VITAMIN REQUIREMENTS OF HANSENULA YEASTS IN RELATION TO THEIR PHYLOGENY

YAYE FURUTANI, R. F. BETZ, AND L. R. HEDRICK

Biology Department, Illinois Institute of Technology, Chicago, Illinois

Received for publication July 24, 1952

Burkholder, McVeigh, and Moyer (1944) determined that *Hansenula anomala* did not require an external supply of vitamins for growth, whereas *H. subpelliculosa* needed thiamin and *H. suaveolens* required biotin. Schultz and Atkin (1947) found that *H. anomala* and *H. lambica*, the latter is also *H. anomala* by synonymy, did not require added vitamins. Wickerham (1951) reported that *H. angusta*, *H. beckii*, *H. californica*, *H. canadensis*, *H. capsulata*, *H. minuta*, *H. silvicola*, and *H. subpelliculosa* required added vitamins for growth; but *H. anomala*, *H. ciferrii*, *H. jadinii*, *H. mrakii*, *H. saturnus*, *H. schneggii*, and *H. suaveolens* did not need added vitamins for growth.

In this study we have investigated the vitamin needs of 35 strains of the vitamin requiring species in order to determine if all of the available strains of the various species were homogeneous in their vitamin needs and to relate their vitamin requirements to their phylogenetic and biochemical patterns.

METHODS

The negative control or vitamin basal medium was the dehydrated "vitamin free yeast base" developed by Wickerham (Wickerham and Burton, 1948; Wickerham, 1951) and produced by Difco. The positive control or complete medium was Wickerham's dehydrated "yeast nitrogen base" plus 10 g glucose per liter. These media are identical, except that the former contains no vitamins. Both media contain the necessary mineral, nitrogen, and carbon sources to support good growth of the yeasts when the needed growth factors are present. These media were prepared in ten times strength and sterilized by passing them through a Seitz or sintered glass filter (the latter is recommended). One-half ml of filtrate was added aseptically to 4.5 ml of distilled water which had been sterilized in 18 mm by 160 mm test tubes at 121 C for 15 minutes. Growth responses for the yeasts, except *H. beckii*, were determined by reading absorbancies in a Coleman spectrophotometer using a wavelength of 420 mμ. For *H. beckii*, millimoles of nitrogen were determined. This yeast produced a considerable amount of mycelia which are present as clumps; thus turbidity readings were not satisfactory.

The individual vitamin solutions were prepared by different methods during this study. Folic acid, inositol, *p*-aminobenzoic acid, riboflavin, and nicotinic acid were sterilized by filtration through a Seitz filter or by autoclaving at 121 C for 15 minutes. Thiamin hydrochloride, pyridoxine hydrochloride, calcium pantothenate, and biotin were dissolved either in distilled water or the vitamin free basal medium and sterilized by filtration using a Seitz or sintered glass filter.

In order to prepare test media which contained the various combinations of vitamins, that is, one or more added and one or more omitted, it was convenient to dissolve the vitamins in distilled water, dilute them to the proper concentration, mix the vitamins under test in distilled water so that each vitamin would be in a ten times concentration, and filter the mixture through a Seitz filter. However, it became apparent that some of the vitamins, especially thiamin, were being partially or completely adsorbed on the Seitz filter pad. An investigation indicated that 4 μg per ml of thiamin when dissolved in 100 ml of distilled water was entirely adsorbed on the filter pad, 40 μg per ml of thiamin in 10 ml of distilled water gave only 2 μg per ml of thiamin in the filtrate, 400 μg gave 355 μg of thiamin per ml in the filtrate. The vitamins also were added to the vitamin free basal medium, pH 4.5 to 5.0, to give a ten times concentration and filtered through a Seitz filter. Under these conditions, only 1.2 μg per ml of thiamin, which is about 30 per cent, of the 4 μg per ml in the ten times solution passed through the Seitz pad. For qualitative experiments, this concentration may be sufficient to support good growth with some yeasts and only fair growth with others. Ob-

viously, any quantitative reports based upon vitamins, especially thiamin, filtered through Seitz filter pads would not be accurate. Subsequently, the vitamins were sterilized by filtration through sintered glass filters. The sterilized vitamins were added to the vitamin free basal medium so that the final concentrations were as follows: biotin, 0.002 μg; thiamin hydrochloride, 0.40 μg; pyridoxine hydrochloride, 0.40 μg; calcium pantothenate, 0.40 μg; riboflavin, 0.20 μg; folic acid, 0.002 μg; *p*-aminobenzoic acid, 0.20 μg; inositol, 2.0 μg; niacin, 0.40 μg. These quantities represent the concentrations before sterilization of the vitamins. Duplicate tubes were prepared in every experiment throughout this study, and incubation was at 25 C.

