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The Effect of Carbon Sources on the Single Cell Proteins and Extracellular Enzymes Production by *Chrysonilia sitophila* (TFB 27441 Strain)

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ABSTRACT

The single cell protein and extracellular enzyme production from a lignocellulolytic fungus, *Chrysonilia sitophila*, using different carbon sources were evaluated. The mycelial dry mass composition showed a high protein (39.2%) and low nucleic acid content (3.3%), as well as carbohydrate, fatty acid, fiber, and ash levels comparable with single cell proteins currently studied. Mycelial protein showed amino acid content similar to or higher than FAO standard requirements. The amino acid, fatty acid, and carotenoid composition, as well as mycelial mass yield and enzyme production, were dependent on the carbon source used. Glucose, saccharose, cellobiose, cellulose, microcrystalline cellulose, lactose, and rice hull as carbon sources were studied.

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The major mycelial dry mass yield was 0.38 g of fungi/g of total substrate with 1.0% glucose as the carbon source in 6-d batch cultures. The maximal extracellular enzyme production was 40 U/L filter paper activity, 20 U/L β-glucosidase, 200 U/L endoglucanase,and 190 U/L protease.

Index Entries: *Chrysonilia sitophila;* single-cell proteins; cellulases; biomass; β-glucosidase; endogluconase; protease.

INTRODUCTION

The large amount of lignocellulosic material produced in the world and its potential conversion to fine products are an open question. The single cell protein and enzyme production using renewable materials have been studied, but further research with new lignocellulolytic microorganisms is still necessary.

The ascomycete *Chrysonilia sitophila* Mont von Arx (TFB-27441 strain) was initially isolated from a xylophagous insect (*1*). This strain is the anamorph stage of *Neurospora sitophila,* that has been utilized in the production of Indonesia "ontjon" for human food (*2–4*), cellulase (*5,6*), and protease production (*7*). The TFB strain, which exhibits higher ligninolytic activity than *Phanerochaete chrysosporium* BKM-F1767, and cellulolytic activity similar to *Trichoderma reesei* QM6a (*8,9*), was efficient to degrade rice hull (*10*) and cellulose (*11*).

Since few lignocellulolytic microorganisms have been studied for direct single cell protein (SCP) production from lignocellulosic materials (*12*), the *C. sitophila* TFB strain as a potential microorganism for production of SCP as well as cellulolytic, proteolytic, and ligninolytic enzymes was investigated using representative carbon sources.

MATERIALS AND METHODS

Chemicals

α-cellulose, microcrystalline cellulose (Avicel), Azocoll, and diphenilamine were from Sigma. All other reagents were of analytical grade.

Fungus

C. sitophila (TFB 27441 strain) was isolated from the xylophagous insect, *Tribolium ferrugineum,* as previously described (*1*). Stock cultures were maintained in Fries-saccharose modified medium agar plates at 5°C.

Fries Modified Medium

This culture medium was adapted (*13*) from Ryan et al. (*14*): $(NH_4)_2C_4H_4O_6$ (ammonium tartrate) 5.0 g/L. $NaNO_3$ 1.0 g/L, KH_2PO_4 1.0 g/L, $MgSO_4$ 0.5 g/L, NaCl 0.1 g/L, $CaCl_2$ 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.2 mg/L, $MnSO_4$ 0.02 mg/L, $ZnCl_2$ 0.15 mg/L, and variable carbon sources at different concentrations. In Petri plate cultures, 1.5% agar was used.

Growth Conditions

The mycelia, obtained from 5 to 10 d old Petri plate cultures with Fries modified medium at pH 6.0, 28°C and variable carbon sources, were submitted to chemical analysis (*see* table legends).

Extracellular enzyme production was carried out in liquid cultures with Fries modified medium, 50 m*M* potassium biphthalate buffer pH 6.0 at 28°C and variable carbon sources at different concentrations (*see* figure and table legends). In the same experiments, mycelial dry mass production, after filtration and drying at 80°C overnight, was determined. For growth in cellulose, mycelium mass was calculated by protein determination of the dry residue (after extraction with 1.0*M* NaOH at 100°C for 10 min), assuming protein in fungi as 40%. Kinetic parameters of cellobiose conversion was carried out in Fries modified medium without buffering control.

Enzymatic Activities

The filter paper (FPA) (*15*) and β-glucosidase (*16*) activities were measured by previously published methods. Protease activity was measured using a modification of the Azocoll method (*17*) at 37°C, pH 6.0, and absorption measurements at 530 nm. Cultures were done in triplicate and average enzymatic activities are presented in the text. Deviations lower than 5% in all cases were observed.

