ready for the experiments.

Medium. The basal medium for submerged culture consisted of: $(NH_4)_2SO_4$, 1.4 g; KH_2PO_4, 2.0 g; urea, 0.3 g; $CaCl_2$, 0.3 g; $MgSO_4$, 0.3 g; NaCl, 0.1 g; $FeSO_4 \cdot 7H_2O$, 5.0 mg; $MnSO_4 \cdot H_2O$, 1.6 mg; $CuSO_4$, 2 mg; $ZnSO_4 \cdot 7H_2O$, 1.4 mg; $CoCl_2$, 2.0 mg; distilled water to 1,000 ml. Proteose peptone, (0.075%), 0.2% Tween 80, and 0.1% yeast extract were added to the basal medium. The initial pH was adjusted to 6.0, which was determined to be optimal for cell growth and cellulase production. Avicel microcrystalline cellulose (1% grade PH 105; FMC Corp., Philadelphia) or pulverized unprinted Finger Lakes Times of Geneva, New York was used as carbon source. The end roll of the newsprint was pulverized in a Buffalo hammer mill with a ¼-in (0.635 cm) mesh size and then passed through a Microjet ultracentrifugal mill (Micro Material Corp., Westbury, N.Y.) with 0.5 mm as final particle size.

Determination of optimal growth temperature. In the study of temperature, 1 drop of evenly dispersed mycelial suspension was inoculated on the center of Difco mycological agar plates by a disposable pipette (24 drops equal to 1 ml). They were incubated at 20, 25, 30, 35, 37, 40, and 45°C, respectively. Each temperature had five duplicate plates. The diameter of the colonies was used as a rate of growth measurement over a period of time.

Preparation of extracellular enzyme extract and biomass. The cell-free enzyme extract was harvested by filtering through a glass fiber filter (Reeve Angel 934 AH; Whatman Inc., Clifton, N.J.) with the aid of suction. Loss of water due to evaporation from shake flasks was compensated for by adding distilled water before running the assay. The extract broth was divided into small plastic vials and frozen immediately at −10°C. The mycelial cells and nondegraded cellulose were washed with 300 ml of distilled water and then were dried in a vacuum oven at 80°C overnight and stored in a desiccator. Biomass was expressed as mycelial nitrogen, which was determined by the Kjeldahl method (6, 18) with Kel-pak no. 5 (Curtin Matheson Scientific, Inc., Houston, Tex.) used as a catalyst.

Protein determination. The extracellular soluble protein was measured by the method of Lowry et al. (8) after precipitation with 10% trichloroacetic acid. Crystalline bovine albumin (Calbiochem, La Jolla, Calif.) served as the standard.

Cellulase enzyme assay. (i) Filter paper activity assay. Filter paper activity was determined by the method of Mandels et al. (11). Dinitrosalicylic acid reagent was prepared as described by Miller (13). Filter paper activity was expressed as milligrams of glucose released per hour per milliliter of filtrate.

(ii) β-1,4-glucan cellobiohydrolase or Cl enzyme (EC 3.2.1.74) assay. By the method of Mandels et al. (12), exoglucanase activity was measured by incubating 2.5 ml of enzyme broth and 2.5 ml of 0.1 M citrate buffer (pH 5.0) with 250 mg of absorbant cotton in a 50°C water bath for 24 h. Dinitrosalicylic acid reagent (3 ml) was used to determine reducing sugar. One unit of Cl activity was expressed as 1 μmol of glucose produced per day.

(iii) β-1,4-glucan glucanohydrolase or carboxymethyl cellulase (EC 3.2.1.4) assay. β-1,4-glucan glucanohydrolase was assayed by mixing 0.5 ml of proper enzyme dilution (to fall within the linear portion of the standard curve) with 0.5 ml of 1% carboxymethyl cellulose (Sigma Chemical Co., St. Louis, Mo.) solution in 0.1 M citrate buffer (pH 5.0) and incubated for 10 min at 50°C in a water bath with moderate shaking (12). Reducing sugar was measured with dinitrosalicylic acid reagent. One unit of enzyme activity was expressed as 1 μmol of glucose liberated per min.

