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Production of mycelial protein and cellulolytic enzymes from food wastes

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SUMMARY

Extracted grape waste material and pressed apple pulp were tested as carbon sources for *Penicillium funiculosum* 515, *Myrothecium verrucaria* 9095 and *Aspergillus niger* TMF-15. They were good growth substrates, especially for *A. niger*. When cultivated on mixed substrate in optimized nutrient medium, *A. niger* accumulated a product of 35% crude protein with a maximum productivity of 0.117 g protein/l/h and cellulose consumption of 90.92%. *A. niger* also produced the highest levels of cellulase activity. Maximum carboxymethyl cellulase and activity against filter paper were 494 units/l and 97 units/l, respectively.

INTRODUCTION

One of the objectives of biotechnology is the utilization of agricultural and food industry wastes for production of protein animal feed, chemicals and energy, and at the same time combating pollution of the environment.

Microbial transformation can be performed in several ways, such as direct bioconversion by monoculture [11,12,14], bioconversion by mixed cultures [2,3] or two-step conversion [13].

Various cellulosic wastes can serve as substrates for direct bioconversion into single-cell protein [11,12,14], but extracted grape waste material and pressed apple pulp have not been examined. These two food-processing wastes are by-products from the wine and juice industries. In this study, they were tested as substrates for single-cell protein and cellulase production.

MATERIALS AND METHODS

Microorganisms

Three microorganisms from our collection were used: *Penicillium funiculosum* 515, *Myrothecium verrucaria* 9095, and *Aspergillus niger* TMF-15. The fungi were maintained on malt agar slants at 4 °C and subcultured every 6 months.

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Substrates

The substrates used were extracted grape waste material, a by-product from the wine industry, and pressed apple pulp, a by-product from apple juice production. The substrates were air dried and then dry milled to 0.25 mm size. Their average chemical composition is given in Table 1.

Culture conditions

Malt agar slant cultures were allowed to develop at 28–30 °C for 4 days and after that they served as stock cultures. They were used for inoculation of Mandels-Weber medium [9] which contained per liter: 1.4 g

TABLE 1

Average chemical composition of extracted grape waste and pressed apple pulp

Component	Percentage of dry weight for:	
	Extracted grape waste	Pressed apple pulp
Moisture	7.42	6.05
Crude protein	15.83	6.35
Acid-insoluble lignin	28.76	17.90
Cellulose	16.20	13.70
Ash	6.15	2.10
Other (pectin, hemicellulose etc.)	25.64	53.90

$(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.3 g $CO(NH_2)_2$, 0.3 g $CaCl_2$, 0.3 g $MgSO_4 \cdot 7H_2O$, 1 g peptone and 1 ml trace elements. The composition of the trace element solution was (g/l): $MnSO_4 \cdot H_2O$, 1.56; $FeSO_4 \cdot 7H_2O$, 5.00; $ZnSO_4 \cdot 7H_2O$, 1.40; $CoCl_2$, 2.00; 15% HCl, 1 ml. The substrate concentration was 10 g/l. The mixed substrate consisted of grape waste and apple pulp in the ratio 1 : 1. pH was adjusted to 5 before the medium was autoclaved.

This medium served for preparation of the inoculum and for main cultivation of the fungi. 5 ml of sterile distilled water was added to a 4-day-old slant and the resulting spore suspension was transferred to a 500-ml Erlenmeyer flask which contained 100 ml of the basal medium supplemented with substrate. The inoculated medium was then incubated on a rotary shaker (200 rpm) at 28–30 °C. After 2 days incubation, 10 ml of this medium was inoculated into 100 ml of the fermentation medium in a 500-ml Erlenmeyer flask. Batch culture experiments were performed under the same conditions as those for inoculum preparation. The fermentation time was 7 days, except for *A. niger* (4 days) when cultivated on the optimized nutrient medium. At defined time intervals, the contents of an entire flask were filtered through glass-fiber filters (G4) and the filtrates were used for enzyme assays. The cell residues were washed with 0.05 M citrate buffer, pH 4.8, and dried at 105 °C to constant weight before analysis.

