# DIAGNOSTIC COLOR DIFFERENTIATION PLATES FOR HEREDITARY RESPIRATION DEFICIENCY IN YEAST

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Received for publication 8 April 1963

ABSTRACT

NAGAI, SUSUMU (National Women's University, Nara, Japan). Diagnostic color differentiation plates for hereditary respiration deficiency in yeast. J. Bacteriol. **86**:299–302. 1963.—Color differentiation between normal yeasts and their respiration-deficient mutants was improved by growing yeast colonies on nutrient agar plates containing several selected dyes and their mixtures. Magdala red (5 to 8 mg/liter) was good for single-color plates, giving deep-red sheen to the mutant colonies in contrast to the normal ones which tinted light red. A mixture of eosin (8 to 15 mg/liter, either Y or B) with trypan blue (15 to 20 mg/liter) was excellent in color and convenient to prepare, giving brilliant purple sheen to the mutant colonies contrasted to the normal ones which tinted grayish violet. These color plates were good over a broad range of *Saccharomyces* species, although the colony shades and suitable dye concentrations varied depending on the species and strains.

Various criteria have been employed for diagnosing hereditary respiration deficiency ("*petite*" or "RD" mutation) in *Saccharomyces* species of yeast since the work by Ephrussi (1953). They include actual oxygen consumption, colony size, cytochromes, utilization of carbon sources (selective culture media), and certain color differentiations by suitable indicators such as triphenyltetrazolium chloride (TTC) and some other dyes, as reviewed recently (Nagai, Yanagishima, and Nagai, 1961). The last-named diagnostic method is convenient particularly for population scoring of such respiratory mutants. This report describes an improved device for better color differentiation with several selected dyes and their mixtures.

## MATERIALS AND METHODS

Single dyes in various concentrations, and mixtures of two dyes (red and blue) in various combinations were added to nutrient agar medium to make color plates. Basal medium and dye solutions were sterilized separately by steaming at 100 C for 75 and 50 min, respectively, and were mixed together after cooling to about 55 C. In routine practice, 20-ml portions of appropriately diluted (to 20-fold of desired final concentration) single-dye solutions and water or two-dye solutions in 16-mm tubes were added to 360 ml of basal agar medium in 500-ml Erlenmeyer flasks to make 400 ml of color medium, which was, after thorough mixing, divided into 12 petri dishes (9-cm). Final nutrient composition was (w/v): glucose, 2.0%; peptone, 0.15%; dehydrated yeast extract, 0.15%; potassium dihydrogen phosphate, 0.15%; ammonium sulfate, 0.15% magnesium sulfate, 0.1%; plus agar, 1.2% (to adequate hardness). *S. cerevisiae* IFO 0044, *S. chevalieri* 0210, and *S. microellipsodes* 1016 were used for general tester organisms. Several other yeasts (see Results) were later used for comparative survey. Mixtures of normal and respiration-deficient (RD) mutant cells were spread on the color plates so as to produce about 100 to 150 colonies per plate, and were incubated at 30 C for 3 to 7 days.

## RESULTS

Table 1 summarizes the tests with selected dyes and their mixtures. Magdala red at 5 to 8 mg/liter gave a most desirable color differentiation (much better for our eyes than for black-and-white photographs), excelling the other dyes when they were applied in single-color plates. The RD mutant colonies appeared in deep-red sheen in contrast to the normal ones which tinted light red (Fig. 1A). Two preparations labeled as Magdalarot and Magdalarot des Handels (both from Grübler, Leipzig, Germany; manu-

TABLE 1. *Dyes and their mixtures selected for color differentiation**

| Red dye | Effective concn of dye in mixtures (mg/liter) | | Blue dye | | | | |
|---|---|---|---|---|---|---|---|
| | Red | Blue | Trypan blue (acid, P) | Nile blue (basic, P) | Methylene blue (basic, NG) | Toluidne blue (basic, NG) | Azine copper blue 2B conc. (acid, P) |
| Magdala red (basic, G) | 5-8 | 8-15 | VG | G | G | G | G |
| Eosin (acid, P) | 8-15 | 15-20 | EX | P | VP | VP | P |
| Rhodamine B (basic, VP) | 3-4 | 15-20 | P | VP | NG | NG | P |
| Neutral red (basic, VP) | 10-15 | 15-20 | P | VP | NG | NG | VP |

