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Effect of Auxins and Herbicides on Enhancement of Protein Synthesis in Fungi

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A number of test compounds were investigated as potential compounds for improving the quantity of fungal protein that can be synthesized from organic wastes. Zinc sulfate was also evaluated in combination with selected test compounds. Where the cost of converting organic wastes to microbial protein may be marginal, the data suggest that low concentrations of test compounds, especially when used in conjunction with $ZnSO_4$, may improve the economic feasibility of fermentation processes to produce protein.

In 1880, Van Sachs postulated that plants are regulated by hormones. In 1934, Kogl and Erxteben isolated the growth regulating substance of plants, the auxin indoleacetic acid (IAA). Some of the same physiological effects of IAA on plants, which could also exert important influences on microorganisms, are cell elongation, root initiation, and induced synthesis of new RNA and protein.

Introduction of the synthesis of new RNA and protein by the exogenous application of IAA has been demonstrated in a variety of tissues, *e.g.*, Rhoeo leaves and bean endocarp (Sacher, 1967), yeast cells (Shimoda *et al.*, 1967), and green pea stem sections (DeHertogh, 1965). With the use of specific inhibitors, this synthesis activity of IAA was proven to be associated with IAA auxin-induced cell wall plasticity and extension. Puromycin, actinomycin D, chloramphenicol, and 8-azaguanine are four inhibitors frequently used in studies on the inhibition of the biosynthesis of RNA and protein.

In studies with herbicides, Ries *et al.* (1967) demonstrated an increase in nitrate reductase activity when suboptimal levels of the herbicide simazine were applied to plants grown with nitrate, but not to plants grown with ammonia as the sole source of nitrogen. An increase in total protein per plant was also confirmed. Rye plants receiving 0.5 to 0.8 μmol of simazine contained up to 45% more water-extractable protein than the controls without simazine. Atrazine, diuron, and terbacil caused similar effects.

Although most of the studies concerned with growth regulators have dealt specifically with plants, there is also some information that describes the action of this group of chemicals on the bacterial and fungal microflor of the soil.

Sikka *et al.* (1965) determined the effect of several concentrations of atrazine on the mycelial growth in liquid media of four common soil fungi: *Trichoderma, Fusarium, Penicillium,* and *Geotrichum.* The herbicide stimulated the growth of the four fungi at concentrations ranging from 1 to 10 ppm. Addition of 10 ppm increased the weight of the mycelium by almost 100%. In general, mycelial growth increased only up to a point with higher concentrations of the herbicides. We conclude from the work of other investigators that auxins and selected herbicides may be effective agents in stimulating the metabolism of nitrogenous compounds in fungi and, thus, in producing fungi with high protein content.

The production of protein from various organic wastes has already been demonstrated with the use of both fungi and bacteria (Callihan and Dunlap, 1971; Rogers and Scarpino, 1972). Dailey (1972) has also reported that one could induce up to an 11.6% increase in protein in *Aspergillus niger* with the use of 10 ppm of IAA, with glucose as

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Table I. A Comparison of the Effect of Six Growth Hormones on the Protein Synthesis of *Trichoderma viride* Using Glucose as a Substrate

Growth regulator, ppm	Av wt of 3 samples, g	Av Kjehldahl of 3 samples, % N	Av % protein of 3 samples	Av g of protein of 3 samples	$T_3 - T_0$, g	% difference	Stat. sample t value (T_3)	Anal. of data,[a] significant levels 95%	90%
0 (control)	0.4161	5.28	33.63	0.1403					
IAA, 20	0.4682	5.64	35.23	0.1647	+0.0244	17.39	3.75	Yes	Yes
FAA, 20	0.4553	5.63	35.21	0.1595	+0.0192	13.63	2.95	No	Yes
MAH, 20	0.4206	5.99	37.46	0.1575	+0.0172	12.25	2.64	No	Yes
2,4-D, 20	0.4337	5.70	35.63	0.1544	+0.141	10.04	2.16	No	No
GA, 20	0.4211	5.87	37.33	0.1532	+0.0129	9.19	1.98	No	No
NAA, 20	0.3727	5.99	37.46	0.1396	−0.0007	0.49	−0.10	No	No

[a] Critical t values: 95% = 3.02, 90% = 2.53; two-tailed test; MS error = 0.0000635.

the substrate. The research presented here thus represents an effort to determine if various levels of selected auxins, herbicides, and/or zinc can stimulate an increase in fungal protein synthesis.

