NICOTINIC ACID REQUIREMENTS OF CERTAIN YEASTS

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Knowledge of the accessory growth factors concerned in the physiology of lactose-fermenting yeasts was needed in an investigation of fermentation that was being conducted by the Bureau of Dairy Industry. Since information on nicotinic acid requirements for the growth of yeasts not fermenting lactose was at best fragmentary and incomplete and information on lactose-fermenting yeasts was completely lacking, a study was undertaken to develop the pertinent knowledge.

It has been known since 1913 that cells of the ill-defined species, *Saccharomyces cerevisiae*, contain nicotinic acid. Funk (1913) obtained nicotinic acid as a constituent of his "vitamine" fraction from yeast. With this knowledge of the nicotinic acid content of yeast, it would seem that investigators would have become interested in the growth response of living cells. However, a careful search of the literature has revealed very few publications on the growth response of yeast to nicotinic acid. Schultz, Atkin and Frey (1938) stated that nicotinic acid has no "bios" effect on strains of *S. cerevisiae*. Fink and Just (1939) showed that *Torula utilis*, an organism which does not ferment lactose, can synthesize the pyridine ring of nicotinic acid from such simple sources as ethanol, acetic acid, fermentable carbohydrates, ammonia nitrogen, and nutritive salts. Kögl and Borg (1941) found that the growth of the Konigsgist or Strain M yeast is not stimulated by nicotinamide alone. Nicotinamide plus thiamine produced a slight stimulation which probably was caused by the thiamine rather than the nicotinamide. Leonian and Lilly (1942) cultivated yeasts which had been trained to grow on a completely synthetic medium devoid of vitamins. As will be shown later in this paper, it is unnecessary to "train" yeasts not fermenting lactose to grow in a medium free of nicotinic acid only.

Koser, Wright, and Dorfman (1942) mentioned, *en passant*, that nicotinamide was one of the factors necessary for rapid growth of one strain of *Torula cremoris*. No other information was found in the literature concerning the growth response of lactose-fermenting yeasts to nicotinic acid.

EXPERIMENTAL

Cultures. A total of 114 strains of lactose-fermenting yeasts was employed in this study. These strains have already been listed by Rogosa (1943). The taxonomic status ascribed to the cultures by the workers who isolated and studied them was provisionally accepted. In addition, the following seven strains of *Saccharomyces cerevisiae*, which do not ferment lactose, were studied: *S. cerevisiae* Hansen American Type Culture Collection #764, *S. cerevisiae*

Hansen A.T.C.C. #765, *S. cerevisiae* Hansen A.T.C.C. #4110, *S. cerevisiae* (*F.B.*) A.T.C.C. #7754, *S. cerevisiae* (*O.P.*) A.T.C.C. #7753, *S. cerevisiae* (*G. M.*) A.T.C.C. #7752, and Yeast "Steinberg" A.T.C.C. #4938.

Medium. The following medium was used in all experiments:

Casein hydrolysate (dry weight)	5 g
Sodium acetate	6 g
Glucose	20 g
l-Asparagine	250 mg
l-Tryptophane	50 mg
l-Cystine	100 mg
Guanine hydrochloride	5 mg
Adenine sulfate	5 mg
Xanthine	5 mg
Uracil	5 mg
Salt solutions A and B; each	5 ml
Thiamine hydrochloride	200 micrograms
Riboflavin	200 micrograms
Ca pantothenate (d)	200 micrograms
Pyridoxine hydrochloride	200 micrograms
P-amino-benzoic-acid	200 micrograms
Inositol	1000 micrograms
Choline chloride	1000 micrograms
Nicotinic acid (when supplied)	1000 micrograms
Folic acid[1] (100 per cent calculated potency)	0.5 microgram
Biotin or biotin methyl ester	0.5 microgram

Lactic acid (0.6 ml.) to yield a pH of 4.8–5.0

Distilled H_2O to yield a total volume of 1000 ml.

