APPLIED MICROBIOLOGY
Vol. 12, No. 4, p. 330-334 July, 1964

APPLIED MICROBIOLOGY

Production of Fungal Mycelial Protein in Submerged Culture of Soybean Whey

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Received for publication 24 February 1964

ABSTRACT

FALANGHE, HELCIO (Northern Regional Research Laboratory, Peoria, Ill.), A. K. SMITH, AND J. J. RACKIS. Production of fungal mycelial protein in submerged culture of soybean whey. Appl. Microbiol. **12**:330–334. 1964.—Various soybean whey media were tested as substrate for seven species of fungi in submerged culture. Very little mycelial growth was obtained with *Morchella hybrida*, *Collybia velutipes*, *Cantharellus cibarius*, and *Xylaria polymorpha*. *Agaricus campestris* failed to grow. *Tricholoma nudum* and *Boletus indecisus* showed the greatest rate of growth and production of mycelial protein and the best utilization of soybean whey solids, with much shorter incubation times compared with those of the other species. *T. nudum* developed as spheres having diameters of about 5 to 8 mm, instead of the usual slurry or yeastlike form, in the presence of added ammonium acetate. *B. indecisus* always developed as spheres. Mycelial yields and production of protein by *T. nudum* greatly decreased with the addition of more than 1% glucose to soybean whey, whereas with *B. indecisus* the yield of protein almost doubled when up to 3% glucose was added. The effect of minerals on mycelial growth was determined. With soybean whey concentrated to 50%, the rate of mycelial growth of *T. nudum* was nearly doubled, but protein content of mycelia was greatly reduced. Mycelial growth and yield of protein of *B. indecisus* grown in concentrated whey were increased greatly. About 4 to 6 g of mycelial protein per liter can be obtained from fermentation in soybean whey, depending upon the medium used. Utilization of soybean whey by fungal fermentation may have economic value in whey disposal and in the production of products of high protein content.

The possibility of growing mushrooms for food and their nutritional requirements in submerged cultures of various media have been investigated (Humfeld, 1948; Humfeld and Sugihara, 1949, 1952). When grown in chemically defined media, *Agaricus campestris* possesses a pleasant nut or cheese-like flavor (Humfeld and Sugihara, 1954). Flavors developed by several species of mushrooms also have been described (Eddy, 1958; Block, 1960; Moustafa, 1960). Mushrooms are rich in amino acids and B-vitamins, and have about the same nutritive value as does torula yeast (Block et al., 1953; Reusser, Spencer, and Salans, 1958*a*). *Morchella esculenta* can synthesize growth-promoting substances that it requires (Szuecs, 1956, 1958). The ability of *Morchella* to utilize various sugars as a source of energy was reported by Litchfield, Overbeck, and Davidson (1963). Protein and fat contents of several mushrooms grown in various media were described by Reusser et al. (1958*b*) and by Falanghe (1962).

Soybean whey is an industrial by-product resulting from the isolation of soybean protein and protein concentrates. Although varying in yield and composition, whole whey accounts for approximately one-third of the defatted meal and contains about 11% of the total meal nitrogen. On a dry basis, whey solids contain 2.5 to 3.5% nitrogen, about 50% of which is protein nitrogen and 25 to 35% of which is soluble sugars (Smith, Schubert, and Belter, 1955; Smith et al., 1962). The essential amino acid content of total whey proteins and heat-coagulable protein is indicative of high nutritional value (VanEtten et al., 1959; Rackis et al., 1961). Because of its low solids content (3 to 4%), recovery of whey is not now economically feasible and it is discarded. Because this practice increases the biological demand of sewage systems, use of the whey solution as a substrate was investigated for production of high-protein products by fungi fermentation in submerged culture. Rate of mycelial growth, protein content of mycelium, and fermentative capacity are reported.