(iv) Cellobiase or β-1,4-glucosidase (EC 3.2.1.21) assay. Cellobiase activity was assayed by incubating 0.2 ml of enzyme extract with 2 ml of 10 mM D-cellobiose solution in 0.05 M citrate buffer (pH 5.0) at 50°C for 1 h. The reaction was stopped by placing the mixture in boiling water for 5 min. The amount of glucose liberated was measured by glucose oxidase reagent (Sigma Chemical Co.). One unit of enzyme activity was expressed as 1 μmol of glucose released per h.

(v) Aryl-β-glucosidase assay. Activity toward *p*-nitrophenyl-β-D-glucoside (PNPG) (Sigma Chemical Co.) was estimated by measuring spectrometrically the release of *p*-nitrophenol from PNPG. Enzyme extract (0.5 ml) was incubated with 2 ml of 2 mM PNPG solution in 0.05 M sodium acetate buffer (pH 5.0) for 10 min. The enzyme activity was stopped by adding 2 ml of 1 M sodium carbonate solution. The yellow color developed during the hydrolysis of the substrate was read at 405 nm in a Cary 219 spectrophotometer. One unit of enzyme activity was expressed as 1 μg of *p*-nitrophenol produced per min. Lignase enzyme assay activity was estimated by the following methods. (i) Gravimetric method: lignase activity was determined by calculating the difference in the lignin content in newsprint inoculated with *V. volvacea* and the uninoculated substrate. Lignin was expressed as acid-insoluble lignin and was determined as described by Browning (1). (ii) Polyphenol oxidase assay: polyphenol oxidase activity was determined by the method of Kirk and Kelman (7). Enzyme extract (1.0 ml) mixed with 1 ml of 40 μM substrate in 1 ml of distilled water, 3 ml of 0.1 M citrate-phosphate buffer at pH 5.5 was incubated in a water bath at 40°C for 1 h. The substrate was either guaiacol or catechol. Polyphenol oxidase activity was expressed as the amount of absorbance change of the product at 465 nm for guaiacol and 395 nm for catechol per ml of filtrate per h. (iii) The oxidase test on the agar plate was done according to the Bavendamm method described by Davidson et al. (5) and the Sundman and Nase method (19) with 0.05% (wt/vol) Indulin AT (a technical lignin; Westvaco Co., North Charleston, S.C.). The Indulin AT was washed with water to remove low-molecular-weight contaminants and then was incorporated into the basal agar medium.

Location of cellulase. *V. volvacea* was cultivated in 100 ml of medium in 500-ml Erlenmeyer flasks as described earlier. Microcrystalline cellulose (0.5%; grade PH 105, FMC Corp.) or carboxymethyl cellulose (Sigma Chemical Co.) was used as primary carbon source. After 108 h of growth, the cells reached the early stationary phase, and the insoluble microcrystalline cellulose residue was completely absent when checked by light microscope. The mycelia were harvested by filtering through a Buchner funnel with

TABLE 1. Effect of temperature on growth of *V. volvacea*

Temp. (°C)	Diam of colony (cm) after (h):	
	48	84
Trial 1		
20	0.00 ± 0.00	0.00 ± 0.00
25	1.08 ± 0.18	1.62 ± 0.08
30	1.60 ± 0.07	3.68 ± 0.20
35	2.60 ± 0.22	6.08 ± 0.26
40	2.32 ± 0.38	6.64 ± 0.25
45	0.00 ± 0.00	0.00 ± 0.00
Trial 2	After 72 h	
35	5.13 ± 0.22	
37.5	6.13 ± 0.05	
40	5.38 ± 0.20	