MATERIALS AND METHODS

Preparation and Maintenance of Fungal Cultures. All species of fungus were of the Solid and Hazardous Waste Research Laboratory collection except *Trichoderma viride* which was obtained from the U.S. Army Natick Laboratory. The fungus evaluated were: *Aspergillus fumigatus,* strain =3, *Aspergillus awamori,* and *Trichoderma viride.* Cultures of these organisms were maintained almost exclusively on BBL saboraud dextrose agar slants, but occasionally they were subcultured in 100 ml of mineral salts containing a suitable substrate. Glucose was the initial substrate, chosen because of its freedom from other substances that could influence growth rate and protein synthesis. Whey waste, obtained from a local milk processor, was the second substrate.

All experiments were conducted in 500-ml erlenmeyer flasks to which were added 2.0 g of glucose or another appropriate substrate and 100 ml of mineral salts solution (Rogers and Scarpino, 1972).

Inoculum. Spores from selected fungi were removed from the surface of agar slants, suspended in a flask containing 250–350 ml of mineral salts solution and from 5.0 to 7.0 g of glucose. This flask was aerated by placing it on a shaker at 100 rpm for approximately 24 hr. Five milliliters (0.1 g dry weight) of this suspension was then used as an inoculum. The experimental flasks, which contained 100 ml of mineral salts, a protein stimulating agent, and inoculum, were then incubated for 3 days at 35° on a New Brunswick gyrotary shaker.

Test Compounds. Six compounds were evaluated for their effect on inducing protein synthesis: indole-3-acetic acid (IAA), gibberellic acid (GA), 1-napthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), maleic acid hydrazide (MAH), and (2-furyl)acrylic acid (FAA). Because of the low solubility of test compounds in water, *N,N*-dimethylformamide, dioxane, and 1 *N* HCl were used as solvents. Volumes of 0.1–0.3 ml of solvents equivalent to those used in the studies were added to control flasks to ensure that stimulation or inhibition of the fungi was not the result of the solvent *per se.* All test compounds (10 mg/1 ml of solvent) were added separately to the autoclaved experimental flasks to avoid possible thermal alteration of properties.

To evaluate the effect of zinc sulfates alone and in conjunction with other test compounds, 1.0 g was dissolved in 100 ml of distilled water and each flask received 0.6 ml for a final concentration of 60 ppm. Test-growth stimulants were added in concentrations from 5 to 60 ppm.

Fungal Mass Analyzed for Increased Protein Synthesis. Following the 3-day incubation period, the flasks were removed from the shaker, autoclaved at 121° for 15 min to kill the fungi as a safety precaution, and then allowed to cool. Each sample was washed twice with 300 ml of tap water and once with 300 ml of distilled water to remove external nitrogenous substances from the fungal mycelium. The samples were then dried for 48 hr at 60° in a hot air oven. Samples were analyzed for nitrogen by Kjeldahl nitrogen method.

Statistical Analysis of Experimental Results. The statistical method selected is one designed to compare each treatment process in an experiment with a control condition. Use of the Dunnet's t-statistic test (Winer, 1962) is appropriate when the level of significance is desired for the set of all comparisons between several treatments and a control.