The yeasts to be studied were transferred from malt extract-yeast extract agar stabs or slants to vitamin free media. After incubation at 25 C for two to seven days, variable amounts of growth occurred. Transfers were made into a second tube of vitamin free medium, and even a third or fourth, until only a slight growth appeared in the tubes. In fact, for a period of eight months, the yeasts were transferred each week into the negative control medium, thus ensuring an ever ready source of inoculum that would give clear cut negative controls in the vitamin free medium. Preliminary experiments indicate that such depleted yeasts may be lyophilized in a vitamin free colloidal suspension, such as a 2 per cent aqueous suspension of medium viscosity carboxymethyl cellulose.[1] Inoculations with either a loop or a drop from a pipette are satisfactory with such depleted inocula.

EXPERIMENTAL RESULTS

Eleven strains of *H. subpelliculosa*, eight strains of *H. capsulata*, ten strains of *H. californica*, and two strains of *H. silvicola* were found to be identical in their vitamin deficiency patterns. *H. subpelliculosa* required thiamin if the tubes were read within four to seven days. However, after two weeks, the negative control tubes gave absorbancy readings of 1.05 as compared with readings of 1.48 for the positive control and vitamin free plus thiamin tubes. This indicates that this yeast is able to manufacture its own thiamin, but at quite a slow rate (figure 1). Thus, thiamin for *H. subpelliculosa* is on the borderline between an essential and an accessory growth factor. This is the proper explanation for the information reported by Furutani and Hedrick (1951) to the effect that *H. subpelliculosa* was able to adapt to biotin and pyridoxine

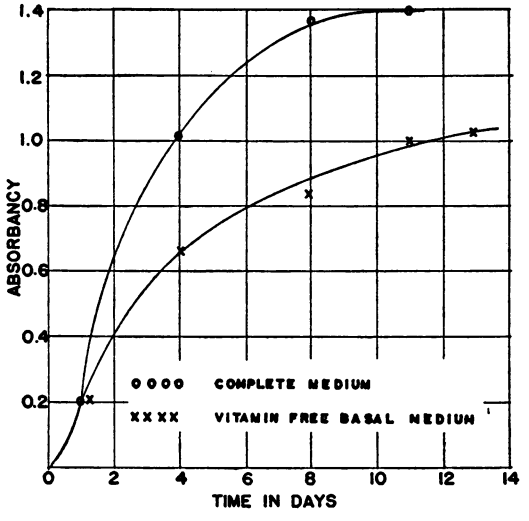

Figure 1. Hansenula subpelliculosa, growth in complete (and basal plus thiamin) and vitamin free media.

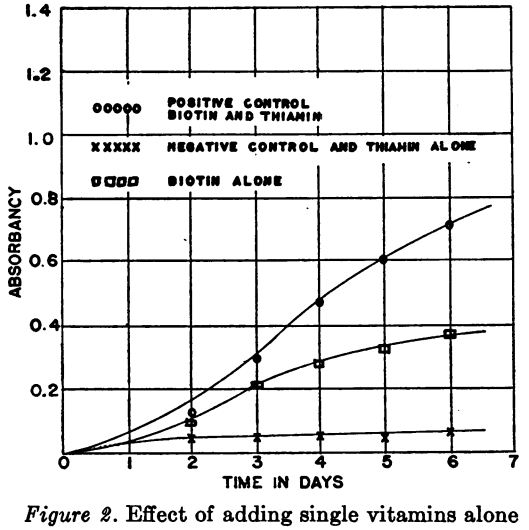

Figure 2. Effect of adding single vitamins alone or together on the growth of *Hansenula minuta*.

in the absence of thiamin during an incubation period of three weeks.

H. angusta and *H. californica* needed only biotin as an added growth factor. In later studies we have not been able to duplicate the so-called adaptation effect of *H. angusta* to thiamin or

[1] Hercules Powder Company.

pyridoxine in the absence of biotin as reported by Furutani and Hedrick (1951).

