

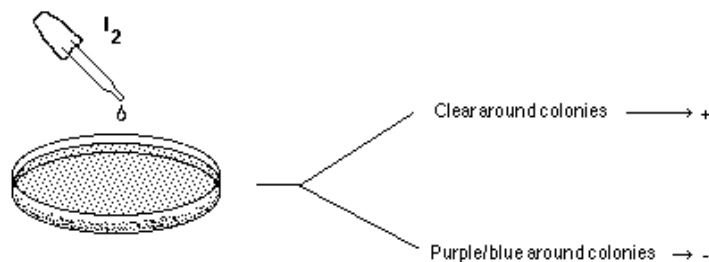
## DIAGNOSTIC TESTS FOR IDENTIFICATION OF BACTERIA

Many different tests have been devised over the years for classification of microorganisms into families, genera, species and even subspecies. Some of these tests are quite simple to perform while others are complicated and may require sophisticated equipment. The tests presented here are among the easier ones that are utilized in major clinical laboratories around the world. These tests are ordered alphabetically in this section. Make sure that you read the complete discussion of each test before you start to perform it.

**IMPORTANT:** Many of the tests mentioned in the following sections are enzymatic reactions. Therefore to get a correct results, one needs to warm up the culture and all test materials to temperatures between 25-40°C for the reactions to proceed. If you have stored your plates, broth culture or test reagents in the refrigerator, you may need to place them at the 37°C incubator for 15-20 minutes before performing the test.

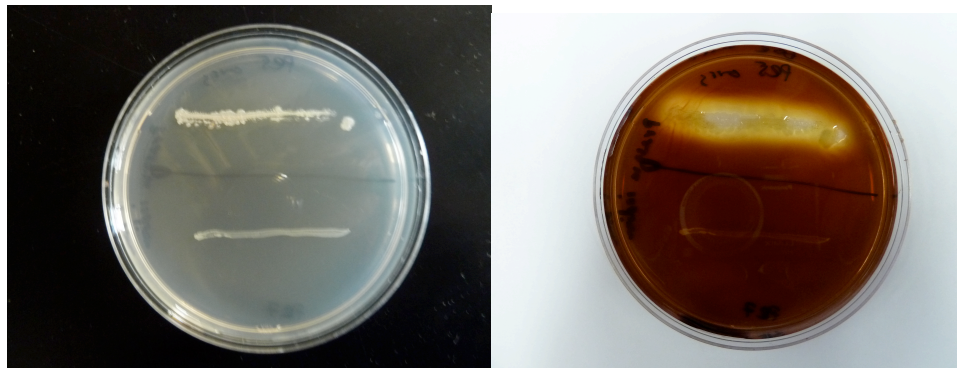
### AMYLASE TEST (STARCH HYDROLYSIS)

- **Objective:** To determine if the organism is capable of breaking down starch into maltose through the activity of the extra-cellular  $\alpha$ -amylase enzyme.
- **Test procedure**
  1. Use a sterile swab or a sterile loop to pick a few colonies from your pure culture plate. Streak a starch plate in the form of a line across the width of the plate. Several cultures can be tested on a single agar plate, each represented by a line or the plate may be divided into four quadrants (pie plate) for this purpose.
  2. Incubate plate at 37 °C for 48 hours.
  3. Add 2-3 drops of 10% iodine solution directly onto the edge of colonies. Wait 10-15 minutes and record the results.



- Interpretation:
  - Positive test ("+" ): The medium will turn dark. However, areas surrounding isolated colonies where starch has been hydrolyzed by amylase will appear clear.
  - Negative test ("-"): The medium will be colored dark, right up to the edge of isolated colonies.

Figure: Two species are inoculated onto a starch plate and incubated at 30°C until growth is seen (plate on the left). The petri dish is then flooded with an iodine solution and photograph taken after 10 minutes (plate on right). Amylase positive species shows a clearing halo around the growth (top line of growth). Amylase negative species does not have this clear halo (bottom line of growth).