RESULTS

Based on the results obtained from preliminary data with *Aspergillus fumigatus,* a concentration of 20 ppm of each auxin was evaluated for its ability to stimulate protein synthesis. In Table I, the effect of the six compounds on protein synthesis with *Trichoderma viride* can be compared. IAA was the only hormone in this experiment to show a statistically significant increase in grams of protein at the 95% confidence interval. MAH and FAA, however, were significant at the 90% level. All compounds except NAA stimulated an increase in weight and in the per cent protein over that of the control flasks.

Effect of Zinc Singly and in Combination with Growth Regulators. A problem occasionally incurred in the preliminary stages of this study was a decrease in fungal mass when plant hormones were added. In an attempt to increase the mycelial mass and protein content of selected fungi, a separate study was done with the use of zinc sulfate. Zinc sulfate at 60 ppm was the optimum concentration necessary for increasing mycelial mass (Rogers and Spino, 1972).

The influence on *Aspergillus fumigatus* strain =3 of zinc in combination with six test compounds is presented numerically in Table II. Gibberellic acid in combination with zinc was the only compound that caused a significant increase in protein at the 95% confidence interval. However, all other test chemicals caused an increase in fungal mass over the control.

The data in Table III show the influence of a combination of zinc and test compounds on protein synthesis with the fungus *Trichoderma viride.* In all cases, except with

Table II. An Evaluation of the Effect of Zinc Alone and in Combination with Six Growth Regulators on the Protein Synthesis of *Aspergillus fumigatus* Strain =3 Using Glucose as a Substrate

Growth regulator, ppm	Av wt of 3 samples, g	Av Kjehldahl of 3 samples, % N	Av % protein of 3 samples	Av g of protein of 3 samples	$T_j - T_0$, g	% difference	Stat. sample t value (T_j)	Anal. of data,[a] significant levels 95%	90%
0 (control)	0.3771	4.96	31.00	0.1171					
GA, 20 zinc, 60	0.5721	5.17	32.31	0.1856	+0.0685	58.45	3.23	Yes	Yes
IAA, 20 zinc, 60	0.5250	5.11	31.92	0.1678	+0.0507	43.29	2.38	No	No
2,4-D, 20 zinc, 60	0.4591	5.51	34.42	0.1575	+0.0404	34.50	1.90	No	No
MAH, 20 zinc, 60	0.5244	4.63	28.98	0.1521	+0.0350	29.88	1.65	No	No
NAA, 20 zinc, 60	0.4868	4.93	30.79	0.1490	+0.0319	27.24	1.50	No	No
FAA, 20 zinc, 60	0.4946	4.75	29.71	0.1468	+0.2097	25.36	1.39	No	No
Zinc, 60	0.4082	4.89	30.54	0.1246	+0.0075	6.40	0.35	No	No

[a] Critical t values: 95% = 3.04; 90% = 2.56 two-tailed test; MS error = 0.0006756.

Table III. Effect of Zinc and Growth Regulators Alone and in Combination with Each Other on the Mass and Protein Synthesis of *Trichoderma viride* Using Glucose as a Substrate