MATERIALS AND METHODS

Whey was prepared from dehulled and undenatured flakes processed from Hawkeye soybeans, 1961 crop year (Fig. 1). Dehulled, defatted meal was prepared by cracking the whole beans between corrugated rolls into six to eight parts and by removing hulls by aspiration in a Eureka seed cleaner; the resulting chips were pressed into flakes between smooth rolls, and oil was extracted with hexane (boiling point, 30 to 60 C). Factors affecting the composition of whey were reported by Smith et al. (1955). Cultures of *Tricholoma nudum* NRRL 2371, *Collybia velutipes* NRRL 2367, *M. hybrida* NRRL 2600, and *Cantharellus cibarius* NRRL 2370 were obtained from C. W. Hesseltine, Northern Regional Research Laboratory; *Boletus indecisus* Gray 168 and *Xylaria polymorpha* Gray 154 were obtained from W. D. Gray, Ohio State University, Columbus; and *A. campestris* was obtained from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Cultures were maintained in the dark on malt agar slants at 25 C.

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Inoculum was produced by aseptic transfer of mycelial bits to 250-ml standard-taper Erlenmeyer flasks containing glass beads and 40 ml of 5% malt extract broth. Flasks were stoppered with cotton plugs and incubated for 10 days at 25 C in the dark. The mycelium was then washed with sterile distilled water; 40 ml of distilled water were added; and the mycelium was broken up by vigorous shaking after replacing the cotton with a glass plug. The resulting suspension was used as inoculum (2 ml per flask).

The cultures were grown in the dark in 500-ml Erlenmeyer flasks containing 200 ml of sterilized soybean whey medium shaken at 200 rev/min in a rotatory shaker having an eccentricity of 2.25 in. (5.72 cm) at 25 C.

Analytical methods. Reducing sugars were determined colorimetrically with the alkaline copper reagent of Somogyi (1945) and the arsenomolybdate reagent of Nelson (1944). Total carbohydrates were determined with anthrone (Morris, 1948). Total solids in the whey media were determined by evaporating to dryness at 110 C. Mycelial weights were obtained by filtering, washing with distilled water, and drying at 60 C under vacuum for 24 hr. Total nitrogen was determined by the micro-Kjeldahl method, and the factor 6.25 was used to calculate crude protein. The reported data are the average of at least two culture runs.

RESULTS AND DISCUSSION

Soybean whey contains appreciable amounts of nitrogen, carbohydrates, and ash. Nitrogen is in the form of proteins, peptides, free amino acids, and other nitrogenous compounds; whey also contains oligosaccharides such as sucrose, raffinose, stachyose, various glycosides, galactans, and hemicelluloses (Markley, 1951). Substantial amounts of phosphorus and minerals, largely as phytate salts, are also present. Whey contains a heat-stable inhibitor of *Rhizopus oligosporus* (Hesseltine, de Camargo, and Rackis, 1963) and a heat-stable trypsin inhibitor(s) (Rackis, 1961). Solids content of whey solution 1 (Fig. 1) is about 3 to 4%.

In initial experiments it was found that diluted whey solution 1, even in the presence of added glucose, did not support good mycelial growth of *T. nudum* and *B. indecisus*; the protein content of mycelium was also greatly low-

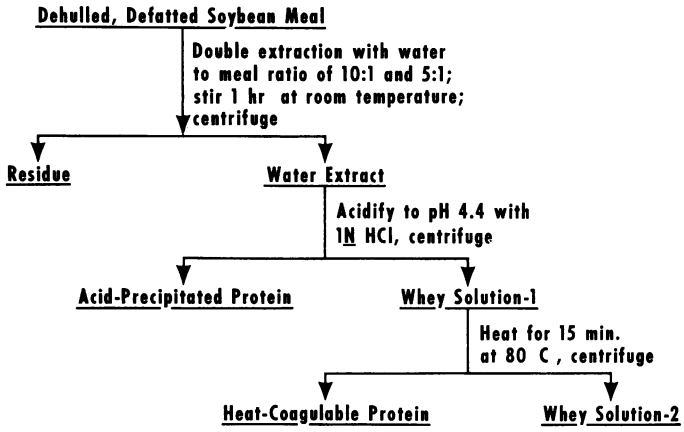

FIG. 1. *Preparation of soybean whey for fungal fermentation.*

TABLE 1. *Growth of fungi in soybean whey medium with added ammonium salts*[a]

