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Single-Cell Protein Production by the Acid-Tolerant Fungus *Scytalidium acidophilum* from Acid Hydrolysates of Waste Paper†

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The bioconversion of waste paper to single-cell protein at pH <1 by *Scytalidium acidophilum* is described. Waste paper pretreated with 72% H_2SO_4 at 4°C was diluted with water to a pH of <0.1 and hydrolyzed. This yielded an adequate sugar-containing substrate for the growth of the fungus. A total of 97% of the sugars (glucose, galactose, mannose, xylose, arabinose) in the hydrolysates were converted to cell biomass. Microbial contamination was not observed. Based on the sugars consumed, *S. acidophilum* produced higher yields in shake cultures than many other *Fungi Imperfecti*. In aerated cultures, productivity increased, and yields of 43 to 46% containing 44 to 47% crude protein were obtained. This compares favorably with *Candida utilis*, a yeast used commercially to produce single-cell protein. The chemical constituents and the essential amino acids of the fungal cells were similar to those of other fungi. The nucleic acid content was characteristic of microbes containing low levels of nucleic acid. The advantages of using *S. acidophilum* for single-cell protein production are discussed.

Cellulose is the dominant constituent of agricultural, wood, and municipal wastes (15), and much research (17) has been devoted to the microbial conversion of these wastes to single-cell protein. A survey of the literature indicates that the following two major methods have been used: (i) inoculation of the wastes with a cellulolytic microbe (6, 7, 27, 29) and (ii) conversion of the cellulose to glucose by means of steam (2), acids (9, 18), alkali (8, 13), or cellulase (10, 23), followed by fermentation.

In the methods described previously, contamination is often a major problem, requiring costly maintenance of aseptic conditions (4, 6, 20, 27). The concern about contamination has been summarized by Callihan and Dunlap (4), as follows: "even if the encroaching organisms happen to be a nontoxic-chemosynthetic bacteria, it probably will not have been approved for food by FDA." In this study the problem of contaminants was minimized by using a fungus that grows well at pH values of <1.0, which is far below the optimal pH value for most microbes. Moreover, we chose to use waste paper as the substrate because it is the largest single constituent in municipal solid wastes and costs billions of dollars a year to collect and dispose of (29).

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MATERIALS AND METHODS

Organism. The fungus used in this study (strain ATCC 26774) was isolated originally by K.C.I. from the acid waters (pH 2.0) of a uranium mine (14). This isolate and two similar isolates (11, 26), also from acidic environments, have been described as a new species, *Scytalidium acidophilum* (24).

Growth medium. The fungus was maintained on medium containing 2.5 g of KOH, 2.0 g of NaOH, 0.1 g of $FeCl_3$, 25.0 g of $MgCO_3$, 100 ml of 10 N H_2SO_4, 5 ml of 29% NH_4OH, 6.3 ml of phosphoric acid (pH 0.5), 2 ml of trace element solution, 12.0 g of glucose, and enough distilled water to bring the volume to 1,000 ml. The trace element solution contained (per liter of distilled water) 120 mg of H_3BO_3, 500 mg of $NaMoO_4 \cdot 2H_20$, 800 mg of $CuSO_4 \cdot 5H_20$, and 150 mg of $MnCl_2$ $4H_20$. In shake cultures (Eberbach rotary shaker; 125 rpm) at room temperature the fungus grew well in this medium, which had a pH of 0.5.

Source and preparation of waste paper. The following three types of waste paper were used: (i) newspaper (with ink) from the *Ottawa Citizen*, purchased locally; (ii) magazines (a mixture of approximately 50% *Time* and 50% *MacLean's*); and (iii) bonded papers (a mixture of discarded letter forms and scientific manuscripts, with and without typing). The papers were Wiley milled (−9 mesh). After the papers were dried overnight at 105°C, samples showed that the moisture content of the newspaper was 5%, whereas the moisture contents of the magazines and bonded papers were 4%.

Paper hydrolysis. A 40-ml portion of 72% H_2SO_4 was added to 20 g of paper in a 2,000-ml beaker in an ice bath. A stainless steel spatula was used to mix the acid

TABLE 1. Amounts of reducing sugars and residues in waste paper hydrolysates

Paper	No. of runs	Wt (g)[a]		%[b]	
		Sugars	Residues	Sugars	Residues
Newspaper	12	11.69 ± 0.32[c]	5.23 ± 0.15	58.5	26.2
Magazine	9	10.26 ± 0.33	7.23 ± 0.07	51.3	36.2
Bonded	11	15.23 ± 0.76	1.30 ± 0.10	76.2	6.5

[a] Grams per 20 g of air-dried substance.
[b] Average values.
[c] Mean ± standard deviation.