Figure 2 indicates that, of the vitamins of the B-complex studied, *H. minuta* required biotin

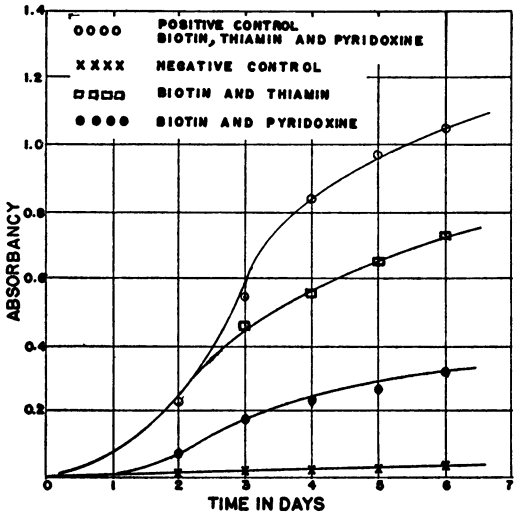

Figure 3. Effect of adding single vitamins alone or together on the growth of *Hansenula capsulata* and *Hansenula silvicola*.

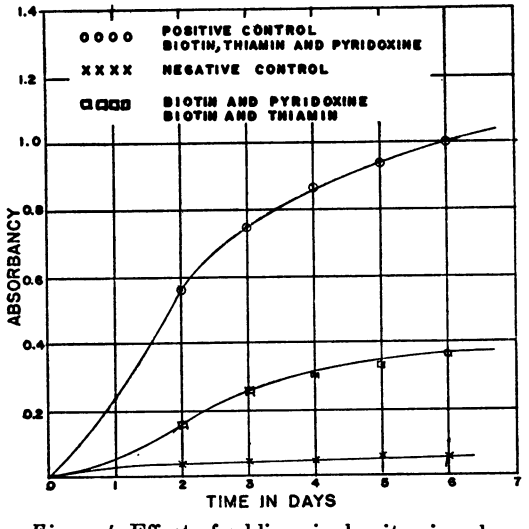

Figure 4. Effect of adding single vitamins alone or together on the growth of *Hansenula canadensis*.

and thiamin, with biotin serving as an essential vitamin and thiamin being a stimulatory growth factor. Figure 3 shows that both *H. capsulata* and *H. silvicola* required biotin and thiamin, but pyridoxine is an accessory vitamin. Figure 4 and table 1 reveal that *H. canadensis* and *H. beckii* need biotin, thiamin, and pyridoxine together for normal growth, as no substantial amount of growth was obtained if any of these vitamins were omitted from the medium.

TABLE 1

Millimoles of nitrogen obtained from a micro-Kjeldahl digestion of mycelium of Hansenula beckii after six days' growth when single vitamins were added either alone or together into an experimental vitamin free basal medium. Results are for two experiments. Hydrochloric acid, 0.021 M, was used in titrating the ammonia formed.

VITAMIN ADDED	MILLIMOLES OF NITROGEN
Positive control	216.3
Negative control	23.7
Biotin	38.1
Pyridoxine	52.3
Thiamin	57.6
Biotin + pyridoxine	54.6
Biotin + thiamin	49.1
Thiamin + pyridoxine	44.5
Biotin + thiamin + pyridoxine	194.6

DISCUSSION

Wickerham (1951) in his monograph on the *Hansenula* restudied the group with respect to their ecological, ploidy, and biochemical relationships. He demonstrated that some free living members of the genus exist exclusively as diploid cells when growing in the vegetative phase, namely *H. ciferrii*, *H. anomala*, *H. schneggii*, *H. jadinii*, and *H. subpelliculosa*. Two species, *H. beckii* and *H. canadensis*, are also entirely diploid. These two live in a restricted environment in association with conifer bark beetles and their larvae, and are considered by Wickerham to have decreased in biochemical activities as they became more dependent upon the trees and beetles on which their existence in nature depends. Free living species which are almost entirely diploid are *H. saturnus*, *H. mrakii*, and *H. suaveolens*. The species of *H. californica*, *H. angusta*, and *H. silvicola* exist in growing cultures as a mixture of haploid and diploid cells. The majority are haploid, but many diploid cells are present. *H. minuta* and *H. capsulata* are exclusively haploid.

For the most part the ability of the species to

ferment a number of sugars is directly related to their tendency toward diploidism. The exceptions are *H. beckii* and *H. canadensis* which have not developed or have lost the ability to form the fermentative enzymes possessed by all the other diploids, due perhaps to a parasitic adaptation to their restricted environment.

The vitamin needs are in general directly related to the tendency of the yeasts toward haploidism, except for *H. beckii* and *H. canadensis*. These latter yeasts require the largest number of essential growth factors, namely, biotin, thiamin, and pyridoxine. None of the other diploids, except one, requires growth factors, and as we indicated above, the thiamin requirement by *H. subpelliculosa* is quite marginal.