fritted disk (porosity fine). The filtrate was free from the cells. The volume of the filtrate was measured with graduated cylinders. The mycelia were washed with 0.05 M citrate buffer (pH 5.0) until the filtrate was free from cellulase activity. The mycelia were suspended in 50 ml of 0.05 M citrate buffer (pH 5.0) and blended in an Omnimixer in an ice bath three times for 3-min intervals at high speed. Sonification of cells was done with a Biosonik IV (Bronwill Scientific Inc., Rochester, N.Y.) operated at maximal power for a total of 10 min with mycelium in an ice-cooled plastic beaker. The temperature did not rise to more than 18°C during the treatment. Half of the sample was treated with 0.2% (vol/vol) Triton X-100 (Sigma Chemical Co.). The suspension was checked under the light microscope. Most of the cells were broken, and the cell fragments and scattered cell constituents could be seen. The suspension was centrifuged in a Beckman L2-65B preparative ultracentrifuge at 30,000 × *g* for 1 h to remove debris. The supernatant was carefully removed, and enzyme activity was assayed as described earlier.

Solid fermentation and saccharification. The end roll of unprinted Finger Lake Times of Geneva was shredded, hydrated, supplemented with 10% wheat bran and 5% $CaCO_3$ and placed in 10-lb (4.5 kg) enameled cans and autoclaved at 121°C for 1 h. A 1,000-ml amount of culture cultivated on cellulose medium (as described earlier) on a New Brunswick shaker in a 2.8-liter Fernbach flask for 4 days at 37°C was inoculated into each can. After 3 days of incubation at 35°C, the can lids were opened and covered with plastic bags with six small holes. The cans were moved to a greenhouse. Daily watering was done to keep the bed moist. After 8 weeks of incubation, the whole mushroom beds were lyophilized in a Virtis freeze dryer and pulverized in a Wiley mill (model 4; Arthur H. Thomas Co., Philadelphia, Pa.) using a 2-mm mesh screen. A 15-g amount of the ground samples was mixed with 300 ml of 0.05 M citrate buffer (pH 5.0) in 500-ml Erlenmeyer flasks capped with a stopper and incubated in a water bath shaker at 50°C for 24 h. The suspension was centrifuged 5 min. A 0.3-ml amount of the supernatant was removed for sugar content estimate with dinitrosalicylic acid reagent.

RESULTS AND DISCUSSION

V. volvacea could grow from 25 to 40°C (Table 1). The mycelium did not grow at all when the temperature was raised to 45 or lowered to 20°C. Active growth occurred from 35 to 40°C with maximal growth at 37°C. The optimal temperature differed from those of Chandra and Purkayastha (2) and Chang-Ho and Yee (4), who recorded 30 and 35°C as optimum, respectively. One unique characteristic of *V. volvacea* was that it did not remain viable when stored at refrigerator temperatures in our laboratory.

V. volvacea is capable of growing in a fairly wide range of pH (Fig. 1). The highest mycelial yield was obtained at pH 6.0. The yield dropped rapidly at pH 5.0. Very little mycelium was obtained at pH 4.5. The optimal growth pH also differed from those of Chandra and Purkayastha (2) and Chang-Ho and Yee (4), who reported 5.5 and 7.0, respectively.

Figure 2 illustrates an optimal pH plateau for cellulase activity of *V. volvacea*. It exists from pH 4.6 to 5.6. pH 4.8 to 5.0 gave the highest activity; therefore, this pH was used for enzyme assay. The optimal temperature for filtrate activity was 50°C. This was much higher than the optimal growth temperature (37°C) of *V. volvacea*. At 37°C, only 50% of maximal filtrate activity was attained.

Our preliminary experiments showed that a supplement of 0.1% yeast extract to Mandels and Reese medium yielded twice as much cellulase and mycelium as the medium without yeast extract. We also found that Tween 80 (sorbitan polyoxyethylene monooleate), a nonionic surface-active agent, increased the cell yield twofold, whereas it enhanced the enzyme yield from 10- to 15-fold when 1% microcrystalline cellulose was in the medium. The 10- to 15-fold-higher enzyme production accompanied by the twofold mycelial yield in the stationary growth phase strongly implies that the primary effect of Tween 80 was on enzyme release through the membrane. This substantiates the findings of Reese and Reese and Maguire (15–17). However, *V. volvacea* failed to grow in a medium containing 0.05% (vol/vol) Triton X-100, a nonionic detergent stronger than Tween 80.

