

Reductases

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Reductases are dehydrogenases (predominantly flavin enzymes) which transfer hydrogen from the substrate to biological acceptors and to dyes. The assay of reductase is chiefly used in food chemistry to determine the freshness (bacterial content) of milk¹⁾. In pure cultures, for example, of lactic acid bacilli, the assay is also used to detect compounds which inhibit the bacterial growth (antibiotics, antiseptics)^{2,3)}. The solution to be examined is added to a sterile culture medium, for example, of milk, which is then inoculated with bacteria and after some time the reductase activity of the medium is compared with a control to which no sample is added.

The methylene blue and resazurin assay methods, which are mainly used for the control of the quality of milk, are described below.

Principle

Reductases catalyse the reactions:



The dyes usually used are triphenyltetrazolium chloride (\rightarrow red formazan), methylene blue (\rightarrow colourless leuco-methylene blue) and resazurin. The latter is a blue dye, which first loses oxygen and is converted to the pink-red resorufin. Further reduction leads to the formation of the colourless dihydroresorufin. According to the reductase activity and the length of the reaction various colour shades are produced, which extend from pastel-blue through blue-violet, red-violet, pink-red to colourless.

Optimum Conditions for Measurements

The pH optimum is at pH 7. Addition of buffer is unnecessary when milk (\approx pH 6.5) is used as both substrate and sample. As resazurin and resorufin are also pH indicators, a possible colour change due to the formation of acid must be allowed for (resazurin is blue at pH $>$ 6.8, red at pH $<$ 5.3; resorufin is red at pH $>$ 6.4, yellow-orange below this pH).

High concentrations of methylene blue and resazurin have a bacteriostatic action. The optimum concentration of methylene blue in the reaction mixture is between 1 in 300000 and 1 in 2000000, and that of resazurin is about 1 in 200000.

Reagents

1. Methylene blue chloride

Crystalline methylene blue as a saturated alcoholic solution in ampoules. Methylene blue cyanate or thiocyanate are used in some countries instead of the chloride.

2. Resazurin

Sodium resazurin, standardized for the reductase assay, in ampoules or as tablets.

3. Ethanol, pure, 96% (w/v)

¹⁾ C. Barthel and O. Jensen, *Milchwirtsch. Zbl.* 41, 417 [1912].

²⁾ C. E. Neal and H. E. Calbert, *The Milk Products J.* 47, 14, 18 [1956].

³⁾ G. Dragon, *Milchwissenschaft* 13, 304 [1958].

Preparation of Solutions

I. Methylene blue:

Shake several grams of methylene blue chloride with 20 ml. ethanol for a few hours. Dilute 5 ml. of the saturated solution to 200 ml. with CO₂-free distilled water. With commercial preparations (tablets or ampoules) follow the instructions given for obtaining a solution.

II. Resazurin:

Dissolve resazurin according to the instructions enclosed with the commercial preparation. In Germany dissolve the contents of a glass ampoule in 1000 ml. hot, distilled water. In the USA dissolve one 0.5 g. tablet (contains 11 mg. dye) in 200 ml. distilled water.

Stability of the solutions

Store the dye solutions in sterile, brown bottles protected from light and air. The methylene blue solution is best prepared freshly each time.

Procedure

The methods for the grading of milk vary from country to country, both in the procedure and also in the choice of reagents. Therefore follow the local procedure for the control of the quality of milk.

Assay methods

Use test tubes or reductase tubes which have been cleaned by boiling (capacity 20, 30 and 50 ml.).

*Methylene blue assay on milk*¹⁾

Pipette

1 ml. methylene blue solution (I) and
40 ml. milk

into a boiling tube (in an assay with 10 or 20 ml. milk use correspondingly less methylene blue solution or dilute the dye solution twice or four-fold). Stopper with a rubber bung which has been previously boiled in water, mix by inverting three times and incubate at 38°C (water bath). Note the time required for decolorization of the mixture. Ignore the colour in the upper third of the mixture because the leuco-methylene blue is re-oxidized by atmospheric oxygen in this part. Instead of continual observation of the decolorization process it may be examined after the first 20 or 30 min. and then every hour. .

*Resazurin assay on milk*⁴⁾

Pipette

1 ml. resazurin solution (II) and
10 ml. milk

into a test tube. Mix by inverting three times, incubate at 37°C (water bath) and examine the colour of the milk after 10 min., 1 hr. or 3 hr. The 10 min. assay is sufficient to detect bad milk quickly, while the 3 hr. assay classifies more accurately samples which have not yet reached stage III (Table 1) after 1 hr.

⁴⁾ Review: *D. W. Watson, Dairy Ind. 13, 751 [1948].*

Evaluation of the results

The quality or bacterial content of the milk can be determined from the experimental results according to Table 1.

Table 1. Grading of the quality of milk on the basis of the reductase assay.
Comparison with the bacterial count⁵⁾

Colour of the resazurin assay after 1 hour	Decolorization time in the methylene blue assay	Approximate bacterial content per ml. milk		Quality grade
		Plate method	Microscopic count	
pastel-blue	5½ hr. and longer	up to 0.5×10^6	up to 0.6×10^6	I good milk
blue-violet	2 to 5½ hr.	0.5×10^6 to 4×10^6	0.6×10^6 to 2.1×10^6	II average milk
red-violet	20 min. to 2 hr.	4×10^6 to 12×10^6	2.1×10^6 to 12×10^6	III poor milk
pink colourless	} less than 20 min.	} above 20×10^6	} above 12×10^6	} IV very poor V milk

Detection of antibiotics in milk⁶⁾**Heat**

9 ml. milk

for 5 min. in a water bath at 80°C and cool to room temperature. Add

1 ml. of an early yoghurt culture,

stopper with a sterile rubber bung, mix and incubate for 2 hr. at 37°C. Then carry out one of the reductase assays described above. If triphenyltetrazolium chloride is used, add 0.3 ml. of a 4% (w/v) aqueous solution. Compare the reduction time with that of a milk guaranteed free from antibiotics which has been treated similarly.

A prolonged reduction time indicates the presence of antibiotics. With triphenyltetrazolium chloride the lower limit of detection is about 0.04 µg. penicillin, 0.2 µg. aureomycin or terramycin and 4.0 µg. streptomycin/ml.

⁵⁾ K. J. Demeter and H. Ordolf. *Milchwirtsch. Forsch.* 12, 1 [1931].

⁶⁾ W. Wodsak, *Nahrung* 3, 729 [1959].