Species	Medium[b]	Time	Mycelium		Dry wt of mycelium[c]	Nitrogen used	Reducing sugars used	Total carbohydrates	Dry matter used	Efficiency[d]	
			Dry wt	Protein content						Sugar used	Original sugar
		hr	*g per liter*	%	%	%	%	%	%	%	%
Tricholoma nudum	1	72	6.0	54.6	51.7	37.4	98.9	96.7	54.1	70.3	69.6
T. nudum	2	72	6.6	53.7	56.0	50.7	98.3	97.7	75.1	76.1	74.8
T. nudum	3	168	6.7	60.7	58.3	40.9	97.2	96.3	64.8	89.3	86.8
Boletus indecisus	1	192	6.9	34.1	59.3	30.8	99.1	96.8	50.6	50.3	49.9
B. indecisus	2	192	6.7	28.4	59.1	26.0	98.7	94.3	54.1	41.1	40.3
B. indecisus	3	192	8.4	33.1	74.3	35.5	98.9	94.4	60.5	60.0	59.2
Morchella hybrida	1	360	4.1	48.3	47.4	20.0	89.4	71.9	32.6	46.9	42.0
M. hybrida	2	360	3.9	54.7	43.4	21.9	87.3	75.2	28.7	52.2	45.5
M. hybrida	3	360	—[e]								
Collybia velutipes	1	240	2.7	36.2	29.2	19.4	71.5	77.5	42.5	29.2	20.9
C. velutipes	2	240	2.8	36.8	30.6	12.4	72.8	76.8	33.9	30.3	22.1
C. velutipes	3	240	—[e]								
Cantharellus cibarius	1	240	2.0	53.8	22.9	12.1	73.7	73.6	35.6	31.3	23.1
C. cibarius	2	240	2.0	49.1	22.1	9.6	73.5	75.7	27.0	28.5	20.9
C. cibarius	3	360	2.8	50.0	30.9	21.3	72.2	74.4	41.6	40.6	29.3
Xylaria polymorpha	1	192	2.0	43.9	17.6	10.5	98.9	95.5	44.2	19.0	18.8
X. polymorpha	2	192	1.4	38.1	12.4	8.0	97.2	93.6	36.9	11.6	11.2
X. polymorpha	3	240	—[e]								

[a] Media contained (per liter): 0.16% nitrogen, 0.47% reducing sugars, 1.20% total carbohydrates, and 2.33% total solids; initial pH was 5.40. All data are average of two flasks.

[b] Media 1, 2, and 3 contained 1 g of added nitrogen as ammonium sulfate, ammonium tartrate, and ammonium acetate, respectively.

[c] Expressed as g per 100 g of total carbohydrate used.

[d] Ratio of g of protein per 100 g of reducing sugars.

[e] No growth.

ered. Dilution and addition of glucose had no effect on mycelial growth of *C. cibarius* and *C. velutipes*. Whey concentrated 50% greatly increased growth but reduced protein content of *T. nudum* and *B. indecisus*. These preliminary results indicated that soybean whey, at its normal concentration, could support adequate growth.

Sterilized whey solution 1 contained suspended particles of heat-coagulated protein, which prevented uniform development of mycelium throughout the medium and interfered with the determination of protein. To avoid this difficulty, sterilized whey was clarified by filtration, which removed approximately half of the nitrogen. Thus, in all other experiments, whey solution 2 was used (Fig. 1). Then, ammonium acetate, tartrate, or sulfate was also added to the media to maintain a suitable ratio of nitrogen to carbon.