The course of cellulase production and the pH profile in the shake-flask cultures inoculated with active suspension of cells are shown in Fig. 3. After a lag of about 36 h, the enzymes began to appear in the medium, and the pH fell rapidly. *V. volvacea* produced protease on skim milk agar plates. Since the medium had proteose peptone as an organic nitrogen source, it probably was used preferentially by the organism. Deamination of amino acids from peptone releases ammonia. This caused the pH to rise in

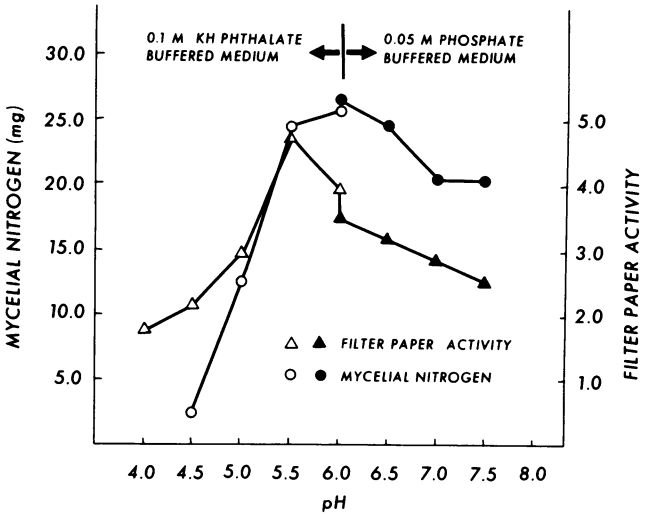

FIG. 1. Effect of pH on growth and cellulase production on *V. volvacea* in buffered medium. Cells and enzyme broth were harvested after 72 h of inoculation.

the first 36 h after inoculation. As active growth occurred (36 h), rapid consumption of nitrogen and cellulose produced a large amount of acid, and hence the pH dropped abruptly. The pH rose gradually after 48 h, probably due to slow cell autolysis. The exponential phase extended to between 48 and 72 h. An exoglucanase activity of 1.16 μmol of glucose produced per ml of filtrate per day and an endoglucanase activity of 2.50 μmol of glucose produced per ml of filtrate per min were obtained within 5 days. The filter paper activity was 3.48 mg of glucose produced per h per ml of enzyme broth. Both extracellular cellobiase and aryl-β-glucosidase activity remained low during filtrate accumulation. Cellulase activity was closely correlated with the amount of protein in the culture filtrate. Reducing sugar was not detected in the culture. The cellulase production on newsprint resembled the pattern on microcrystalline cellulose (Fig. 4). In shake culture, *V. volvacea* produced dispersed suspensions of short mycelial threads, various sizes of pellets and balls, and sometimes large white mat-like pieces. The submerged cultivated mycelium had an earthy flavor and was not equivalent to the mushroom flavor possessed by the sporophore.

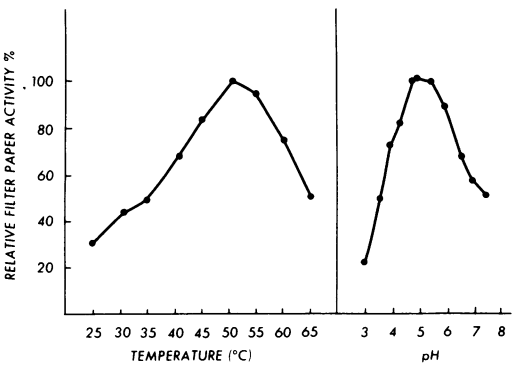

FIG. 2. Effect of temperature and pH on relative filter paper activity of cellulases produced by *V. volvacea*.