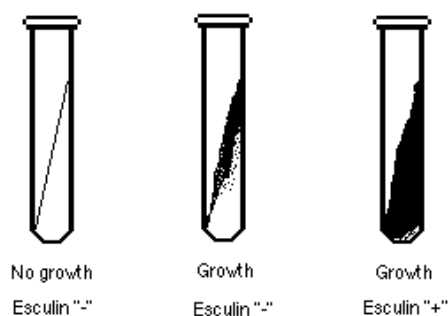
### BILE ESCULIN TEST

- Objective: To determine the ability of an organism to hydrolyze the glycoside esculin to esculetin and glucose. Esculetin reacts with iron to form a dark brown to black complex. The medium contains 40% bile. Some streptococci that are capable of splitting esculin cannot tolerate an increased concentration of bile. So this is basically two tests: (a) growth on 40% bile and (b) hydrolysis of esculin.
- Test procedure
  1. Obtain a bile esculin slant. With a sterile loop, touch a colony of your pure culture to obtain a light inoculum. Uncap the tube and flame the lip of the tube. Insert the loop to the bottom surface of the agar. Touch the agar and gently slide the loop in a zigzag fashion along the surface of the agar as you pull the loop out. Flame the lip of the tube again and put the cap back on.

2. Incubate the slant at 37°C for 48 hrs and check the results. If the results seem negative, continue incubation for up to 96 hrs before reporting the results as negative.

- Interpretation

- Positive: Half or more of the medium is blackened (black to dark brown) in any time interval.
- Negative: Less than half of the tube is blackened after 96 hrs.



**CARBOHYDRATE FERMENTATION TESTS**

- Objective: To determine the ability of an organism to ferment (degrade) a specific carbohydrate in a basal medium producing acid or acid with visible gas. The acid would change the color of the medium in a positive test. The following carbohydrate semi-solid media tubes are available at our lab:

- Arabinose
- Glucose
- Glycerol
- Inulin
- Maltose
- Sorbitol
- Trehalose
- Xylose

- Test procedure

1. Using a sterile needle, stab the tube within 1/4 inch of the bottom with medium inoculation.
2. Incubate for at least 48 hrs. Bacteria that are known to be slow growers should be given up to 96 hours.

- Interpretation:

- Fermentation

Positive: Any yellow color (not orange). It does not necessarily have to be the whole tube. A positive result is referred to as ("+") or (A) or (Acid), as fermentation forms acidic products.

Negative: A red, pink or orange color - no yellow at all.

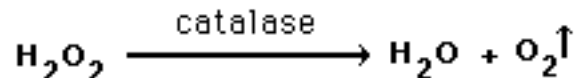
- Gas production

Positive: Significant bubbling in semisolid medium (one small bubble is generally negative, caused by the stab). Gas may also cause the medium to get separated from tube. Record as (G) for positive gas production.

Negative: No gas bubbles except those produced by stabbing.

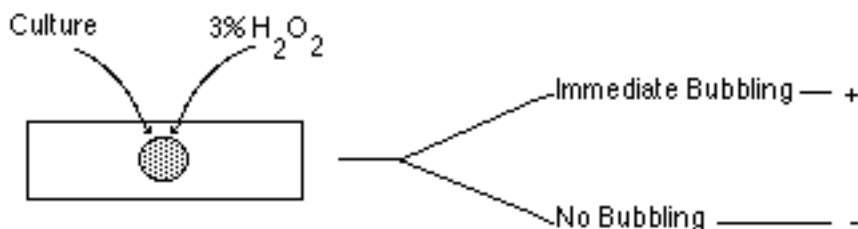
CATALASE TEST

- Objective: To test for the presence of the enzyme catalase.



- Test procedure

1. If the plate is refrigerated, it should be allowed to warm up to room temperature and then incubated for 15 min at 37°C before performing the test. Pick a loopful of colonies from a not-too-old pure culture plate and place on a clean glass slide. Do not take your colonies from a blood agar plate. Blood contains catalase; therefore a false positive reaction would be obtained.
2. Add one or two drops of 3% H<sub>2</sub>O<sub>2</sub> and wait 10-15 seconds to observe.





- Interpretation
  - Positive test: immediate bubbling ( $O_2$  formed).
  - Negative test: no bubbling.
- Precautions
  - a. When doing the slide test, always add organism to the slide first and then add the reagent since platinum used in the inoculation needle may produce a false positive result. Nichrome wire does not cause bubbling.
  - b.  $H_2O_2$  is very unstable when exposed to light.  $H_2O_2$  decomposition also increases as temperature increases due to dissolved oxygen. Therefore it is important to keep this reagent in the refrigerator at all times when not in use and to shake before it is used.