Of the seven species of fungi inoculated in soybean whey media containing different ammonium salts, only six developed mycelia (Table 1). *A. campestris* did not develop mycelia in any of the three soybean whey media. The addition of ammonium acetate to soybean whey medium completely inhibited growth of *C. velutipes*, *M. hybrida*, and *X. polymorpha*; increased the lag period of *T. nudum*; and increased mycelium growth of *B. indecisus*. *T. nudum* exhibited slurry or yeastlike growth in whey media containing ammonium sulfate and ammonium tartrate, whereas, with ammonium acetate, formation of spheres of 5 to 8 mm in diameter was a distinct advantage in filtering and harvesting the mycelium. *T. nudum* and *B. indecisus* showed the most rapid rate of growth and the highest production of mycelial protein with shorter incubation periods, compared with the other species. Depending upon the type of media, *T. nudum* produced 3.1 to 4.1 g of protein per liter, and *B. indecisus*, 1.9 to 2.8. Fermentative efficiencies and conversion of whey solids and nitrogen into mycelial protein were also high for these two organisms. Even after 360 hr of incubation, the other species did not produce nearly as much mycelia. The formation of spherical forms of *T. nudum* and *B. indecisus* and their rapid rate of growth were the criteria used to select these organisms for additional study.

TABLE 2. *Growth of various fungi on soybean whey media containing ammonium acetate and added glucose*

Fungus	Glucose added	Incubation time	Mycelium		Protein produced	Dry wt of mycelium[a]
			Dry wt	Protein content		
	%	*hr*	*g/liter*	%	*g/liter*	
Tricholoma nudum[b]	0[c]	144	7.4	55.1	4.1	64.5
	0	288	7.4	55.1	4.1	64.5
	1	144	7.7	55.2	4.3	36.6
	1	288	7.6	52.7	4.0	36.4
	2	144	2.7	49.5	1.3	9.8
	2	288	10.2	61.0	6.3	31.1
	3	144	2.8	48.2	1.4	7.3
	3	288	9.0	60.2	5.4	20.1
Boletus indecisus[b]	0	192	8.0	34.9	2.8	70.6
	0	216	8.9	33.6	3.0	78.7
	1	192	14.4	33.9	4.9	69.1
	1	216	13.2	27.0	3.6	63.6
	2	192	18.8	28.8	5.4	57.8
	2	216	17.9	25.0	4.5	54.9
	3	192	17.2	27.4	4.7	53.4
	3	216	22.2	25.3	5.6	50.0
Collybia velutipes[d]	0	216	5.4	45.9	2.5	38.9
	0	288	4.1	37.4	1.5	29.8
	1	216	4.8	41.5	2.0	32.3
	1	288	7.1	42.9	3.0	40.6
	2	216	5.4	48.9	2.6	22.3
	2	288	7.1	38.2	2.7	24.3
	3	216	5.7	40.7	2.3	20.5
	3	288	7.7	34.1	2.6	18.6

[a] Expressed as g per 100 g of total carbohydrate used.

[b] Initial pH was 5.10.

[c] The medium without added glucose contained (per liter): 0.11% nitrogen, 0.58% reducing sugar, 1.18% total carbohydrates, and 2.00% total solids; with 1% added glucose: 1.76% reducing sugar, 2.12% total carbohydrate, 2.85% total solids; with 2% glucose: 2.90% reducing sugar, 3.30% total carbohydrate, 3.80% total solids; with 3% glucose: 4.08% reducing sugar, 4.50% total carbohydrate, and 4.86% total solids. All media contained 0.56 g of nitrogen as ammonium acetate; initial pH was 5.10. All data are average of two flasks.

[d] Initial pH was 4.50.

