

Plate Assay of Thiamine

I. Using *Kloeckera brevis*

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Lactobacillus fermenti strain 36 is the most frequently used microorganism for the assay of thiamine both in the tube method, (Sarett and Cheldelin, 1944; Fitzgerald and Hughes, 1949), and in the plate method, (Bacharach and Cuthbertson, 1948; Jones and Morris, 1949). Other microorganisms have more recently been suggested for the tube assay; Hoff-Jorgensen and Hansen (1955) used the yeast *Kloeckera brevis*, and Deibel *et al.* (1958) used *Lactobacillus viridescens*.

Difficulties are often encountered in the tube methods (Analytical Methods Committee, 1954) but the improved medium of MaciasR (1957) overcomes some of these. However, when the level of thiamine in samples is reasonably high, such as in multivitamin preparations, the *L. fermenti* plate method may be used; this is far less susceptible to variations than the tube assay with the same microorganism. Plate assays also have the advantages of simplicity and high throughput and, when in use as a routine, the large 12 in. x 12 in. plates, as used by Lees and Tootill (1955) and Simpson and Lees (1956), give assay values with a standard error of ± 10 per cent. The present paper describes a plate assay with *Kloeckera brevis* adapted from the tube method of Hoff-Jorgensen and Hansen. This plate assay has been in use in these laboratories for the past year in parallel with the *L. fermenti* plate assay.

MATERIALS AND METHODS

Culture and Inoculum

Kloeckera brevis strain B 768¹ is kept as a slope on malt agar, stored at room temperature, and subcultured fortnightly. For the inoculum, the growth from slopes, which have been incubated at 30 C for 18 hr, is washed off with sterile saline, 0.9 per cent, centrifuged, washed once with saline and finally resuspended in saline to give an optical density reading of 0.51 at 650 m μ in the 1-cm rectangular cell of the Unicam spectrophotometer,² or by plate count, 125 million yeasts per ml.

Cultures not older than 10 days were used for inoculum; older cultures stored from 10 days up to 3 weeks

¹ National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, England.

² Unicam Instruments Ltd., Arbury Works, Cambridge, England.

gave slightly diffuse zones, and cultures older than this gave very diffuse zones.

The composition of the basal medium is shown in table 1. The medium is steamed to dissolve the agar, distributed into flasks in the required amounts (usually 160 to 220 ml), and then steamed for an additional 20 min. It may be stored at room temperature for up to 3 months.

Hoff-Jorgensen and Hansen found that the inclusion of sulfite treated yeast extract in their medium enhanced the growth of *K. brevis* in the tube assay. Several batches of plate assay medium were made including this yeast extract, but no differences in assay values on the two media were found, and no "drift" occurred in the assay of natural products such as yeast. In all cases, the medium without the yeast extract gave zones with much sharper edges, and it was therefore omitted from the basal medium.

Assay Procedure

The (2 + 2) assay is carried out on large 12 in. x 12 in. plates with 8 x 8 quasi Latin-square design, or 6 x 6 Latin-square design as described by Lees and Tootill (1955) and Simpson and Lees (1956), or on Petri dishes. The required amount of medium is melted in a steamer, and then maintained at a temperature of 48 to 52 C for between one-half and one and one-half hr; if held

TABLE 1
Composition of the basal medium

Vitamin free acid hydrolyzed casein*...	5.0 g
Glucose.....	40.0 g
Trisodium citrate.....	7.5 g
Asparagine.....	0.4 g
Inositol.....	0.02 g
Choline chloride.....	0.004 g
Biotin.....	10 μ g
Nicotinamide.....	1000 μ g
Pyridoxine HCl.....	1000 μ g
Calcium pantothenate.....	1000 μ g
Salts E†.....	100 ml
Volume to (with glass distilled water)...	1000 ml
	pH 5.0
Agar*.....	20.0 g

* Oxoid, Oxo Ltd., London, England.

† Hoff-Jorgensen and Hansen (1955).

at this temperature for longer periods, the growth zones tend to become diffuse. For plates requiring 160 ml of medium, 8 ml of the inoculum suspension per plate gave the best results. A heavier inoculum gave rise to small zones and a lighter inoculum to larger, diffuse zones. The suspension is well mixed with the melted medium and poured on a flat even surface, where the plates are allowed to set. Ten-mm holes are cut to an 8 x 8 or 6 x 6 design, and the agar discs are removed.

The dose response curve for thiamine HCl was linear over the range 0.5 to 16 μg per ml and the diameters of the growth zones usually measured from 18 to 27 mm over this range. Levels of 1 and 4 μg per ml were used for the standard, and samples were diluted accordingly. Each 8 x 8 plate accommodates two standards and six samples, and the 6 x 6 plate accommodates one standard and two samples. Samples and standard are added to the holes according to pattern by means of standardized dropping pipettes, within one-half to two hr after the agar has set, and the plates are incubated for 16 to 20 hr at 30 C. The diameters of the growth zones are measured and results worked out in the usual manner. Preincubation of the plates before adding the standard and samples, or refrigeration after, gave rise to very diffuse zones of growth.