Salt solution A: $K_2H\,PO_4$, 25 g; KH_2PO_4, 25 g; KI, 2.5 mg; H_3BO_3, 25 mg; H_2O to 250 ml.

Salt solution B: $MgSO_4 \cdot 7H_2O$, 10 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 4H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 2.5 mg; $ZnSO_4$, 25 mg; H_2O to 250 ml.

Preparation of medium. Labco "Vitamin-Free" casein was refluxed for 3 hours 3 successive times with 95 percent ethanol. An alternative procedure was to re-dissolve and re-precipitate the casein a minimum of 4 times. The alcohol treatment proved to be just as efficient and more convenient to use. The casein was dried and 100 g. was hydrolyzed with 500 ml. of 25 per cent H_2SO_4 by heating in the autoclave for 10 hours at 15 lbs. pressure. After cooling, the hydrolysate was stirred for 20 minutes with Norit-A (10 mg./g.) at a pH of approximately 1.0. This adsorption was repeated 3 times. Excess H_2SO_4 was removed by means of $Ba(OH)_2 \cdot 8H_2O$ until a pH of 5.2 was attained. The hydrolysate was diluted to a volume of 1 liter. C.P. glucose was used and in some experiments C.P. glucose in solution was treated with Norit-A and re-crystallized twice. C.P. l-asparagine was dissolved to a concentration of 10 mg./ml. l-Cystine (2 mg./ml.) was dissolved in distilled H_2O without heating by adding enough HCl to effect solution. Guanine hydrochloride, adenine sulfate, xanthine, and uracil were dissolved in a concentration of 1 mg./ml. by prolonged heating in distilled H_2O acidified with enough HCl to effect solution.

[1] Supplied through the courtesy of Dr. R. J. Williams of the University of Texas.

Thiamine hydrochloride was dissolved in acetate buffer (pH 4.5); the other vitamins in distilled H_2O. All solutions were preserved under toluene in the dark at 10°C. In all experiments in which the growth response of an organism was to be tested to increasing concentrations of nicotinic acid, the nicotinic acid solutions were made on the day of the experiment.

Every lot of medium was made personally by the writer subject to a system of rigid controls for each reagent. The medium was tubed in 8 ml. quantities in ordinary bacteriological test tubes, which were plugged with cotton, and sterilized for 15 minutes at 15 lbs. steam pressure.

Glassware. All glassware employed was scrupulously clean and in final critical experiments was washed by the usual laboratory facilities and subsequently rinsed at least 10 times in tap H_2O and 3 times in distilled H_2O.

Inoculum and incubation. The cultures used for inoculum were between 48 and 72 hours old at the time of inoculation. Both serial transfer and washed cell techniques were employed. Serial transfer was carried out by loop through at least four passages in the basal medium with and without the presence of nicotinic acid. In washed cell techniques the cells were centrifuged and washed 3 times in phosphate buffer at pH 6.5. The cells were then resuspended in phosphate buffer equivalent to 10 times the original volume of the culture. This cell suspension was shaken for a minute or two in a mechanical shaker. *One drop* of this suspension was used for inoculum and inoculations were carried out quickly in order to avoid settling of cells at the bottom of the pipette. All cultures were incubated without agitation at 30°C. in the dark.

Measurement of growth responses. Measurements of growth responses were made photometrically by means of a photoelectric photometer (Type F, American Instrument Company) with filter 51. When the tubes were ready for photometric measurements, they were removed from the incubator and placed at 0°C. for at least 20 minutes to stop growth. A series of tubes was corked, shaken, the bubbles of gas allowed to dissipate (approximately 1 minute), the contents of the tubes were poured into matched tubes, and the photometric measurements determined as percentage of light transmitted through the tube. These readings were converted to percentage of light absorbed by subtracting them from 100.

Repetition of experiments. Each experiment was performed at least 3 times and some experiments were repeated as many as 8 times.