Protein, Carbohydrate, Alcohols, Fatty Acid, Pigment, Nucleic Acid, Ash, and Fiber Determination

The protein determinations were carried out by the modified Lowry method (*18*), and the amino acid distribution determined by the amino acid analyzer (Beckmann 119 CL) after hydrolysis for 24 h with HCl 6*N* at 105°C. The carbohydrates were measured by Somogyi-Nelson (*19*) or phenol-sulfuric acid methods (*20*), and glucose by glucotest. The alcohols were extracted from the culture broth with ethyl ether, previously saturated with NaCl, and then analyzed by GC with a FFAP 20% Chromosorb W (80–100 mesh, 6 ft×1/8 inch) column at 75°C, with a Perkin Elmer Gas Chromatograph 3920. With the same instrument, methylated fatty acids

were determined using a DEGS 20% Chromosorb W (80–100 mesh, 6 ft × 1/8 in.) column. Carotenoid extract (*21*) was analyzed in a Waters Co. HPLC with an ultraviolet-visible detector 441 and a Superspher RP-8 column, using acetonitrile-tetrahydrofuran-hexane (130:30:15) as eluent. Total nucleic acids were extracted with perchloric acid (*22*) and determined by 280 nm absorption value (*23*), and DNA content by reaction with diphenylamine (*22*), RNA content was calculated by the difference of total nucleic acid and the DNA contents. Ash and Fiber were determined by AOAC method (*24*). Chemical determinations were carried out in triplicate, and the results showed in the tables are the average value followed by its deviations. Fatty acid, pigment, and amino acid determinations showed less than 2% deviations for the standard mixtures used.

RESULTS AND DISCUSSION

Single Cell Protein

The mycelial dry wt yields (g of dry mycelium/g of total substrate) obtained in different carbon sources after 6 d of growth were: glucose (1.0%) 0.38, cellobiose (1.0%) 0.30, saccharose (1.0%) 0.24, cellulose (1.5%) 0.14, rice hull (3.0% and 10-d-old culture) 0.05, and lactose (1.0%) 0.02. These results demonstrate the potential of this fungus to grow on representative lignocellulosic carbon sources (cellobiose, α-cellulose and rice hull) as compared to characteristic carbon sources for SCP production (glucose and saccharose). The *C. sitophilia* mycelial mass yield from 6-d-old glucose cultures was similar to that of *Rhodocyclus gelatinosus* (0.44), and lower than that of *Rodobacter sphaeroids* (0.54) from 3-d-old cultures using the same substrate. These bacteria are efficient for Cassava waste conversion to SCP (*25*). The yield for saccharose conversion (0.24) was lower than torula yeast (0.52) cultured in an optimized continuous fermentation process (*26*). The chemical composition of mycelium grown in Fries-1.5% saccharose agar plates was determined (Table 1). The mycelial protein amino acid composition using different carbon sources as substrates was determined, since protein syntheses are affected by growth substrate (*27*) (Table 2). The total protein content (around 40%) is in the same range as those for *Chaetomiun cellulolyticum* (45%) (*28*) and various yeast species (34–50%) used in SCP production (*29*). Our strain has higher carbohydrate, fatty acid and fiber, and lower ash content than SCP from *Candida utilis* (torula yeast) (*26*) used for food production. The total nucleic acid content (3.3%) is lower than those produced by traditional microorganisms utilized in single cell protein production (e.g., yeast 6–12%, algae 3–8%, bacteria 8–16%) (*30*). The DNA content (0.7%) is similar to that observed for *P. chrysosporium* (0.9%) (*22*). Table 2 shows that the amino acid composition of mycelial *C. sitophila* protein is not constant for different substrates. For more recalcitrant material (microcrystalline cellulose and rice

Table 1
Chemical Composition of Mycelia from *Crysonilia sitophila* (TFB 27441 Strain) Grown in Saccharose as a Carbon Source

Components	Percentage (w/w)
Protein	39.2 ± 1.5
Carbohydrate	29.3 ± 2.2
Ether extract	12.1 ± 1.6
Fiber	3.3 ± 1.5
Ash	5.6 ± 1.0
Nucleic acids	3.3 ± 0.2
Ribonucleic acid	2.6 ± 0.4
Deoxyribonucleic acid	0.7 ± 0.2

Table 2
Aminoacid Distribution of *C. sitophila* (TFB 27441 Strain) Mycelial Protein (g of a.a./100g of protein)