RESULTS

As Hoff-Jorgensen and Hansen found for the tube assay, the pyrimidine and thiazole moieties of thiamine are not utilized by the test organism either singly or together, at least up to concentrations 20 times the maximum concentration of thiamine in the standard, nor do they have an inhibitory effect. However, in the presence of thiamine, pyrimidine plus thiazole gave rise to zones larger than those obtained from thiamine alone, but only when both were present in at least 10 times the concentration of the thiamine.

Thiamine appeared to be the only limiting factor

TABLE 2

Comparison of thiamine assays by the *Kloeckera brevis* and the *Lactobacillus fermenti* plate methods

Sample	Thiamine Content	
	<i>K. brevis</i>	<i>L. fermenti</i>
	mg per g	mg per g
Fortified yeast tablets.....	0.560	0.570
Vitamin tablets.....	0.680	0.680
Vitamin tablets containing iron....	0.730	0.750
	mg per ml	mg per ml
Liquid multivitamin preparation...	99	96
Vitamin syrup.....	0.159	0.163
Vitamin syrup containing liver extract.....	0.156	0.159

for the growth of *K. brevis* in the plate assay since assay results obtained from samples diluted in water or in the treated yeast extract agreed well within the limits of error of the test.

Recoveries of thiamine added to solutions of fortified yeast tablets, vitamin tablets, or vitamin preparations were 93 to 115 per cent when the proportion of thiamine present in the samples to thiamine added was 1:1, 1:2, 1:10 or 1:50 (wt:wt).

No stimulation of *K. brevis* was found on the addition of lactose up to eight times the thiamine concentration in solution. Neither ascorbic acid up to 1000 times, nor ferrous sulphate up to 100 times the thiamine concentration appeared to affect the assay.

Many natural products such as flour or meat have too low a thiamine content for assay by plate methods which require a much higher vitamin level than tube methods. The assay method described has been used mainly for the estimation of thiamine in liquid multivitamin preparations for injection or oral use, and for tablets containing vitamins of the B group with or without the addition of yeast, yeast extract, ascorbic acid, and iron salts.

Table 2 gives a comparison of the assay values for some of these preparations by two plate methods; results are the mean of assays made on two different days. The limits of error ($P = 0.05$) for large plates using an 8 x 8 design were 91.8 to 108.9.

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SUMMARY

A plate method for the assay of thiamine using the yeast *Kloeckera brevis* is described. The pyrimidine and thiazole moieties of thiamine are not utilized, either singly or together, and assay results are not affected by lactose or ascorbic acid in relatively large amounts.

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Plate Assay of Thiamine

II. Using *Lactobacillus fermenti*

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An improved medium for the tube assay of thiamine using *Lactobacillus fermenti* strain 36 has been reported by MaciasR (1957). Starting with the original medium of Sarett and Cheldelin (1944) he investigated each ingredient and described a medium which he stated nearly doubled the growth response of *L. fermenti* within the assay range, and afforded greater reproducibility of assay results and better recoveries of added thiamine.

Many of the problems encountered with the tube assay are not met in the plate assay with the same microorganism. For the past 10 years, therefore, it has been our practice to use plate assays in preference to tube assays for thiamine using a modified Sarett and Cheldelin (1944) and Jones and Morris (1949) basal medium.

This paper reports the results obtained in plate assays with *L. fermenti* 36 on our routine medium compared with MaciasR's medium, together with results obtained by varying some of the ingredients of the basal medium.

EXPERIMENTAL METHODS

The tube assay medium (MaciasR, 1957) was modified for plate assays by using the medium single strength and solidifying with 2 per cent agar and with acid hydrolyzed casein, (Oxoid)¹ substituted for Casamino Acids (Difco).² After preliminary experiments in which no improvement was found with the addition of xylose, this was then omitted from the medium. In general, cystine was used in place of cysteine as this gave satisfactory results; steaming the medium for 20 min after

distribution into flasks was found preferable to autoclaving.

The composition of the basal media used are given in table 1.

L. fermenti 36³ was kept in a stab medium consisting of 1 per cent yeast extract (Difco), 1 per cent glucose and 2 per cent agar; after autoclaving at 15 lb pressure for 15 min, 10 µg sterile thiamine was added per tube. Subcultures were made every 2 weeks and, after incubation at 37 C for 18 hr, the stabs were stored in the refrigerator until required for use.

In this series of experiments, the inoculum was at first prepared in Difco micro inoculum broth and grown at 37 C overnight. After a few weeks, however, little and then no growth occurred in this inoculum broth. Whereas Malgras, Meyer, and Pax (1957) had found that mutants which would grow in the absence of pantothenic acid were produced when using the Difco micro inoculum medium, our experience with this medium was that there was often little or no growth in the broth. No further investigations were made in the case of thiamine, and the inoculum was thereafter grown on the riboflavin basal medium (Jones and Morris, 1950) with added riboflavin. This procedure had already proved satisfactory over a long period for routine plate assays of thiamine in these laboratories.

The overnight culture was centrifuged, washed twice in sterile 0.9 per cent saline solution and finally resuspended in sterile saline to give an optical density reading of 0.28 at 650 mµ in the 1-cm rectangular cell of the Unicam spectrophotometer,⁴ or by plate count,

³ American Type Culture Collection No. 9338.

⁴ Unicam Instruments Ltd., Arbury Works, Cambridge, England.

¹ Oxo Limited, London, England.

² Difco Laboratories, Inc., Detroit, Michigan.