Most of the carboxymethyl cellulase and cellobiase were found to be cell free, with little bound to mycelia (Table 2). The enzyme yields were considerably less with soluble carboxymethyl cellulose than with insoluble microcrystalline cellulose as a substrate. Treatment of sonicated mycelia with 0.2% (vol/vol) Triton X-100 did not cause an increased activity in either enzyme. More cell-free carboxymethyl cellulase was found in medium containing Tween 80. Detectable amounts of glucose were released when cells were ruptured by sonication. Cellulase was induced in the presence of β-1,4-glucosidic linkage. The highest cellulase yield was obtained when growth was on insoluble microcrystalline cellulose. Weak cellulase activity was also detected when cells were grown on cellobiose and lactose.

V. volvacea failed to grow on either gallic acid or tannic acid malt agar in the Bavendamm test. Only very faint brown discoloration formed under the inoculum mat in gallic acid malt agar. There was no reaction at all in tannic acid malt agar. The organism grew quite well on Sundman

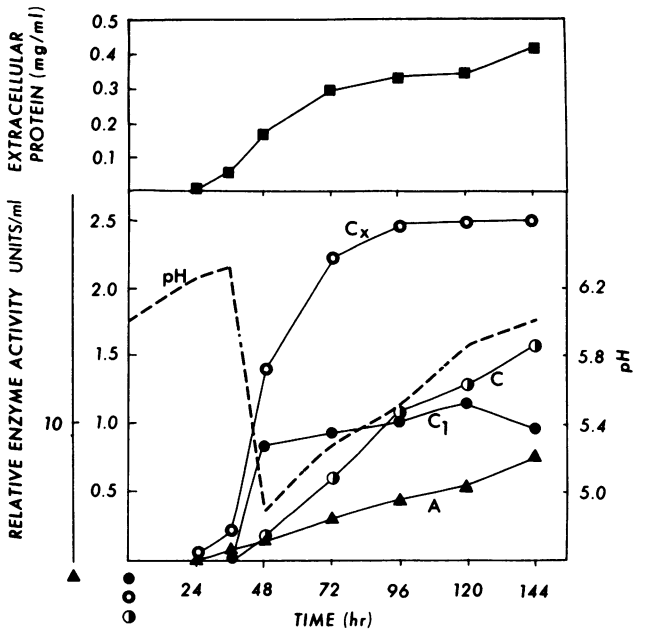

FIG. 3. Cellulolytic enzyme and pH development of *V. volvacea* on microcrystalline cellulose. Abbreviations: C1, exo-β-1,4-glucanase; Cx, endo-β-1,4-glucanase; C, cellobiase; A, aryl-β-glucosidase. Units of enzyme activity are defined in the text.