TABLE 2. Sugar compositions of the waste paper hydrolysates

Sugar	% In:		
	Newspapers	Magazines	Bonded paper
Glucose	80	71	60
Mannose	2	3	4
Galactose	2	1	1
Xylose	15	22	32
Arabinose	1	3	3

with the paper and compress the mixture into a spongy mass. After standing overnight at 4°C, the mixture was left at room temperature for 4 h and diluted with 950 ml of distilled water, and the final hydrolysis was carried out at 116°C for 15 min. The hydrolysate was vacuum filtered through tared filter paper in a Buchner funnel and washed with four 10-ml portions of distilled water. A 5-ml sample of the hydrolysate was removed to determine reducing sugars, and the residue was dried to a constant weight at 105°C.

Fermentation and harvesting of biomass. Each filtered hydrolysate (approximately 960 ml, pH <0.1) was placed in a 2,000-ml Erlenmeyer flask, and the salts and trace elements of the growth medium were added. The flasks were inoculated with 2 ml of a 7-day-old culture in growth medium. Flasks containing the cultures were either incubated on the rotary shaker for 10 to 14 days at room temperature or remained static and received filtered air blown through the inoculated hydrolysate at about 2 bubbles per s. In the latter cultures evaporation losses were compensated for daily. The incubating cultures were checked periodically for possible contaminants by streaking loopfuls on potato dextrose agar plates adjusted to pH 1 and 2 with H_2SO_4. The total biomass was determined as dry weight of washed mycelia after filtration, as described above. The residual reducing sugars in the filtrates were determined, and the percent yield of biomass was calculated as follows: (dry weight of fungus produced/amount of sugar utilized) ×100. The dried product was ground to a fine powder with a mortar and pestle.

Analytical procedures. Protein was estimated from the N content (N × 6.25; Kjeldahl procedure). Amino acids were determined by hydrolyzing the material with boiling 6 N HCl for 19 h and then analyzing the hydrolysate with a Beckman model 101M amino acid analyzer; nucleic acid was estimated by extracting the material with 0.5 N $HClO_4$ (3) and using calf thymus DNA and deoxyribose as standards. Total lipids were determined by ether extraction, and crude fiber was determined by acid and base extractions (1). Dry matter and ash were determined by heating in a vacuum at 105°C and combustion in a muffle furnace at 600°C, respectively (1). Lignin or lignin-like substances were estimated by determining the methoxyl content (1).

Total carbon was determined by using dry combustion. For metal analyses, samples were digested in a mixture containing nitric and perchloric acid, and the metals were measured with an atomic absorption spectrophotometer. The phosphorus in the solution was determined by the ammonium molybdate method (1).

Total reducing sugars were determined colorimetrically with *O*-toluidine, using Harleco glucose reagent (American Hospital Supply Corp., Gibbstown, N.J.) as a standard. The neutral monosaccharides were determined by using ion-exchange chromatography and gas chromatography (22). X-ray diffraction was performed by using Fe-filtered cobalt radiation on a Philip diffractometer and infrared analysis with a Beckman model 4250 analyzer.

RESULTS AND DISCUSSION

Composition of waste paper. Under the hydrolysis conditions used, the total content of reducing sugars released from the newspaper (Table 1) was 58.5% of the air-dried weight, or about 61% of the oven-dried weight. This agrees closely with previous reports (16) of the reducing sugar content of newsprint (approximately 56%) and the cellulose content (28) of the *Wall Street Journal* newspaper (61%). The magazines and bonded paper contained about 7% less and about 20% more reducing sugars, respectively, than the newspaper. These differences in hydrolyzable sugar contents appeared to be related to the amounts and compositions of the unhydrolyzable residues. The magazines, which had the lowest sugar content, had the highest residue content (approximately 36%). The methoxyl, X-ray, and infrared analyses showed that the residue was composed of about 20% lignin-like substances plus a substantial amount of the clay mineral kaolinite [$Al_2Si_2O_5(OH)_4$] and a trace amount of talc [$Mg_3Si_4O_{10}(OH)_2$]. From the Al and Mg contents of the perchloric acid-nitric acid digest of the residue, we calculated that the kaolinite and talc contents were 61 and 1%, respectively. These minerals, which are called

TABLE 3. Weights, percent yields, and protein contents of fungal mycelia derived from fermentation of waste paper hydrolysates[a]

