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Production of Vitamin B_{12} by *Micromonospora*

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Various species of *Micromonospora* produced yields of vitamin B_{12} activity as high as 11 μg/ml under conditions of shaken flask fermentation.

Vitamin B_{12} has been isolated from fermentation broths of numerous microorganisms, including antibiotic-producing actinomycetes (3). Organisms of the genus *Micromonospora*, order *Actinomycetales*, some of which also produce antibiotics, were investigated for the elaboration of the vitamin B_{12}-like activity. Seventy-six cultures of *Micromonospora*, including 69 unclassified species, were grown and analyzed for the presence of vitamin B_{12}. The identified species also examined in this manner were *M. purpurea* (NRRL 2953), *M. echinospora* (NRRL 2985), *M. echinospora* var. *ferruginea* (NRRL 2972), *M. echinospora* var. *pallida* (NRRL 2997), *M. halophytica* (NRRL 2998), *M. fusca* (CBS or NRRL B943), and *M. chalcea* (ATCC 12452).

Lyophilized cultures of these micromonosporal species were used as seed and were grown in submerged fermentations in 100 ml of a medium contained in 300-ml Erlenmeyer flasks on rotary shakers at 240 rev/min. This medium consisted of beef extract (Difco), 0.3%; tryptose, 0.5%; glucose, 0.1%; soluble starch, 2.4%; yeast extract, 0.5%; and calcium carbonate, 0.1 to 1%. Since cobalt is an essential component of vitamin B_{12}, it was at first added to the medium with the purpose of enhancing the vitamin B_{12} activity produced by these organisms. However, this was found unnecessary because of sufficiently high levels of cobalt in the constituents of the medium. The flasks were incubated on a rotary shaker at 37 C for 96 hr. A 5% transfer was made into the same medium which was incubated on a rotary shaker at 26 C for 72 hr. A 5% transfer was again made into a fermentation medium containing 3% soybean meal (A. E. Staley Manufacturing Co., Decatur, Ill.), 4% glucose, 1% calcium carbonate, and 0.0003% cobalt chloride, and the organisms were grown for 7 days on a rotary shaker at 26 C.

The vitamin B_{12} content of the filtered broths was determined microbiologically, employing *Lactobacillus leichmannii* ATCC 7830 (*U.S. Pharmacopeia*, 17th rev.), or by the agar diffusion assay with *L. leichmannii* ATCC 4797 (1). Samples were extracted and prepared for assay by the procedure of Perlman and Barrett (4). The assay data (Table 1) demonstrate that all of the strains tested produced vitamin B_{12} activity.

Further experiments were carried out with *M. purpurea* in an effort to isolate and identify vitamin B_{12}. The antibiotic substances coproduced by the organism were removed by adsorption on IRC 50 resin (Rohm and Haas), and the broth was treated essentially as described by Weinstein (U.S. Patent 3,169,100).

TABLE 1. *Vitamin B_{12} assay of Micromonospora species*

Species	Vitamin B_{12} assay (μg/ml)
M. purpurea (NRRL 2953)	5.6
M. carbonacea (NRRL 2972)	0.3
M. echinospora (NRRL 2985)	3.5
M. halophytica (NRRL 2998)	2.8
M. fusca (CBS)	1.7
M. chalcea (ATCC 12452)	6.0
Micromonospora species (ATCC 10076)	0.93
69 Unclassified strains	0.01-11.5
Uninoculated control	0

TABLE 2. *Comparative paper chromatography*

Compound	R_F values[a] with two solvent systems[b]	
	A	B
Isolated B_{12}	0.35	0.50
Vitamin B_{12} standard	0.35	0.50

[a] Bioautographed zones (*L. leichmannii* ATCC 4797).

[b] Systems: (A) 2-butanol (77 ml), distilled water (23 ml), 5% KCN solution (0.25 ml), $NaClO_4$ (100 mg); (B) 2-butanol (100 ml), distilled water (50 ml), 5% KCN solution (0.25 ml), concentrated NH_4OH (1.0 ml).

The isolated red compound was compared with crystalline vitamin B_{12} by a modification of the ionophoresis method of Holdsworth [2; Whatman no. 3MM paper, 0.5 M acetic acid containing 0.02% KCN (w/v), 10 v/cm, 18 hr, *L. leichmannii* ATCC 4797) and also by descending chromatography on Whatman no. 1 paper (U.S. patent 3,021,262).

The isolated red solids and the vitamin B_{12} standard which were compared in the two paper chromatographic systems described were found to be identical (Table 2). Electrophoretically, both samples moved the same distance (5.5 cm) from the origin, further confirming their identity to be the same.

The absorption spectra of the material isolated from the *M. purpurea* fermentation were compared with those of a sample of pure vitamin B_{12} in the visible, ultraviolet, and infrared regions, and in each instance the spectra were found to be the same as those of the reference compound.

These data indicate that the *Micromonospora* group of organisms resembles the *Streptomyces* in its ability to synthesize vitamin B_{12}.

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