* Acid or basic property of each dye and its effectiveness in single-color plate reaction are given in parentheses. Degrees of effectiveness are expressed as follows: EX, excellent; VG, very good; G, good; P, poor; VP, very poor; NG, ineffective. Dyes were tested with *Saccharomyces cerevisiae* IFO 0044, *S. chevalieri* 0210, and *S. microellipsodes* 1016. All dyes were reagent-grade preparations for biological work [appearing in Conn's (1961) monograph], except azine copper blue 2B which was a technical-grade preparation for dyeing.

factured before World War II) were collaterally tested and found to be similarly good, but the latter gave a little deeper shade. The contrast was emphasized further (considerably improved for photographs) by addition of trypan blue, the RD colonies being bluish violet (Fig. 1B). However, this supplement is not recommended because violet dye aggregate appeared in the agar when more than 15 mg/liter of trypan blue were added, probably owing to binding between two sorts of oppositely charged pigment ions.

Mixture of eosin with trypan blue out of a number of blends gave excellent color differentiation (sharp for both eyes and photographs), better than that given by Magdala red. RD colonies appeared in brilliant reddish-purple sheen in contrast to normal ones which tinted light grayish violet (Fig. 1 C and D). Colonies sectored or variegated with RD regions were also clearly detected. Eosins Y (yellowish, water-soluble) and B (bluish) proved to be almost equally good, but a little deeper reddish shade was obtained with Y than with B. Furthermore, addition of 7 to 10 mg/liter of tartrazine or Sirius yellow G (both yellow acid dyes, technical-grade preparation) to the above mixture rendered the violet background color grayish and improved the contrast between the yeast colonies and the plate medium. Those yellow dyes in that range of concentration did not significantly affect the colony shades.

Mixture of rhodamine B and neutral red, respectively, with trypan blue gave color differentiation appreciably better than that anticipated, because of very poor differentiation by these red dyes in single-color plates. Difference in the colony shade was to some extent similar to that caused by the eosin-trypan blue mixture. However, the background color was too heavily red for visual comparison, and the contrast developed very slowly. Palatin scarlet A, another red acid dye (not shown in Table 1; technical-grade preparation), at 20 to 30 mg/liter, also gave fairly good differentiation between light red (normal) and deep red (RD). But the RD colonies had far less sheen than those colored with Magdala red, and the contrast developed very slowly.

Mixture of eosin with trypan blue also developed good color contrast in the following yeast species and strains: *S. bayanus* IFO 0206; *S. carlsbergensis* 0565; *S. cerevisiae* 0203, 0275, 2005, 2017, and 2023; *S. cerevisiae* var. *ellipsoideus* 0233, 0234, 0245, 0263, 0490, 2324, and 2361; *S. exiguus* 0215; *S. italicus* 0725; *S. oviformis* 0262; *S. pastorianus* 0539; *S. peka* 0301; *S. robustus* 0224; *S. rosei* 0252; *S. steineri* 0253; *S. uvarum* 0289; and *S. willianus* 0614. Colony shades and suitable dye concentrations varied depending on the species and strains in the ranges from 6 to 15 mg/liter for eosin, and from 15 to 25 mg/liter for trypan blue. A mixture at 12:15 mg/liter was convenient for primary trials. Magdala red (5 to 10 mg/liter) was also good for most of these yeasts, but was poor (totally deep red) for a few, such as 0224, 0245, 0262, 0263, and 2017.

Most color plates were prepared as described in Materials and Methods to make the actual nutrient composition and dye concentrations