Apart from cultivation of *A. niger* on the Mandels-Weber medium, it was also cultivated on another medium which was previously optimized [15]. The composition of the optimized nutrient medium was (g/l): $(NH_4)_2HPO_4$, 1.5; KH_2PO_4, 1.5; $CO(NH_2)_2$, 0.3; $CaCl_2$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; malt sprouts, 3.68; peptone, 1.0; and 1 ml of the trace element solution. The concentration of mixed substrates was 20 g/l.

Analytical methods

The dry matter content of samples was determined by filtering on a glass-fiber filter (G4) and drying at 105 °C to constant weight.

Crude protein content was estimated by determining the Kjeldahl nitrogen content and converting it to protein by multiplying by a factor of 6.25. Reducing sugars were estimated by the dinitrosalicylic acid method [10]. Cellulose was determined using the anthrone method [7]. The ash content was estimated as the material remaining after heating at 600 °C in a muffle furnace [6]. Lignin was assayed as acid-insoluble material using a standard method TAPPI (T-13m-54) [6].

Cellulolytic activities, as carboxymethyl cellulase

TABLE 2

Bioconversion of extracted grape waste material and pressed apple pulp by monocultures

Parameter	Grape waste			Apple pulp		Mixed substrate		
						Mandels–Weber medium		Optimized medium
	P. funiculosum	*M. verrucaria*	*A. niger*	*P. funiculosum*	*A. niger*	*P. funiculosum*	*A. niger*	*A. niger*
Maximum specific growth rate (h^{-1})	0.016	0.018	0.026	0.016	0.030	0.015	0.019	0.041
Maximum productivity (g protein/l/h)	0.015	0.028	0.047	0.032	0.053	0.069	0.096	0.117
Maximum cellulose consumption (%)	67.07	70.70	77.59	96.70	96.22	96.43	95.43	90.92
Maximum crude protein content (% of total dry weight)	26.60	21.80	29.60	20.11	27.33	26.69	31.75	35.00
Maximum CMC activity (units/l)	200	90	360	48	346	90	384	494
Maximum FP activity (units/l)	78	17	138	9	83	23	140	97

(CMC-ase) and as activity against filter paper (FP), were determined by standard methods [5]. Filter paper activity was determined by incubating 0.5 ml of culture filtrate with 1 ml of 0.05 M citrate buffer pH 4.8, containing a 1-cm × 6-cm strip (50 mg) of Whatman No. 1 filter paper. After incubation for 1 h at 50 °C, the reaction was terminated by adding 3 ml of dinitrosalicylic acid reagent. The tubes were placed in a vigorously boiling water bath for 5 min, then cooled by placing them in a cool water bath for 5 min. Then, 20 ml of distilled water was added to every tube and after 40 min the absorbance was read at 540 nm. One unit of filter paper activity was defined as 1 μmol glucose equivalent released per min.

Carboxymethyl cellulase activity (1,4-β-D glucan 4-glucano-hydrolase) was determined by incubating 0.5 ml of the culture filtrate with 1 ml of 1% (w/v) carboxymethyl cellulose (CMC 71.2, Hercules Inc.) in 0.05 M citrate buffer, pH 4.8, at 50 °C for 30 min. The reaction was terminated by adding 3 ml of dinitrosalicylic acid reagent. The subsequent procedure was the same as for the filter paper activity. One unit of CMC-ase activity was defined as 1 μmol glucose equivalent released per min.

RESULTS AND DISCUSSION

The results obtained by testing extracted grape waste material and pressed apple pulp as substrates for cultivation of *P. funiculosum*, *M. verrucaria* and *A. niger* are shown in Table 2. Grape waste was a better substrate for *A. niger* than for *P. funiculosum* and *M. verrucaria*. *A. niger* generated the highest levels of cellulase activity while the quantities of crude protein found in the fungal products of *A. niger* and *P. funiculosum* were 29.60% and 26.60%, respectively. *A. niger* exhibited a higher specific growth rate (0.026 h^{-1}), productivity (0.047 g protein/l/h and cellulose consumption (77.59%) than the other two fungi.

M. verrucaria was eliminated from further investigations because of the poorer results obtained with this species. When apple pulp was used as a substrate for *A. niger* and *P. funiculosum*, a remarkable difference was perceived between the cellulase activities of the two molds. *A. niger* yielded 27.33% crude protein and was a better protein producer than *P. funiculosum*. This might be explained by the pectolytic properties ($70 \cdot 10^3$ IU/l polygalacturonase activity) of the crude enzyme complex of *A. niger* [8]. It is likely that pectinases act synergistically with cellulases, digesting materials of the cell lamella; making the cell wall more vulnerable to cellulolytic attack [1].

