POSSIBLE USE OF ROSE BENGAL AGAR AS DIFFERENTIAL MEDIUM FOR THE ISOLATION OF BACTERIA IN THE FAMILY RHIZOBIACEAE

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The rose bengal agar developed by Smith and Dawson (Soil Sci., **58**, 467–471, 1944) to count soil fungi has a number of advantages over the normal acidified medium. In addition to having a neutral pH, the dye in the medium will confine the growth of the fungi, particularly those showing aerial and spreading growth. These authors note, however, that a few bacteria are tolerant of the rose bengal used in this differential medium and characterized them as "soft, raised, glistening colonies that would not be confused with the fungal colonies."

Mold counts using rose bengal agar were made in this laboratory involving approximately 500 plates. Although no accurate check of the numbers of bacterial colonies was made, the incidence of these colonies provoked a preliminary study. After isolating about 50 of the colonies, all were found to be small, gram-negative rods. Good growth was obtained on ordinary glucose nutrient agar. Further tests showed utilization but no acid or gas on lactose, glucose, or sucrose. These preliminary tests suggest that most if not all of the bacterial colonies observed on the rose bengal agar used for isolating molds belong to the family *Rhizobiaceae*. There is a possibility that this medium may prove useful as a differential medium for isolation of bacteria in this Family.

ISOLATION OF BACTERIAL, CELL-FREE, STARCH SACCHARIFYING ENZYMES FROM THE MEDIUM AT 70 C[1]

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Militzer, Sonderegger, Tuttle, and Georgi (Arch. Biochem., **24**, 75, 1949; **26**, 299, 1950) and Militzer and Tuttle (Fed. Proc. Am. Soc. Biol. Chem., **10**, 224, 1951) described a heat stable malic dehydrogenase, cytochrome oxidase, cytochrome c and adenosinetriphosphatase, obtained at 65 C from a stenothermophilic bacterium, strain no. 2184. They were not able to demonstrate amylase or protease formation in the medium at this temperature because the protein precipitates were without activity.

Imsenecki, Solntzewa, and Kuzyurina (Mikrobiologiya, **11**, 29, 1942) reported

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the isolation of amylases of *Bacillus diastaticus* at 60 C, showing good liquefying activity at 100 C and some saccharifying activity at 85 C.

In this laboratory, cell-free enzymes of *Bacillus stearothermophilus*, ATCC no. 7954, and of strains belonging to the same species are obtained routinely at 65 C. This communication describes the isolation and the starch saccharifying activity of a cell-free preparation obtained from the medium at 70 C and tested at 90 C.

Bacillus stearothermophilus, ATCC no. 7954, was grown in 1.0 per cent trypticase, 0.5 per cent yeast extract broth for 15 hours at 65 C. Ten ml were added to 500 ml of 2 per cent trypticase, 2 per cent yeast-extract, 0.5 per cent soluble starch (Merck) broth in a 2.5 liters "low form" flask, giving a shallow layer. Incubation was at 70 C for 24 hours in a covered water bath. Growth occurred but was not abundant. The final pH was 6.4.

The entire medium was centrifuged for one-half hour at 1,800 rpm at room temperature. The supernatant liquid was dialyzed in cellophane bags against

TABLE 1

Formation of reducing sugar at 90 C by a cell-free enzyme preparation obtained from the medium at 70 C

(Average of two determinations)