Growth regulator, ppm	Av wt of 3 samples, g	Av Kjehldahl of 3 samples, % N	Av % protein of 3 samples	Av g of protein of 3 samples	$T_j - T_0$, g	% difference	Stat. sample t value (T_j)	Anal. of data,[a] significant levels 95%	90%
T. viride, control	0.4350	5.44	34.00	0.1479					
Zinc, 60	0.5680	5.79	36.19	0.2057	+0.0578	39.08	3.98	Yes	Yes
2,4-D, 20	0.5108	5.67	35.44	0.1809	+0.0330	22.31	2.30	No	No
2,4-D, 20 zinc, 60	0.5324	5.87	36.69	0.1932	+0.0453	30.62	3.16	Yes	Yes
GA, 20	0.5221	5.41	33.98	0.1773	+0.0294	19.87	2.05	No	No
GA, 20 zinc, 60	0.5684	5.78	36.15	0.2022	+0.0543	36.71	3.78	Yes	Yes
IAA, 20	0.5128	5.44	33.93	0.1744	+0.0265	17.91	1.85	No	No
IAA, 20 zinc, 60	0.6244	5.75	35.94	0.2243	+0.0764	51.65	5.32	Yes	Yes
FAA, 20	0.5240	5.31	33.19	0.1738	+0.0259	17.51	1.80	No	No
FAA, 20 zinc, 60	0.6253	5.60	34.88	0.2185	+0.0706	47.73	4.92	Yes	Yes
MAH, 20	0.4552	5.27	32.94	0.1619	+0.0140	9.46	0.98	No	No
MAH, 20 zinc, 60	0.5650	5.54	34.65	0.1957	+0.0478	32.31	3.30	Yes	Yes
NAA, 20	0.3569	5.99	37.46	0.1337	+0.0142	9.60	−0.99	No	No
NAA, 20 zinc, 60	0.4169	6.09	38.06	0.1586	+0.0107	7.23	0.75	No	No

[a] Critical t values: 95% = 3.01; 90% = 2.57, two-tailed test; MS error = 0.0003092.

NAA plus zinc, the combination of test compounds plus zinc was significant at the 95% confidence interval. More importantly, the combinations of test compounds and zinc resulted in significant increases in fungal mass over that given by compounds alone.

Although the results of these last three studies (in Tables I–III) conflict in some ways, clearly zinc, in combination with other test compounds, effects an increase in the weight of the fungal mass and thus an increase in protein when compared with that of the control values. The increase in protein yield with combinations of zinc and growth regulators over that of the controls is significant in most cases at the 95% confidence interval.

The weight and sometimes the protein content of samples that contained test compounds only increased over that of the controls, but not to the extent of samples that contained growth regulators plus zinc. NAA was the only test compound that appeared to have little effect on pro-

Table IV. Average Protein Values for Two Experiments Designed to Determine the Concentration of a Growth Regulator Needed to Promote Optimum Protein Synthesis in *Aspergillus awamori* Using Glucose as a Substrate

Growth regulator, ppm	Expt 1, av g of protein/ 3 samples	Expt 2, av g of protein/ 3 samples	Av g of protein/sample for both expts
0 (control)	0.1257	0.1279	0.1268
IAA, 5	0.1461	0.1281	0.1371[a]
IAA, 10	0.1401	0.1268	0.1335
IAA, 15	0.1376	0.1190	0.1283
IAA, 20	0.1344	0.1131	0.1238
IAA, 25	0.1355	0.1187	0.1271
IAA, 30	0.1322	0.1102	0.1212
GA, 5	0.1315	0.1200	0.1258*
GA, 10	0.1327	0.1153	0.1240
GA, 15	0.1287	0.1164	0.1126
GA, 20	0.1299	0.1174	0.1237
GA, 25	0.1303	0.1187	0.1245

[a] Concentrations of IAA and GA at which greatest stimulation occurred.

tein yield of the various fungi. Values for other growth regulators were conflicting but showed a trend toward stimulation of protein yield.

Concentration of Test Compound Needed for Optimum Protein Yields. *Aspergillus awamori* (another promising fungus) was grown in concentrations of IAA and GA of from 5 to 30 ppm to determine the concentration of test compound needed to yield the greatest protein increase on a glucose substrate. The results of this experiment are shown in Table IV. The highest protein yield occurred at the 5-ppm concentration.

In general, when the test compounds were evaluated using whey as a substrate, they did not produce as great an increase in protein yield at 5 ppm as was the case when glucose was the substrate. The reason for this occurrence is not clear, but it could be because whey, being a more complex substrate than glucose, could mask the action of test compounds at 5 ppm. Table V shows that the greatest influence of test compounds occurs at 10 ppm concentration for only IAA.