Productivity and cellulose consumption were higher when apple pulp was used, whereas cellulase production and crude protein content were higher when the grape waste was used (Table 2). These differences are due to the composition of the two-food processing wastes. Pressed apple pulp, rich in non-cellulosic polysaccharides and with a lower lignin content, supported higher productivity and cellulose consumption, while the grape waste, with its higher cellulose content, was a better cellulase inducer.

The molds were also cultivated on mixed substrate which contained grape waste and pressed apple pulp providing both non-cellulosic polysaccharides and cellulose in the culture medium. *A. niger* utilized this mixture better than *P. funiculosum*, yielding 31.75% crude protein with a productivity of 0.095 g/l/h. The cellulase activities were about the same as achieved when only grape waste was employed.

Since *A. niger* grew better than *P. funiculosum*, it was also cultivated on the optimized nutrient medium. Its kinetic parameters and cellulase activities are given in Table 2. The growth curves are shown in Figs. 1 and 2.

When cultivated on the optimized nutrient medium, the cells of *A. niger* contained 35% crude protein, which is about 10% higher than when the organism was cultivated on non-optimized medium with mixed substrate (Table 2), while in respect to the average initial protein content of the mixed substrate (11.5%), it represented an enrichment of about three times (Tables 1 and 2).

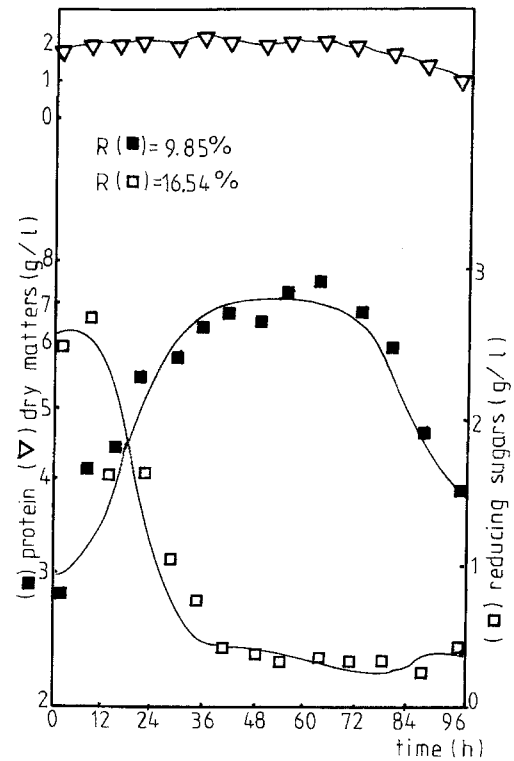

Fig. 1. Concentration of crude protein, reducing sugars and dry matter as a function of the cultivation time of *A. niger* on optimized nutrient medium with a 1 : 1 mixture of grape waste and apple pulp as carbon source.

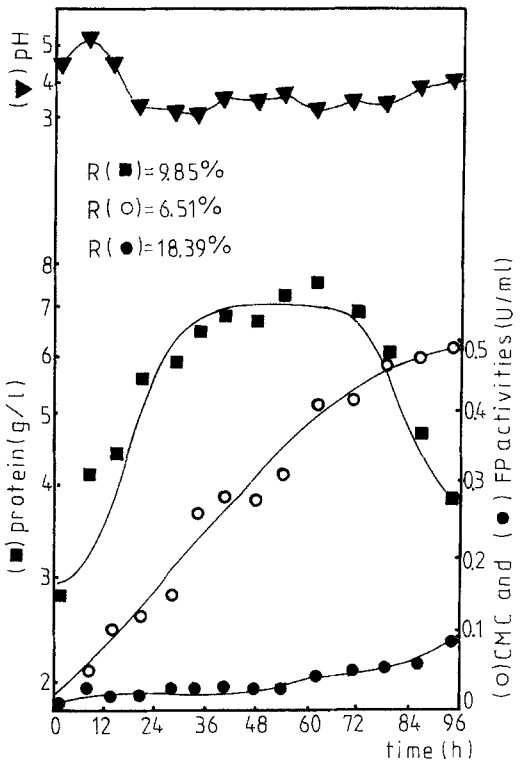

Fig. 2. Biosynthesis of crude protein and cellulases by *A. niger* cultivated on optimized nutrient medium with a 1 : 1 mixture of grape waste and apple pulp as carbon source.