**DNASE TEST**

- Objective: DNase mediates the hydrolysis of DNA. Methyl Green indicator is stable at pHs above 7.5 but becomes colorless at lower pHs. The hydrolysis of DNA in the agar by bacterial DNase reduces the agar pH.
- Test procedure
  - 1 Using a sterile loop, inoculate a DNA+Methyl Green agar plate with the fresh bacterial culture. Use a heavy streak line for each bacterial strain to be tested. Be sure to label the plate bottom properly for each strain. You can share a single plate with 4 other students who want to do the same test on their own unknowns.
  2. Incubate at  $37^\circ C$  for 48 hrs.
- Interpretation
  - The test is positive if clearing develops around the areas of growth. If the color of the agar around the growth is unchanged, the test is negative (i.e., the organism is not able to produce DNase).

### GELATINASE TEST (GELATIN LIQUEFACTION)

- Objective: To determine the ability of an organism to produce proteolytic-like enzymes (gelatinases) which break down gelatin. Gelatinase destroys (hydrolyzes) the gel and causes its collapse and liquefaction.
- Test procedure
  1. Obtain two solidified gelatin butts but keep them in the refrigerator until just prior to inoculation. Pick up a heavy inoculum from your pure culture and stab one of the butts to a depth of 2 inches. The other tube should not be inoculated and used as a control.
  2. Incubate both the test and control tubes simultaneously at the optimal growth temperature for the organism for 48 hours to 14 days.
  3. At the end of each 48-hour period, place both tubes (test bacterium and control) in a refrigerator for about 1 hour to determine whether digestion of gelatin (liquefaction) has occurred. Make the transfer from incubator to refrigerator without shaking the tubes. Check tubes daily up to 2 weeks unless liquefaction occurs sooner.
- Interpretation

Gently tilt the tubes. The test is positive if the medium of the test organism is liquefied (gelatin breakdown) while that of the control has remained solid (lack of gelatin hydrolysis). The result of the test is negative if the medium of the test organism is as solid as that of the control.
- Precautions
  - Always run a control tube in parallel with organism being tested.
  - Gelatin is solid when incubated at 20°C or less and liquid at 30°C or greater. Gelatin changes from a gel (solid state) to a liquid at about 28°C. Therefore, if gelatin tubes are incubated at 30°C or greater, they must first be placed in a refrigerator for an hour and cooled before an interpretation of liquefaction is made.
  - Do not shake gelatin tubes while warm since growth and liquefaction of gelatin frequently occur only on the surface layer. If the gelatin is shaken and allowed to be mixed with the warm fluid of the medium, there is a possibility that a positive result may be overlooked, and thereby a false negative result may be obtained.

### HEMOLYTIC REACTIONS

- Objective: Some pathogens are able to produce exoenzymes called hemolysins which lyse red blood cells and thus their action can be demonstrated on a blood agar plate.
- Test procedure
  1. Using a sterile loop, inoculate a blood plate (SBA) with the pure culture of the organism to be tested using the quadrant method. Also stab the medium in the second quadrant with your loop. (Some hemolysins show their effects better under lower oxygen concentrations.)
  2. Incubate for 48 hours at optimum temperature for the organism.
- Interpretation

Interpret by noting the reaction around isolated colonies as follows:

Alpha ( $\alpha$ ) hemolysis: formation of a green or brown zone around the colonies (due to loss of potassium from the red cells).

Beta ( $\beta$ ) hemolysis: complete lysis of cells and reduction of released hemoglobin; a clear zone appears around isolated colonies.

Gamma ( $\gamma$ ) hemolysis: no hemolytic reaction (no change of the medium surrounding isolated colonies).

- Precautions
  - The reaction should be checked only around isolated colonies. If you do not have isolated colonies on the blood agar, a lighter inoculation should be streaked and the test repeated.

### MANNITOL SALT AGAR TEST

- Objective: To determine the ability of an organism to grow in 7.5% NaCl and ferment mannitol.
- Test procedure
  1. Streak an MSA plate with a light line of inoculum from the pure culture of the test organism using a sterile loop.
  2. Incubate at 30°C for at least 48 hours.

- Interpretation

Any significant growth indicates the organism is a *Staphylococcus* species. The phenol red indicator changes to yellow at low (acid) pH, which is a product of fermentation. Therefore, fermentation of mannitol will change the color of agar to yellow. Orange is negative.