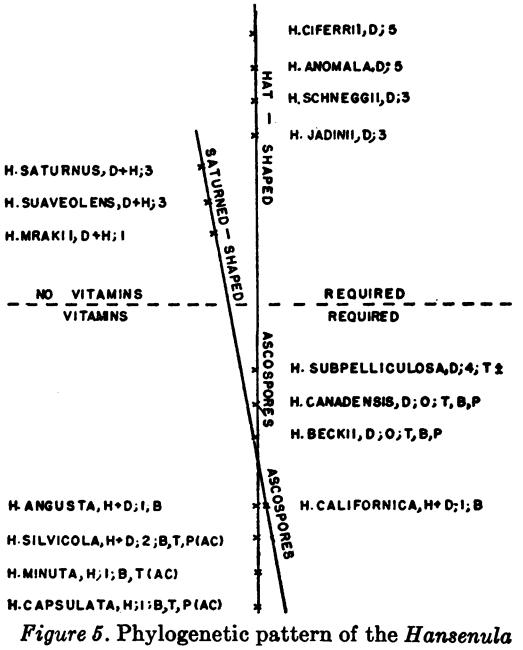

Figure 5. Phylogenetic pattern of the *Hansenula* and their vitamin requirements. Legend: D, diploid; D + H, mostly diploid, some haploid; H + D, mostly haploid; H, all haploid. B, T, P, biotin, thiamin, and pyridoxine required, respectively. (AC) indicates that the vitamin is accessory, not essential. Numerals refer to the number of sugars fermented.

Of the predominantly haploid yeasts, *H. angusta* and *H. californica* require only biotin, and *H. silvicola* needs biotin and thiamin as essential vitamins with pyridoxine being an accessory growth factor. Since *H. silvicola* has been isolated only from gummy exudate of the wild cherry tree, this material probably has supplied the yeast with the growth factors needed. The strictly haploid *H. minuta* needs biotin as an essential and thiamin as an accessory vitamin, while *H. capsulata* requires biotin and thiamin with pyridoxine as an accessory growth factor.

In general, for members of this genus, the need for vitamins parallels quite closely their other biochemical and ploidy characteristics. Superimposed upon the phylogenetic pattern are ecological factors which modify the vitamin requirements. The yeasts living in the most restricted environments require the largest number of vitamins for normal growth. These general relationships are outlined in figure 5.

ACKNOWLEDGMENT

The authors wish to thank Dr. Lynferd Wickerham for supplying the cultures of species and strains of the *Hansenula* yeasts; Difco Laboratories for some complimentary samples of Wickerham's synthetic media; Hercules Powder Company for the cellulose gum.

SUMMARY

Forty-six strains of yeasts representing fifteen species of the genus *Hansenula* were studied and the specific B-vitamin requirements of thirty-five strains of eight vitamin requiring species were determined.

The individual vitamin requirements of these yeasts corresponded quite generally to their ploidy characteristics except for *Hansenula beckii* and *H. canadensis*, diploids which merit special consideration due to their ecological relationships with bark beetles in conifer trees. All the other diploids, except *H. subpelliculosa*, did not need added vitamins, and this latter yeast had a marginal requirement for thiamin. The predominantly haploid yeasts, *H. angusta* and *H. californica*, required biotin, while *H. silvicola* needed biotin and thiamin with pyridoxine as an accessory vitamin. Of the exclusively haploid yeasts, *H. minuta* required biotin with thiamin as a stimulatory factor, whereas *H. capsulata* needed biotin and thiamin with pyridoxine as an accessory factor.

The ecologically restricted diploids, *H. beckii* and *H. canadensis*, required biotin, thiamin, and pyridoxine as essential vitamins.

REFERENCES

BURKHOLDER, P. R., MCVEIGH, I., AND MOYER, D. 1944 Vitamin requirements of certain yeasts and bacteria. J. Bact., **48**, 385–391.

FURUTANI, Y., AND HEDRICK, L. R. 1951 Vitamin requirements as factors in the phylogeny of species of genus *Hansenula*. Bact. Proc., **1951**, p. 67.

SCHULTZ, A. S., AND ATKIN, L. 1947 The utility of the bios response in yeast classification and nomenclature. Arch. Biochem., **14**, 369–380.

WICKERHAM, L. J. 1951 The classification and taxonomy of yeasts. U. S. Dept. Agric. Tech. Bull. no. 1029, 1–56.

WICKERHAM, L. J., AND BURTON, K. A. 1948 Carbon assimilation tests for the classification of yeasts. J. Bact., **56**, 363–371.