The effect of added glucose on growth of various mushrooms in whey media containing ammonium acetate is shown in Table 2. With an incubation period of 144 hr, 1% glucose had little or no effect on mycelial growth, protein content, and yield of protein of *T. nudum*. More than 1% glucose in a period of 144 hr greatly inhibited mycelial

TABLE 3. *Growth of Tricholoma nudum in soybean whey media containing different ammonium salts and added iron, sulfur, and zinc*

Soybean whey medium*	Incubation time	Mycelium	
		Dry wt	Protein content
	hr	*g/liter*	%
With ammonium tartrate	72	6.8	53.4
	144	5.5	52.1
	216	4.6	49.9
Plus Fe, S, and Zn	72	6.9	55.8
	144	5.8	52.4
	216	4.9	49.1
With ammonium acetate	72	—†	
	144	7.0	55.9
	216	5.7	50.7
Plus Fe, S, and Zn	72	—†	
	144	—†	
	216	—†	

[a] Final concentration of nitrogen in all media was 0.11%, including the addition of 560 mg of nitrogen either as ammonium tartrate or ammonium acetate; when added, media also contained 111 mg of S as sulfuric acid, 0.6 mg of iron as ferrous sulfate, and 0.6 mg of zinc as zinc sulfate per liter. The pH of media containing ammonium tartrate was 4.50; that for ammonium acetate was 5.20.

[b] No growth.

growth and reduced protein content slightly. Yields of mycelial protein were also drastically reduced. However, with an incubation period of 288 hr and 2% added glucose, maximal mycelial growth and protein content were obtained: about 6.3 g of protein per liter compared with about 4.1 g of protein in the absence of glucose.

With *B. indecisus*, maximal mycelial growth and production of protein were obtained with 3% added glucose and 216 hr of incubation. Protein content of mycelium tended to decrease slightly with increasing concentration of glucose. *B. indecisus* yielded 5.6 g of protein per liter of whey media containing 3% glucose, compared with about 3 g in the absence of glucose.

Added glucose and 288 hr of incubation increased mycelial growth of *C. velutipes*, but had very little effect on yield of mycelial protein. The effect of added glucose on mycelial growth and protein content depends upon the species and incubation time.

Addition of iron as ferrous sulfate, sulfur as sulfuric acid, and zinc as zinc sulfate in the concentrations used had practically no effect on growth of *T. nudum* in soybean whey media containing ammonium tartrate (Table 3). These minerals, however, completely inhibited growth of *T. nudum* in whey containing ammonium acetate even after 216 hr of incubation.

Phosphorus as phosphoric acid greatly inhibited mycelial growth and protein yield of *T. nudum* (Table 4). Potassium as potassium hydroxide showed small losses in mycelial growth and yield of protein, whereas magnesium, as the sulfate, increased mycelial growth by 17% and yield of protein by 11%.

The effect of doubling whey concentration in the media on mycelial growth, protein content, and fermentative efficiency of *T. nudum* and *B. indecisus* is shown in Table 5. Maximal mycelial growth of *T. nudum* occurred after 144 hr of incubation, and was nearly twice as great as that obtained with normal whey media (Tables 1 and 2) at comparable incubation periods. Because protein content of the mycelia in concentrated whey was decreased, yield of protein (3.5 to 4.0 g per liter) was about the same as that obtained with the normal whey media (3.1 to 4.3 g per liter). Growth of *B. indecisus* in concentrated whey media was greatly increased. The amount of mycelia and protein produced with *B. indecisus* was comparable to that obtained with normal whey media in the presence of 2 and 3% added glucose (Table 2), and was nearly twice as great as that obtained with whey media described in Table 1. Thus, both *T. nudum* and *B. indecisus* converted whey nitrogen to mycelial protein more efficiently in concentrated whey. The differences in protein yield of these two species, and the type of soybean whey media used, will have important economic effects on soybean whey utilization. Differences in fermentative efficiency, as well as differences in the amount of whey solids utilized during fermentation, will also have to be considered in disposal of whey.

Recovery of coagulable whey protein and use of whey solids in fungi fermentation should reduce the biological oxygen demand of the liquor after harvesting the mycelia. In practice, recovery of the heat-coagulated protein separately may be unnecessary, because it could serve as part of the culture media and be recovered in part with the mycelia.