Culture	Paper	No. of runs	Dry wt of mycelia (g/flask)	% of yield (% of sugar consumed)	% Protein[b]
Shake	Newspaper	4	4.32 ± 0.13[c]	37.7 ± 1.2	35.38 ± 1.13
	Magazine	4	3.79 ± 0.03	38.4 ± 0.6	34.88 ± 0.38
	Bonded	5	5.55 ± 0.81	37.2 ± 1.1	35.18 ± 1.34
Aerated	Newspaper	4	4.72 ± 0.22	43.0 ± 3.0	44.24 ± 2.85
	Magazine	4	4.85 ± 0.15	48.9 ± 1.8	47.31 ± 1.13
	Bonded	4	8.20 ± 0.41	55.5 ± 2.6	45.76 ± 5.02

[a] All preparations were fermented for 12 days.
[b] Determined as described in the text (N × 6.25).
[c] Mean ± standard deviation.

fillers by the paper-making industry (5), are added to paper stock to increase strength and smoothness. The bonded paper had the lowest residue content (approximately 7%), which was composed of 63% kaolinite and 7% talc. No lignin-like substances were detected. The newspaper residue was X-ray amorphous and contained about 63% lignin-like substances. Since newsprint does not require the density and strength of bonded or magazine paper, it is generally made from pulp which has a higher lignin content (5).

An analysis of the carbohydrate fractions of the hydrolysates (Table 2) showed that they consisted of the usual five wood sugars (5). The high percentage of glucose indicates that the carbohydrate was principally cellulose. Xylose, which is derived from hemicellulose, was the next most abundant sugar and, proportionally, was present in larger quantities in the magazines and bonded paper than in the newspapers. Pulps that are high in hemicellulose make stronger papers (5) for magazines and bonded papers.

Fungal growth and yields. Contaminants were never a problem. The streaked agar plates showed only the presence of *S. acidophilum*. After harvest, we found that the percentage of reducing sugars consumed per flask ranged from 94.6 to 99.4%. Freshly harvested dried and ground mycelia had a yeast odor. However, after a few days the mycelia became virtually odorless.

Our average fungal yields (38%) and their protein contents (35%) from the shake cultures (Table 3) were higher than the average values reported for 175 *Fungi Imperfecti* grown similarly on glucose (12). In the latter study, 53% of the fungi had yields ranging from 33 to 50%, and 40% had yields of less than 16 to 33%. Moreover, the protein contents of the top 10 cultures, based on the efficiency of sugar utilization, ranged from 14 to 34%.

The yields and protein contents of *S. acidophilum* from the aerated cultures (Table 3) were substantially higher than those from the shake cultures. In the former systems, the fungus produced yields of 43 to 56%, with protein contents of 44 to 47%. These values compare favorably with the values for the well-known single-cell protein producer *Candida utilis* (20), a yeast which produces yields of 45 to 52% containing 45 to 54% protein when it is grown on seven different wood hydrolysates (23) and has a protein content of 47 to 55% when it is grown on cannery wastes, molasses, and spent sulfite liquor (30).

The higher yields from the aerated systems are in agreement with the findings of Crawford et al. (7), who observed that *Thermomonospora fusca*, a celluloytic thermophilic actinomycete, degraded far more pulping fines and produced more biomass in a 40-liter fermentor equipped with an air sparger than in 500-ml shake flasks. These authors speculated that the better results in the aerated systems were due to a more efficient oxygen supply. In our shake cultures, yields comparable to those in the aerated systems might have been obtained if the flasks had been baffled. Baffled flasks (21) supply more oxygen for microbial cultures than unbaffled flasks.

It is also noteworthy that in our aerated systems (Table 3), the highest fungal yields (56%) were from bonded paper hydrolysates. This was probably due to the fact that during operation of these systems the average room temperature increased from about 23 to 29°C because of spring weather conditions.

Because of the copious amounts of biomass (yields up to 46%) produced in the aerated cultures of the bonded paper hydrolysates (Table 3), we suspected that *S. acidophilum* was utilizing other sugars besides glucose. Additional experiments showed that at pH 0.5, *S. acidophilum* consumed xylose, arabinose, mannose, and galactose. Furthermore, this fungus consumed galacturonic and glucuronic acid, which are also structural constituents of hemicellulose.

TABLE 4. General chemical composition and trace element content of fungal mycelia[a]

Component	µg/g	%
P	9,270 ± 2,325[b]	
Mg	660 ± 178	
K	8,975 ± 2,307	
Cu	13 ± 0.7	
Fe	98 ± 38	
Zn	87 ± 31	
Mn	1 ± 0.3	
Lipids		2.6 ± 0.4
Crude fiber		13.3 ± 0.7
Dry matter		98.3 ± 0.2
Ash		3.5 ± 1.2
Carbon		46.8 ± 0.2

[a] Based on two harvests from newspaper hydrolysates and two harvests from magazine hydrolysates.
[b] Mean ± standard deviation.