RESULTS

Characteristic growth responses to nicotinic acid of the washed cells of a yeast not fermenting lactose, *Saccharomyces cerevisiae* (F.B.), and a lactose-fermenting yeast, *Mycotorula lactis* #130, are shown in figure 1.

Seven strains of *S. cerevisiae* were transferred by loop through 3 passages in a medium free from nicotinic acid. From the third passage, loop transfers were made into 2 sets of media, one containing adequate nicotinic acid and one free from nicotinic acid. Growth responses were determined after 16 hours of incubation. The results, which are summarized in table 1, show that it was un-

necessary to furnish nicotinic acid for the growth of these yeasts not fermenting lactose.

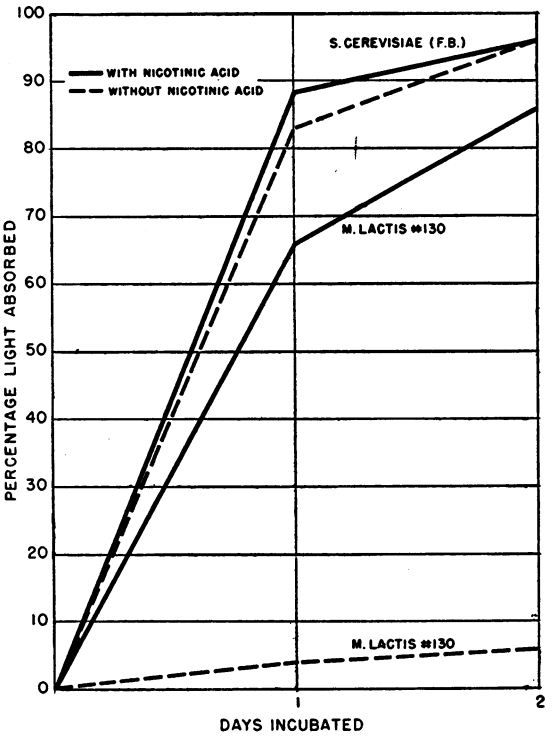

FIG. 1. GROWTH RESPONSES OF SACCHAROMYCES CEREVISIAE AND MYCOTORULA LACTIS IN THE PRESENCE AND ABSENCE OF NICOTINIC ACID

TABLE 1

Growth response of Saccharomyces cerevisiae in the fourth serial loop passage incubated for 16 hours in the presence and absence of nicotinic acid

CULTURE	PERCENTAGE LIGHT ABSORBED (UNINOCULATED CONTROL = 0)	
	Medium free from nicotinic acid	Complete medium
Saccharomyces cerevisiae Hansen #764	63	50
Saccharomyces cerevisiae Hansen #4110	68	66
Saccharomyces cerevisiae Hansen #765	53	61
Saccharomyces cerevisiae (old process) #7753	50	40
Saccharomyces cerevisiae (Gebrüder Mayer) #7752	44	45
Saccharomyces cerevisiae (F.B.) #7754	62	65
Yeast "Steinberg" #4938	49	46

One hundred fourteen strains of lactose-fermenting yeasts were transferred serially by loop through two parallel sets of media, one containing adequate nicotinic acid and one free from nicotinic acid. Incubation was for 7 days in each passage and transfers were made at the end of this time regardless of the

abundance or paucity of growth. Typical growth responses of 12 representative strains in the fifth serial loop passages are shown in table 2. These results show that an external source of nicotinic acid is indispensable for the continued growth of these lactose-fermenting yeasts.

TABLE 2

Growth response of lactose-fermenting yeasts in the fifth serial loop passage incubated for 7 days in the presence and absence of nicotinic acid*

CULTURE	PERCENTAGE LIGHT ABSORBED (UNINOCULATED CONTROL = 0)	
	Medium free from nicotinic acid	Complete medium
Saccharomyces anamensis #145	2	93
Saccharomyces lactis #131	2	90
Saccharomyces fragilis #15	2	86
Type F #93	6	93
Monilia pseudotropicalis (Castellani) #32	4	95
Mycotorula lactis #130	7	90
Zygosaccharomyces lactis #27	8	93
Zygosaccharomyces lactis #90	5	90
Torulopsis kefir #149	1	80
Torula cremoris #2	6	87
Torula lactosa #168	2	88
Torula sphaerica #13	7	95

* Seven days incubation between each transfer.