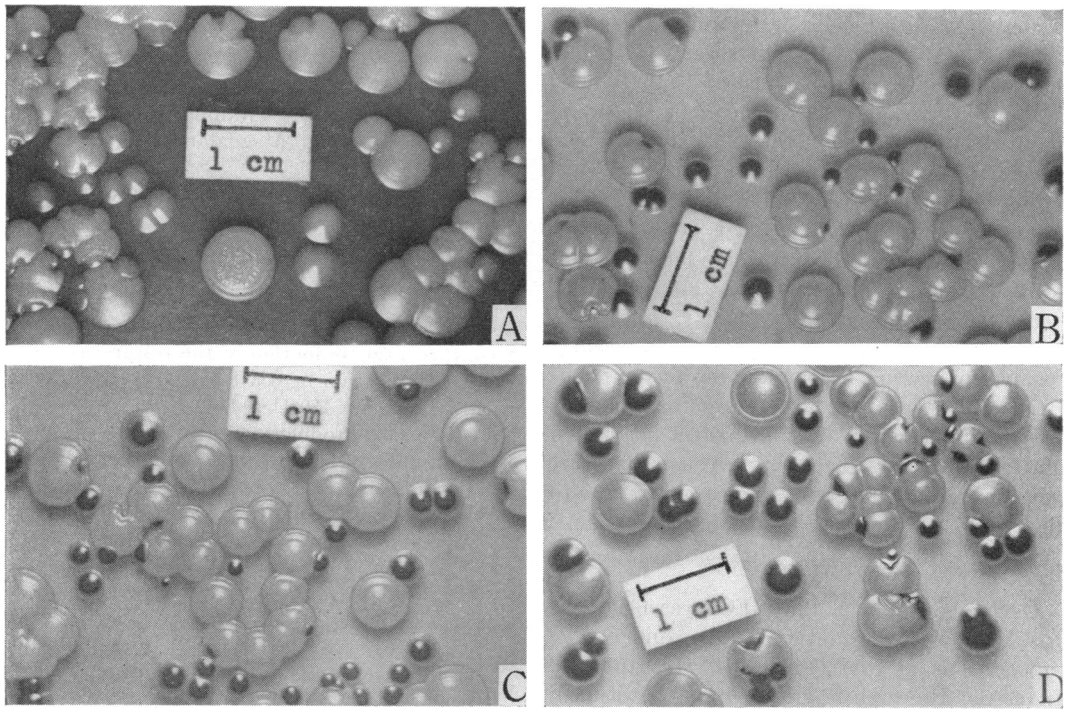


FIG. 1. *Differentiation by color plates between normal (large, light) and respiration-deficient (small dark) colonies. Saccharomyces cerevisiae IFO 0044 grown on Magdala red "des Handels," 5 mg/liter (A); Magdala red plus trypan blue, 8:15 mg/liter (B); and eosin Y plus trypan blue, 15:15 and 15:20 mg/liter, respectively (C and D). See Results for actual shades.*

exactly as specified. But it was later found to be a permissible approximation to add a few milliliters of concentrated dye solutions (2 to 4 mg/ml) over 200 or 400 ml of regularly prepared basal agar medium.

## Discussion

Color differentiation between normal yeast and RD mutants was tried earlier with TTC (Raut, 1953) and leuco base of methylene blue (Gause, 1958), both added to nutrient agar. Later came the TTC agar overlay technique, which gives quick and clear-cut differentiation between normal (deep red) and RD (remaining white) colonies (Ogur, St. John, and Nagai, 1957; Nagai, 1959, 1962). However, its drawback is that two steps, namely, growing sample colonies on normal agar medium and subsequent overlay with TTC-agar, are necessary to accomplish the color differentiation. Improved color plates presented here facilitate the diagnosis by one-step operation, developing color contrast more clear-cut than that by earlier indicator media.

For a color medium to be extensively accredited, it should: (i) develop color contrast as good as possible not only between normal and RD colonies but also between the yeast and background agar, (ii) neither disturb yeast growth nor produce RD mutants during the growth (Nagai, 1959, 1962), (iii) neither form dye aggregate nor fade away at least in a month or so, (iv) be good over a broad range of yeast species and strains, and (v) be easy to prepare with well-standardized, preferably inexpensive, dye preparations commonly available on the market. Our eosin-trypan blue medium appears to be most recommendable with regard to the requirements discussed above. It probably has definite but yet unrecognized physiological rationale, although it has been brought to use through empirical trials. It is certainly a good counterpart of the well-known eosin-methylene blue medium for differentiation of the enteric

bacteria and their biochemical mutants. Magdala red is also very good. Bright sheen of the RD colonies may be somewhat related to the usefulness of this dye as a vital fluorochrome (Drawert, 1955). However, it has been sold only by a few companies from Germany under somewhat confusing commercial designations (Drawert, 1955; Conn, 1961; Nagai, 1962), and is not so readily available to order. Dyes which are found only in technical-grade preparations are also less convenient in this respect.

The improved color plate method is sufficiently reliable for scoring RD mutants in mixed populations. It is suggested, however, not to apply this method immediately after ultraviolet irradiation and other drastic treatments because the yeast might suffer complications by heavy exposure to these dyes, which are otherwise harmless.

## ACKNOWLEDGMENTS

Generous supply of the "IFO" yeasts by the Institute for Fermentation, Osaka, and of dye preparations by the Osaka City Institute for Industrial Research is gratefully acknowledged.

This work was supported in part by a grant (No. 4062, to J. Ashida) from the Ministry of Education, Japan.

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