TABLE 5. Average amino acid composition of fungal mycelia[a]

Amino acid	Amt (g/100 g of protein)
Cysteic acid	0.3 ± 0.1
Aspartic acid	8.1 ± 0.6
Threonine	5.2 ± 0.4
Serine	4.6 ± 0.2
Glutamic acid	11.1 ± 0.9
Proline	3.9 ± 0.3
Glycine	4.5 ± 0.2
Alanine	5.8 ± 0.5
Half-cystine	1.4 ± 0.2
Valine	4.9 ± 0.4
Methionine	1.4 ± 0.4
Isoleucine	3.9 ± 0.4
Leucine	6.1 ± 0.4
Tyrosine	3.3 ± 0.2
Phenylalanine	3.4 ± 0.2
Glucosamine	0.5 ± 0.1
Galactosamine	3.4 ± 1.7
Lysine	5.4 ± 0.4
Histidine	2.2 ± 0.2
Ammonia	1.5 ± 0.4
Arginine	5.4 ± 0.3

[a] Based on three harvests from shake cultures and four harvests from aerated cultures.

Analysis of the fungal mycelia. Table 4 shows that of the eight elements determined, C, P, K, and Mg (in that order) were the most abundant elements in *S. acidophilum* mycelia. This agrees with many other fungal analyses (19) which have shown that the carbon content of fungi is approximately 50% and that P is usually the most abundant nonmetallic element found in the ash, whereas K and Mg are the most abundant metals. As in yeast extract, Fe and Zn are present at levels about one-tenth that of Mg, and Mn is present in small amounts only. The lipid and ash contents of *S. acidophilum* are about 2 to 3% lower than those of *C. utilis*, whereas the crude fiber content is about 7% higher (30).

Analyses of the protein contents of the mycelia from seven different hydrolysates (Table 5) showed that the amino acid composition of *S. acidophilum* was relatively constant. Except for tryptophan, which could not be determined in the acid hydrolysates, the essential amino acids were present in about the same concentrations as in the actinomycete *T. fusca* (7) and the fungi *Trichoderma viride* and *Gliocladium deliquescens* (6). Compared with the yeast *Saccharomyces cerevisiae* (17) and the bacteria *Alcaligenes faecalis* and *Cellumonas* sp. (13), the isoleucine, leucine, phenylalanine, and lysine contents were lower. As with other analyses of fungal mycelia, the presence of glucosamine, galactosamine, and ammonia in the tissues lowered the crude protein content. The average nucleic acid content (two determinations) for shake culture fungal growth was 6.2% (about 6 g/100 g of crude protein), which is characteristic of microbes containing low levels of low nucleic acid (17).

Economics. Since the production of single-cell protein by *S. acidophilum* cultured in strong acid-catalyzed hydrolysates of waste paper has not been practiced commercially, cost estimates are at best speculative. However, based on preliminary considerations of yield, price of H_2SO_4, and competitive products (e.g., soybean meal), commercial exploitation of *S. acidophilum* deserves consideration.

The use of *S. acidophilum* has the following inherent advantages over the use of other microbes. (i) There would be no need to have costly aseptic conditions. (ii) A wide range of sugars can be fermented. (iii) Studies with our isolate and ATCC 26772 (11) showed that *S. acidophilum* also grows well at pH 0.5 in media adjusted with HCl. Thus, hydrolysates obtained by treating wastes with 1% HCl (23) could be utilized. (iv) The filamentous growth of *S. acidophilum* permits low-cost filtration methods for mycelium recovery. (v) There would be no need to dilute the acidic hydrolysates to pH values that are generally favorable for the growth of most fungi. This requires a dilution factor of 100- to 1,000-fold. (vi) After the biomass was harvested, the acids could be reused with more substrate. (vii) Because of the tolerance of *S. acidophilum* to high salt concentrations (11, 25), the pH of strongly acidic hydrolysates could be adjusted by adding strong bases and $MgCO_3$. In the present work, tests showed that fermentation proceeded in a medium containing 45 g of $MgCO_3$ per liter, which is equivalent to 39 g of H_2SO_4. In the past, neutralization of acid media has been accomplished by adding $Ca(OH)_2$ or $CaCO_3$ (9, 23). However, this entails the added

steps of removing the $CaSO_4$ precipitate. In our system there is the added advantage that the carbonates and bases (e.g., $MgCO_3$, KOH, and NH_4OH) supply essential elements required by the fungus.

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