A.A.	Glucose (1.0%)	Cellobiose (1.0%)	Saccharose (1.5%)	Microcrystalline cellulose (1.5%)	Rice hull (1.0%)	FAO
Asp	11.29	12.47	13.06	7.59	10.96	-
Thr	5.01	5.19	5.12	3.88	19.43	2.8
Ser	5.18	5.60	5.23	3.61	6.07	-
Glu	13.88	10.63	20.12	6.73	12.42	-
Pro	7.76	5.80	6.51	3.83	8.24	-
Gly	6.14	5.23	7.40	22.37	9.81	-
Ala	8.81	7.47	8.79	4.51	12.70	-
Val	8.17	7.99	9.46	5.98	0.01	4.2
Met	2.12	0.99	1.89	1.61	0.01	2.2
Ile	0.01	9.40	1.45	4.94	3.98	4.2
Leu	12.93	9.36	7.90	5.94	10.67	4.8
Tyr	1.16	3.74	1.07	2.74	0.01	2.8
Phe	3.32	4.28	4.12	2.55	0.01	2.8
Lys	6.33	5.65	3.03	0.01	3.71	-
His	2.14	1.53	0.01	4.63	0.01	-
Arg	4.10	4.66	4.82	19.46	2.00	-

Table 3
Fatty Acids from Mycelia of *C. stophila*
(TBF 27441 Strain) Grown in Glucose and Saccharose Carbon Sources*

Carbon Source	Fatty Acid												
	C6	C8	C10	C12	C14	C16	C16:1	C18	C18:1	C18:2	C18:3	C22	Others
Glucose	ND	ND	ND	ND	ND	9.6	ND	4.8	25.8	29.9	8.5	9.6	11.8
Saccharose	2.7	2.6	1.1	3.5	4.0	7.4	6.0	11.6	18.0	13.6	9.5	ND	20.0

*Total ether extract were: 12.4% in glucose and 12.1% in saccharose substrate. ND: not detected.

hull), the change in amino acid composition was significant. For microcrystalline cellulose, the mycelia shows higher glycine and arginine, and lower lysine content than in glucose substrate. For rice hull, higher threonine and isoleucine, and lower content of valine, methionine, phenilalanine, and histidine content than mycelia obtained from glucose substrate were observed. These results indicate that protein syntheses was affected when different substrates were utilized by the fungus in a similar way as observed previously by Bellamy (27). Apparently, in the latter paper, the amino acid composition of protein produced during growth of *Thermoactinomyces* on feedlot waste result from culture, to time of harvest, and to substrate (e.g., methionine from a value of 0.77 to 2.21% depending on the substrate) (27). Most of the amino acids in all mycelial protein obtained from different carbon sources showed concentration values similar to the FAO standard requirement, but methionine, valine, tyrosine, and histidine were significantly deficient when rice hull as carbon source was utilized.

Table 3 shows the mycelium fatty acid composition obtained from glucose and saccharose substrates. In general, in both carbon sources, a high unsaturated fatty acid percentage was observed (64.2% and 47.1% for glucose and saccharose, respectively). The relative carbohydrate distribution of *C. sitophila* grown in saccharose was the following: mannose 10.4%, fructose 1.5%, galactose 6.9%, xylose 0.8%, and a high glucose content 80.3%. As an illustration of the vitamin content, thiamine showed 1.93+0.08 mg/100 g of dry mycelium that is almost twice that for torula yeast (*26*).

Figure 1 shows the mycelium pigment distribution when grown in different carbon sources. Xanthophylls (retention time 0–5 min.), apoxanthines (5–10 min), and carotenoids [trans β-carotene (11.5 min) and 9-*cis*β-carotene (12 min)] were observed. The carotenoid distribution was carbon source dependent as for amino acids and fatty acids, since xanthophyll was predominant for growth in saccharose and cellobiose. Apoxanthines were predominant when glucose was the carbon source.

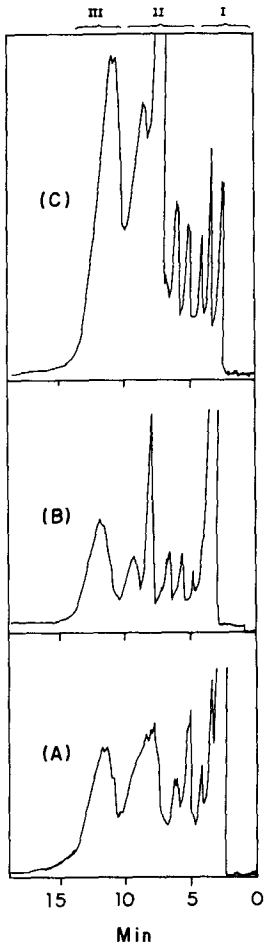

Fig. 1. Carotenoids distribution from mycelial mass of *C. sitophila* grown in different carbon sources. (A) Saccharose 1.0%; (B) Cellobiose 1.0%, and (C) Glucose 1.0%. (I) Xantophills, (II) Apoxanthines, (III) Carotenoids.