The decreasing effect, result of a longer lag phase, of increased ratios of carbon to nitrogen on mycelium growth of *T. nudum* is similar to that observed with other species in other media (Reusser et al., 1958*b*; Moustafa, 1960; Litchfield et al., 1963). However, the effect was not observed with *B. indecisus* and *C. velutipes*.

Differences in fermentative efficiency obtained with *T. nudum* and *B. indecisus* in concentrated soybean whey

TABLE 4. *Growth of Tricholoma nudum in soybean whey containing ammonium acetate and added minerals*[a]

Soybean whey medium[b]	Mycelium	
	Dry wt	Protein content
	g per liter	%
Control	6.5	54.9
Added phosphorus[c]	1.4	64.2
Added potassium[d]	6.1	49.1
Added magnesium[e]	7.6	53.1

[a] Incubation time in all media was 144 hr.

[b] All media contained 0.56 g of nitrogen as ammonium acetate; 0.11% final concentration of nitrogen (pH 5.60).

[c] Amount added was 150 mg of phosphorus per liter as phosphoric acid.

[d] Amount added was 300 mg per liter as potassium hydroxide.

[e] Amount added was 20 mg per liter as magnesium sulfate.

TABLE 5. *Growth of Tricholoma nudum and Boletus indecisus in soybean whey concentrated to 50%**

Species	Incubation time	Dry wt of mycelium†	Crude protein dry matter	Nitrogen used	Reducing sugars used	Total carbohydrates used	Dry matter used	Efficiency‡	
								Sugar used	Original sugar
	hr	*g/liter*	%	%	%	%	%	%	%
Tricholoma nudum	72	9.0 (44.2)	39.1	65.6	97.8	96.9	63.7	66.0	64.5
T. nudum	96	10.4 (50.6)	36.9	70.0	98.2	97.4	67.3	71.4	70.1
T. nudum	144	11.4 (53.4)	34.4	72.7	98.3	98.0	72.8	72.9	71.6
T. nudum	192	10.4 (50.7)	39.2	73.2	98.5	98.2	72.8	76.3	75.2
Boletus indecisus	192	16.3 (76.3)	27.6	80.2	98.7	97.3	74.0	42.7	42.2
B. indecisus	216	17.0 (79.3)	26.6	80.2	98.7	97.4	74.3	42.7	42.2
B. indecisus	240	16.8 (78.6)	26.4	79.3	98.8	97.4	74.0	42.0	41.5
B. indecisus	264	17.1 (80.0)	27.1	79.3	98.8	97.4	74.0	44.0	43.4

* Soybean whey medium contained: 1.08% of reducing sugars, 2.15% total carbohydrates, 0.10% nitrogen, and 3.81% total solids (pH 4.40). All data are average of two flasks.

† Figures in parentheses indicate amount expressed as g per 100 g of total carbohydrates used.

‡ Ratio of g of protein per 100 g of reducing sugars.

as the sole source of nitrogen and in whey containing ammonium salts were not very large.

Far better efficiencies were obtained with *T. nudum* and *B. indecisus* cultured in concentrated soybean whey and in whey containing ammonium salts, than in media containing molasses or waste sulfite liquor (Reusser et al., 1958*b*) and in some vinasse media (Falanghe, 1962). Fermentative efficiency of *T. nudum* was higher than that of *B. indecisus*.

T. nudum usually forms yeastlike or slurry mycelium (Falanghe, 1962; Cirillo, Hardwick, and Seeley, 1960). This form of mycelium was obtained with *T. nudum* in all soybean whey media, except those containing ammonium acetate; but, in it, mycelium developed as uniform spheres, which was a definite advantage in filtering and harvesting. *B. indecisus* formed spheres in all soybean whey media.

ACKNOWLEDGMENT

We are indebted to L. I. Wilson for technical assistance.

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