DISCUSSION

There are many possibilities as to the cause of the stimulation or inhibition of protein synthesis by growth regulators in fungi. In plants, it has been suggested that regulators may promote the synthesis of specific enzymes by unmasking the appropriate preformed messenger RNA (Spirin, 1966; Yung and Mann, 1967) or by changing a particular variety of transfer RNA needed to initiate synthesis (Armstrong, 1966).

Sikka *et al.* (1965) did not identify the mechanism that might be involved in stimulating *Trichoderma* growth in the presence of 10 ppm of atrazine. They did, however, postulate that atrazine may have influenced an increase in the rate that fungus utilized sugar in the medium. Kaiser and Reber (1970) concluded that stimulating fungi with small doses of atrazine resulted in the same increased consumption of nitrate that Ries observed in plants.

Without any apparent change in morphology or changes in concentrations of extracellular esterase activity, results from this study clearly show that growth regulators in low concentrations (parts per million) will increase the fungal protein content at significant levels over that of the control.

It was further demonstrated that zinc, in combination with chemical growth regulators, will increase both mycelial mass and fungal protein content. Although the produced protein varied from 7.23 to 39.08% (see Table III), the usual value was about 10%, similar to that observed by Dailey. Stimulation of protein synthesis in fungi was more apparent with glucose than when a more complex substrate such as whey was used. The interest in enhancing the yields of protein in microorganisms is of considerable importance, and higher yields could improve the economic feasibility of single protein production processes. At present, most microbial production processes that utilize organic wastes are economically marginal, and further efforts to achieve viable operations may depend more on a physiological rather than engineering breakthrough.

Table V. Effect of Indole-3-acetic Acid, Gibberellic Acid, and Zinc on Mass and Protein Synthesis of *Aspergillus awamori* Using Whey Waste as a Substrate

Growth regulator, ppm	Av wt of 3 samples, g	Av Kjehldahl of 3 samples, % N	Av % protein of 3 samples	Av g of protein of 3 samples	$T_1 - T_0$, g	% difference	Stat. sample t value (T_1)	Anal. of data,[a] significant levels 95%	90%
0 (control)	0.5527	5.92	36.98	0.2042					
IAA, 5	0.5491	6.08	38.00	0.2086	+0.0044	2.15	0.92	No	No
IAA, 5 zinc, 60	0.5857	5.83	36.45	0.2130	+0.0088	4.30	1.85	No	No
IAA, 10	0.5574	6.25	39.44	0.2196	+0.0154	7.54	3.24	Yes	Yes
IAA, 10 zinc, 60	0.5698	6.18	38.60	0.2199	+0.0157	7.68	3.30	Yes	Yes
GA, 5	0.5485	6.23	38.96	0.2136	+0.0094	4.60	1.98	No	No
GA, 5 zinc, 60	0.5713	6.05	37.81	0.2158	+0.0116	5.68	2.44	No	No
GA, 10	0.5364	6.20	38.77	0.2079	+0.0037	1.81	0.78	No	No
GA, 10 zinc, 60	0.5634	5.95	37.21	0.2096	+0.0054	2.64	1.13	No	No
Zinc, 60	0.5530	6.03	37.66	0.2081	+0.0039	1.90	0.82	No	No

[a] Critical t values: 95% = 3.07; 90% = 2.60 two-tailed test; MS error = 0.000034.

The results from this study clearly show in many cases predetermined concentration of test compounds can improve the protein yields of processes to recycle organic wastes when the wastes are used as substrates to produce microbial protein. These or other agents may also enhance protein yields in on-going fermentation processes that utilize hydrocarbons as feed.