TABLE 3

Logistic model[a] for mathematical representation of the batch culture data presented in Figs. 1 and 2

Coefficient	Reducing sugars	Protein	CMC activity	FP activity
K	2.7000	7.6000	0.5000	0.1000
a_0	−2.4319	0.6352	2.8094	3.6300
a_1	−0.3455	−0.2072	−0.1183	−0.3962
a_2	0.0488	0.0141	0.0016	0.0236
a_3	−0.0019	−0.0005	−1.3880	−0.0006
a_4	0.0000	6.3430	1.8093	6.8673
a_5	−2.6827	−2.0001		−2.8081
a_6	8.4720			

[a] $Y = \dfrac{K}{1 + \exp[F(t)]}$; $F(t) = a_0 + a_1 + \cdots + a_n t^n$;

K, a_n, constants; t, time;

$$R = \frac{\sum_{i=1}^{N} \left| \dfrac{Y_{\text{calc. i}} - Y_{\text{obs. i}}}{Y_{\text{obs. i}}} \right|}{N} \cdot 100\,(\%);$$

R, average relative error; N, number of measurements

After a short lag phase, the quantity of crude protein continuously increased, up to 7.22 g/l (35%), while the content of soluble sugars decreased (Fig. 1). The mold utilized 90.92% of the cellulose content of the mixed substrate (Table 2). During the batch cultivation, there was a continuous increase in cellulolytic activities, so that maximum CMC-ase activity (494 IU/l) and FP activity (97 IU/l) were achieved by the end of the 4th day (Fig. 2). The mold reached maximum cellulase activities after growth had stopped and the cells had started to autolyse.

All curves given in Figs. 1 and 2 are presented with an adequate mathematical model (Table 3) and derived parameters (Fig. 3) are obtained differentiating the fitted equations [4].

The maximum specific growth rate of 0.041 h^{-1} was achieved by the 17th h of cultivation (Fig. 3). The value obtained was considerably higher than the specific growth rates attained by the fungus when cultivated on the other substrates. This value may be compared to the specific growth rates of *Chaetomium cellulolyticum*, one of the most prominent cultures for bioconversion of lignocellulosic substrates. Moo-Young et al. [11] reported that the specific growth rate of *C. cellulolyticum* varied from 0.02 to

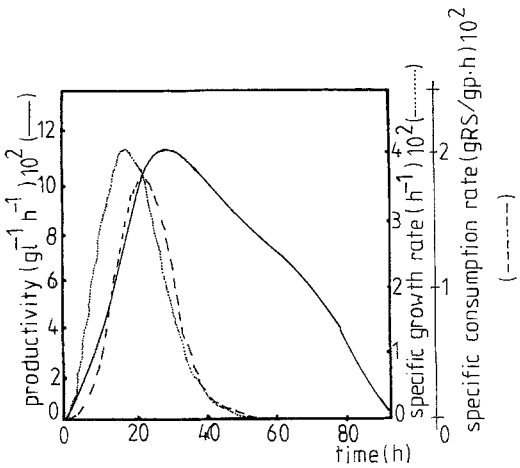

Fig. 3. Growth parameters of *A. niger* cultivated on optimized nutrient medium with a 1 : 1 mixture of grape waste and apple pulp as carbon source.

0.19 h^{-1} depending on the pretreatment of the lignocellulosic raw materials and the period of growth.

The specific rate of consumption of soluble sugars reached a maximum of 0.0185 g reducing sugars/g protein/h during the exponential phase (Fig. 3). According to the maximum productivity of 0.117 g protein/l/h, *Aspergillus niger* can be considered as an organism with a good

production capacity with respect to protein biosynthesis [11,12].

Grape waste and apple pulp, especially when used as a mixture, may be regarded as potential substrates for *Aspergillus niger* TMF-15, both for single cell protein and cellulase production.

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