In summary, the protein from this fungus is of high quality with essential amino acids, but dependent on the carbon source utilized. The fatty acid and carbohydrate compositions, as well as the low nucleic acid content, indicate that this fungus is a good candidate not only for ruminant food (proteic enhancement of lignocellulosic materials), but also for food production for human consumption, when saccharose or glucose are used as carbon source.

Production of Extracellular Enzymes

Table 4 shows the FPA, β-glucosidase, endoglucanase, and protease production in different carbon sources. The enzymatic activities are all dependent on the carbon source used. In general, cellulolytic activities were similar and, in few cases, lower than those of *T. reesei* QM6 strain

Table 4
Effect of Substrate on Cellulase and Protease Production by *C. sitophila* (TFB 27441 Strain)[a]

Substrate	Protein (mg/ml)	FPA (U/l)	β-glucosidase (U/l)	Endo-glucanase (U/l)	Protease (U/l)
Glucose(1.0% b)	0.36	16.0	20.0	50.0	40.0(c)
Cellobiose(1.0% c)	0.23	4.0	20.0	ND	110.0
Cellobiose(0.1% c)	0.08	4.0	2.0	ND	190.0
Saccharose(1.0% b)	ND	4.3	ND	ND	10.0(c)
μ-cellulose(1.5% c)	0.39	40.0	10.0	200.0	ND
ucryst.cell.(1.5% b)	0.50	3.0	18.0	74.0	ND
Rice hull(1.0% b)	2.10	6.0	13.0	ND	ND

[a] At the 6th day of culturing.
[b] Stationary conditions.
[c] Agitated conditions at 150 rpm.
ND: not determined.

(*31*). A high FPA was obtained when cellulose was used as carbon source, but β-glucosidase was maximal in glucose and cellobiose as carbon source.

The protease activity measured by the Azocoll method (2.52 U/mg) showed a higher specificity than that of pepsins (0.67 U/mg), and a lower value than pancreatin (4.10 U/mg) (*17*) when 0.1% cellobiose carbon source was used. The production of proteases was highly dependent on the carbon source and its concentration. Cellobiose appears as a protease enhancer and when low concentration was used, protease was the major enzyme produced. In order to justify the protease production, it has been suggested that some cellulases are secreted in the inactive zymogen form and then activated through proteolytic enzyme digestion. Another role of protease produced by cellulolytic fungi could be to cleave off cell wall-bound cellulases and, hence, release these enzymes into the extracellular fluid (32) Eriksson (33) has demonstrated that protease treatment of endocellulases derived for *Sporotrichum pulverulentum* resulted in a four-fold increase of its activity. This part of our work is actually in progress. The protease production is specially interesting for high protein content substrates (e.g., peanut) conversion to SCP, since these enzymes are directly involved in protein solubilization (*7*).

In order to study kinetic parameters of cellulosic material conversion, cellobiose was used as a representative substrate. Figure 2 shows the relationship between mycelial dry wt, cellobiose uptake, glucose formation, and glucosidase production during fungal growth. The cellobiose hydrolysis is very rapid after the third day, and glucose accumulation in the

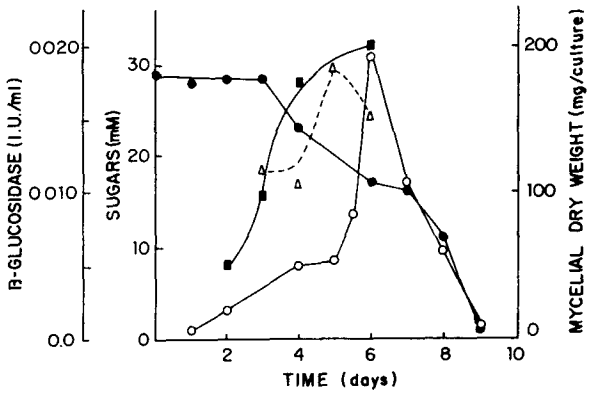

Fig. 2. Kinetic parameters of cellobiose (1.0%) conversion by *C. sitophila* in agitated cultures at 28°C. (—△—) mycelial dry wt; (—■—) β-glucosidase activity; (—○—) glucose formation and (—●—) total sugar consumption.

medium shows a maximum at the sixth day, followed by a rapid consumption until ninth day. In this period, a pH increase in the culture was observed from 6.1 to 7.5 between the fourth and the sixth day. The β-glucosidase activity was in agreement with cellobiose conversion in this period.

Another extracellular enzyme produced by this fungus that plays an important role in lignocellulosic material conversion is the ligninase (67 U/L) (*8*).

The production of alcohols during growth was evaluated, and showed a concentration of 0.15% of ethanol in culture filtrates of 1.0% cellobiose degradation at the third day of growing. In the presence of glucose, ethanol and other alcohols with long carbon chains were detected.

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