REACTION TIME IN HR	MG MALTOSE PER 5 ML AS DETERMINED	MG MALTOSE PER 5 ML MINUS ZERO HR	INCREASE MG MALTOSE PER HR
0	1.95	—	—
1	2.40	0.45	0.45
2	2.70	0.75	0.30
3	2.90	0.95	0.20
4	3.10	1.15	0.20
9	3.90	1.95	0.16
12	4.30	2.35	0.13

running tap water for 24 hours. Ten per cent toluene, by volume, was added as antiseptic. The bags were pervaporated (Kober, J. Am. Chem. Soc., **39**, 944, 1917) in a 65 C forced draft incubator. After 6 hours the volume had decreased from 365 ml to 110 ml. Two volumes of 95 per cent ethanol were added, the mixture was stirred for one hour, and then centrifuged for one-half hour at 1,800 rpm at room temperature. The clear supernatant was discarded. The precipitate was dried *in vacuo* over calcium chloride for one and one-half days at room temperature. The 110 ml of solution yielded 2.02 g of a dry, yellowish, finely granular powder. Before use it was dissolved in 60 ml of boiled, distilled water.

The substrate was a 2 per cent soluble starch (Merck according to Lintner) suspension prepared in Sorensen's M/15 phosphate buffer, pH 7.0. Substrate and enzyme solution were mixed in equal portions, using 50 ml of each in a 500 ml Erlenmeyer flask. The reaction mixture was placed into a covered water bath at 90 C. Samples were removed after 0, 1, 2, 3, 4, 9, and 12 hours of incubation. The amount of reducing sugar was determined by the method of Underkofler, Guymon, Rayman, and Fulmer (Iowa State Coll. J. Sci., **17**, 251, 1943) and expressed as maltose. Results are shown in table 1.

The freshly inoculated fermentation medium had 7.5 mg of maltose per 5 ml. The fermented medium after 24 hours at 70 C contained 14.6 mg of maltose per 5 ml. The dissolved ethanol precipitate had 3.9 mg per 5 ml. Since it was mixed with the substrate in equal portions, the mixture at zero hour contained 1.95 mg of maltose per 5 ml. This amount was deducted from each determination.

It must be emphasized that this experiment was conducted under conditions which were not necessarily optimum for the enzyme. Preliminary results with enzyme preparations obtained from the medium of cells grown at 65 C and tested at 85 C showed much better results than those previously described. It is hoped that even better activity will be obtained as soon as the optimum conditions have been worked out.

The identity of the enzyme preparation presents a problem. It might be *alpha*-amylase, maltase, or a mixture of enzymes. Whatever the case, an active enzyme preparation has been isolated from the medium of stenothermophilic bacteria at 70 C, the upper limit of growth for this particular strain; and activity at 90 C was ascertained.

THE ACTION OF MEMBERS OF THE GENUS ACHROMOBACTER ON TRIMETHYLAMINE OXIDE AND RELATED COMPOUNDS

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Members of the genus *Achromobacter* have considerable significance as causes of spoilage of fresh foods, including especially marine products. Identification of an unknown organism as an *Achromobacter* and its separation from closely related forms are sometimes difficult. The work here reported was conducted to ascertain whether the action of members of the genus on trimethylamine oxide, choline, and betaine might have some diagnostic importance, as has been found to be the case among the *Enterobacteriaceae*. The factors involved in the production of the amine from the various compounds tested were also studied.

Twenty cultures were selected for study: *Achromobacter stationis*, *A. thalassius*, *A. aquamarinus*, *A. lipidis*, *A. butyri*, *A. guttatum*, *A. hartlebii*, *A. lipolyticum*, *A. amylovorum*, *A. liquefaciens*, *A. alcaliaromaticum*, *A. delicatulum*, *A. superficiale*, *A. eurydice*, and 6 unnamed species isolated from marine sources and designated as strains C96, M8, M14, M23, N43, and N45. *Shigella alkalescens* and *S. dysenteriae* were used as positive and negative controls, respectively, on trimethylamine oxide reduction.

Replicate cultures to test the ability of each species to reduce trimethylamine oxide were grown in a medium of the following composition: peptone "C" 0.5 per cent, NaCl 0.5 per cent, $MgSO_4 \cdot 7H_2O$ 0.1 per cent, KH_2PO_4 0.1 per cent, glucose 0.25 per cent, and trimethylamine oxide 0.1 per cent. pH adjusted to