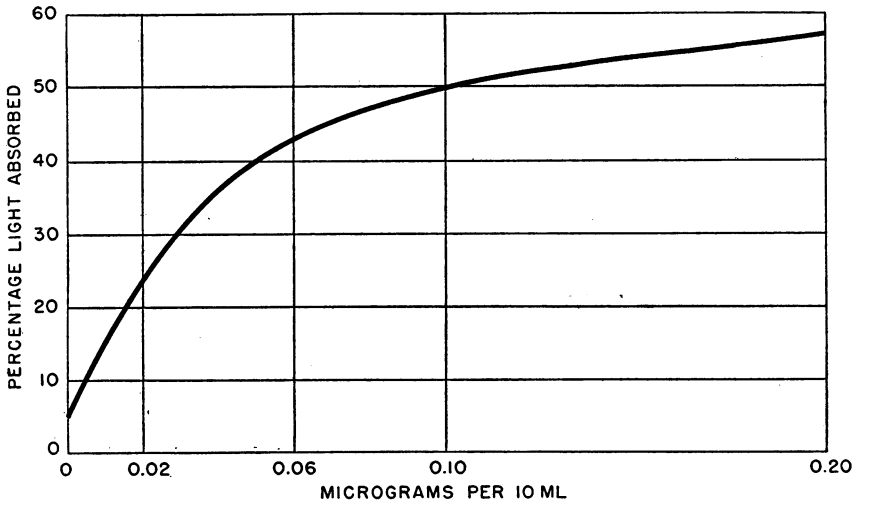

FIG. 2. GROWTH RESPONSE OF MONILIA PSEUDOTROPICALIS #32 TO INCREASING CONCENTRATIONS OF NICOTINIC ACID. INCUBATION 24 HOURS AT 30°C.

To obtain further evidence of the essentiality of nicotinic acid for the growth of lactose-fermenting yeasts, cells of *Monilia pseudotropicalis* (Castellani) #32 were washed 3 times and inoculated into a series of tubes containing graded concentrations of nicotinic acid. Inoculations were made into each concentration

in quadruplicate and tested after incubation for 24 hours. The result, which was characteristic of all lactose-fermenting strains tested, is shown graphically in figure 2.

DISCUSSION

Generally, growth of an organism may proceed for 1 or even 2 transfers in a deficient medium. This fact suggests the possibility of cell storage of vital materials that enable the cell to function until the vital materials are exhausted. For this reason serial transfer techniques were employed in addition to washed cell techniques. With some strains slight growth was noted on prolonged incubation in a medium free from nicotinic acid when washed cells were used for inoculum. On passing the cultures through serial transfer, there were only traces of growth at best. In general, however, the techniques yielded corroborative results.

The data show that lactose-fermenting yeasts cannot synthesize sufficient nicotinic acid to satisfy the normal needs of reproduction and indicate that yeasts which do not ferment lactose readily synthesize sufficient nicotinic acid for optimum growth.

The role of nicotinic acid or nicotinamide in coenzyme systems has been reviewed and emphasized by Schlenk (1942). In view of the differences in exogenous nicotinic acid requirements between lactose-fermenting and yeasts not fermenting lactose, it is probable that the vitamin-enzyme relations of the cell in fermentation are dissimilar for the two groups of organisms.

SUMMARY

Yeasts which do not ferment lactose do not require an exogenous source of nicotinic acid for growth. Lactose-fermenting yeasts, in contrast, require an exogenous source of nicotinic acid for growth.

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