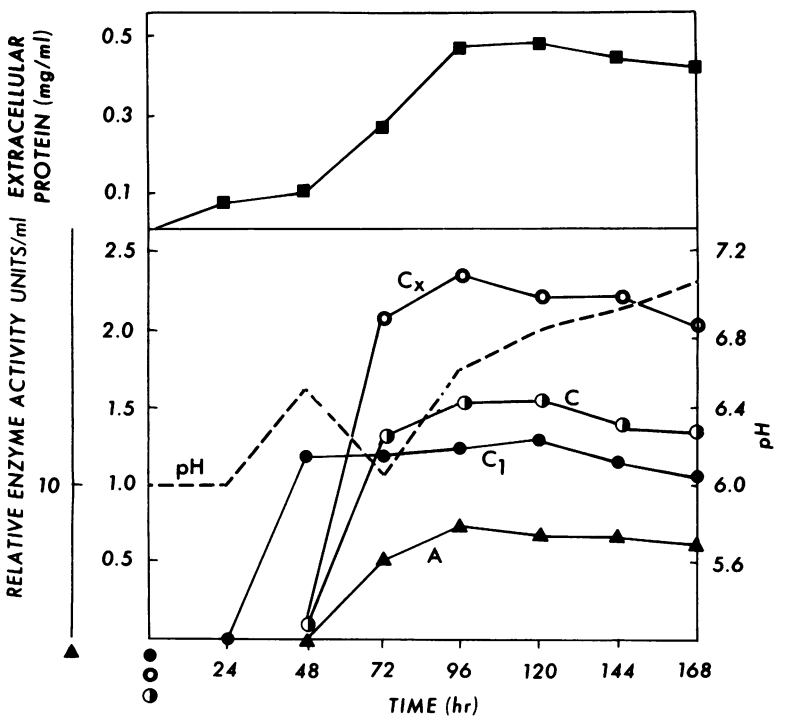

FIG. 4. Cellulolytic enzyme and pH development of *V. volvacea* on Finger Lakes Times newsprint. For abbreviations see the legend to Fig. 3.

TABLE 2. Distribution of enzyme activity of *V. volvacea*

Carbon source	Cell-free activity[a] in total culture fluid		Cell-bound activity[a] in total culture fluid		Cell-bound activity[a] after 0.2% Triton X-100 treatment	
	CMCase	Cellobiase	CMCase	Cellobiase	CMCase	Cellobiase
Microcrystalline cellulose[b]	18.7	31.3	2.5	1.8	2.5	1.8
Carboxymethyl[b] cellulose	6.1	0.4	Neglig.	Neglig.	Neglig.	Neglig.
Microcrystalline cellulose[c]	38	32.1	2.5	1.5	2.5	1.5
Carboxymethyl[c] cellulose	8.37	0.5	Neglig.	Neglig.	Neglig.	Neglig.

[a] Carboxymethyl cellulase (CMCase) activity is expressed as 1 mg of glucose produced per min. Cellobiase activity is expressed as 1 mg of glucose produced per h.
[b] Without Tween 80.
[c] With Tween 80.

and Nase agar plates. A clear zone around and under the fungus mat was shown after flooding with $FeCl_3$ and K_3 $Fe(CN)_6$) reagent, indicating that phenolic compounds were utilized to some extent by *V. volvacea*. However, the organism did not digest any of the lignin portion of newsprint in agitated submerged culture. The filtrate also showed a negative reaction toward guaiacol and catechol in the polyphenol oxidase assay. Approximately 5% (wt/wt) of reducing sugar could be produced per day from saccharification of dry mushroom bed in 0.05 M citrate buffer at pH 5.0 (Fig. 5). This was disappointingly low.

Although *V. volvacea* could hydrolyze 1% microcrystalline cellulose in shake flasks in 4 days, its cellulase activity in the cell-free filtrate was lower than the most active cellulase-producing strains of *Trichoderma reesei* (10). Activity toward cotton (C1 activity) was not of practical significance despite the fact that it could be cultivated on cotton waste in solid fermentation. Rapid decomposition of cellulose by the organism during growth was not necessarily associated with high cellulase production in the filtrate. The organism failed to degrade lignin in submerged shake culture. Quantitative studies of lignocellulase production in submerged cultures showed *V. volvacea* to be a poor lignocellulolytic organism.

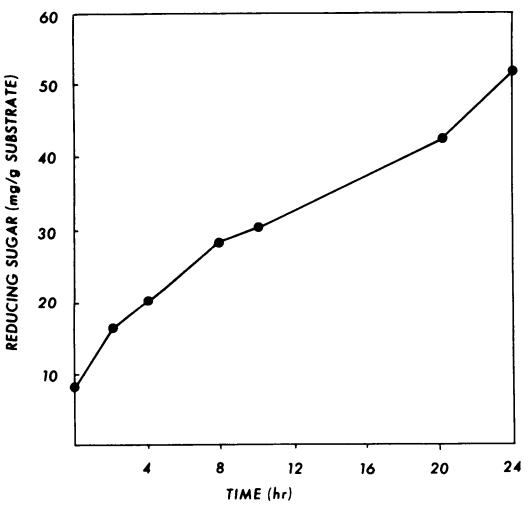

FIG. 5. Glucose production from mushroom bed in 0.05 M citrate buffer (pH 5.0).

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