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Extraction of Protein, Low in Nucleic Acid, from *Saccharomyces fragilis* Grown Continuously on Crude Lactose

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Conditions for the maximum recovery of protein with a low nucleic acid content from yeast *Saccharomyces fragilis* using sodium hydroxide (0.4%), sodium chloride (4%), or water were established. Homogenization of a 5% cell suspension in NaOH (0.4%) in the presence of glass beads gave maximum extraction, *i.e.* 83% of Kjeldahl protein compared to 55% when water was used. Maximum yield of protein was obtained by acid precipitation (pH 4.3) of the alkaline extract, *i.e.* 88% *vs.* 86% from the aqueous extract. Total cell protein recoveries were 65 and 26% for alkaline and aqueous extracts, respectively. The yeast protein isolates contained >83% protein and less than 5% nucleic acids. Negligible amounts of nucleic acid occurred in yeast protein precipitated from a water extract at pH 6.0 and 80°.

The resistance of cell walls to rupture or digestion and the presence of nucleic acids are two major problems impeding the large scale use of single-cell protein (SCP) in food products. The indigestibility of the cell wall limits the availability of cell components thereby reducing the nutritional value of intact yeast cells (Tannenbaum *et al.,* 1966; Tannenbaum and Miller, 1967). The amino acid composition of cell wall is unbalanced, lacking sulfur and aromatic amino acids (Enebo, 1968; Ikawa and Snell, 1956; Salton, 1964). In order to improve nutritional value, the cytoplasmic protein must initially be extracted from microbial cells and then concentrated (Hedenskog and Ebbinghaus, 1972).

Several attempts were made for the direct extraction of protein from intact microbial cells (Mitsuda *et al.,* 1969; Mitsuda *et al.,* 1971; British Petroleum Co., 1970; Kyowa Hakko Kgyo, 1966). With intact cells an extensive chemical treatment or physical rupture is necessary to render the cell contents available to the extractant (Curran and Evans, 1942). When cells are first disintegrated, cell components can be extracted using mild chemicals, *i.e.,* water, dilute salts, and alkaline solutions.

Many have suggested the use of enzymes to digest the cell wall of yeast (Carenburg and Haden, 1970; Monreal and Reese, 1968; Sugimoto and Yokotsuka, 1968; Yamamoto *et al.,* 1972). However, these methods are limited because of extensive lysis and indiscriminate proteolysis. Very effective disintegration of yeast cell wall has been achieved using mechanical methods (Hedenskog *et al.,* 1969, 1970; Hedenskog and Ebbinghaus, 1972; Hedenskog and Mogren, 1973; Linnane and Vitols, 1962; Nossal, 1953; Novotny, 1964; Rehacek *et al.,* 1969; Wimpenny, 1967). Hedenskog *et al.* (1970) indicated that extraction of disintegrated yeast cells gave higher protein yields than non-disintegrated cells.

The nucleic acid content of microbial cells is regulated by microbial growth rate (Herbert, 1961; Kjelgaard and Kurland, 1963; Neidhardt and Fraenkel, 1961; Neidhardt and Magasanik, 1960). Nucleic acid content of yeast cells can be reduced by decreasing the growth rate (Vananuvat and Kinsella, 1975a,b), but this is not practical since rapid growth is obligatory in most processes for economic cell production. Thus, reduction of the nucleic acids present is necessary (Hedenskog and Ebbinghaus, 1972). Heat activation of endogenous yeast nucleases has potential (Maul *et al.,* 1970). Ohta *et al.* (1971) investigated the optimization of this process. A combination of a heat-shock process followed by dialysis or washing with phosphate solution, at an alkaline pH, was evaluated by Canepa *et al.* (1972). Other enzymatic methods in which exogenous ribonuclease was added to a suspension of yeast cells were described (Castro *et al.,* 1971; Dekloet *et al.,* 1961; Necas, 1958; Schlenk and Dainko, 1965). Chemical extraction of nucleic acids with acid, alkali, phenol, salts, and detergents has been used for analytical determination (Arnstein and Cox, 1966; Chargaff *et al.,* 1950). Although these methods are successful in reducing the nucleic acid content of protein extracts, they are complicated and expensive.

A limited amount of information is available concerning

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