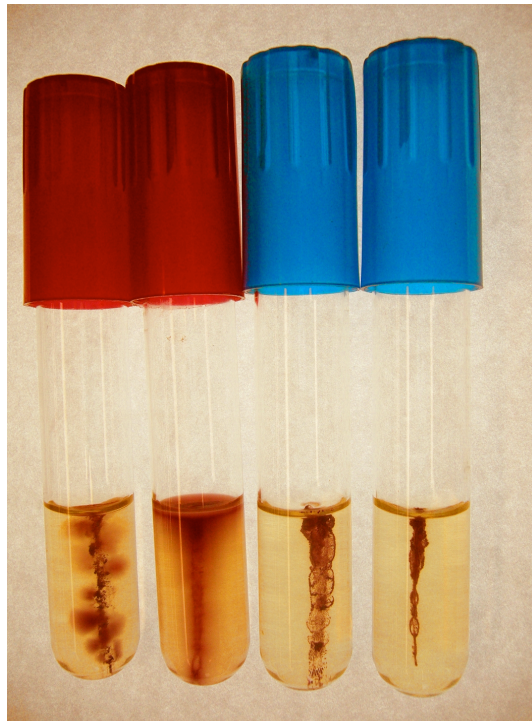
Positive: Growth, yellow color (mannitol "+").

Negative: Growth or no growth; red or orange color (mannitol "-").

**MOTILITY TEST**

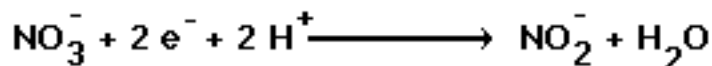
- Objective: To determine whether an organism is motile. See the chapter on the use of motility butts.
- Test procedure and Interpretation
  1. See section “Motility Butts” in the “Detection of Bacterial Motility” chapter. If the results of using a wet mount and motility butt are still doubtful, one may also try the agar plate method.

Figure: The two left and the two right motility butts were stabbed with two motile and non-motile species, respectively and tubes were incubated for 48 hours. Feathering is clearly seen of the left tubes but not on the right ones.

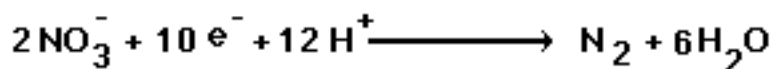


**NITRATASE TEST (NITRATE REDUCTION; DENITRIFICATION)**

- Objective: To determine the ability of an organism to reduce nitrate to nitrite which is then reduced to free nitrogen gas. The nitrogen in nitrate serves as an electron acceptor. The result of the denitrification process is the production of nitrite:



We can test for the presence of  $\text{NO}_2^-$  using Reagents A and B as described below. Denitrification may also produce  $\text{N}_2$  gas:



In this case, all the  $\text{NO}_3^-$  will be converted to  $\text{N}_2$  gas which escapes to the atmosphere. We can test for this step by looking for the absence of  $\text{NO}_3^-$  through the addition of Zn powder as described below.

- Test procedure
  1. Inoculate a nitrate agar slant with your pure culture using a sterile loop to transfer a rather heavy inoculum.
  2. Incubate at 37°C for at least 48 hours.
  3. Add 2-3 drops of Reagent A and 2-3 drops of Reagent B to your tube. Reagent A is 0.8% sulfanilic acid in 30% acetic acid and Reagent B is 0.6% N,N-dimethyl- $\alpha$ -naphthylamine in 30% acetic acid (CAUTION: Reagent B is a potential carcinogen, so work in the hood and avoid inhaling it or allowing for contact with skin; wash hands thoroughly after work).

- Interpretation

Reduction of nitrate to nitrite is indicated if a red color develops quickly (within 1-2 minutes). If no color develops, add a very small amount of zinc powder (~20 mg) to the tube containing the reagents. If a pink to dark red color develops after adding the zinc powder within 5 min., the test is negative (nitrate is present and is not reduced by the organism but zinc has reduced it to nitrite). If no color develops, the test is positive (the organism was able to reduce all the nitrate to nitrite and further to  $\text{N}_2$  which escaped from the tube).

- Precautions

- If tubes are stored in the refrigerator, they should first be brought back up to the optimum temperature of the growth condition of the organism.
- When performing the nitrate reduction test using  $\alpha$ -naphthylamine, the color produced in a positive reaction may fade quickly. Interpret results immediately, particularly when performing a number of tests.
- A strong nitrate-reducing organism may exhibit a brown precipitate immediately after the addition of the reagents. This is due to the effect of excess nitrite upon the p-amino group of the azo-dye and may be reduced by using dimethyl- $\alpha$ -naphthylamine.
- Some organisms are capable of reducing nitrate to nitrite, yet they destroy the nitrite as fast as it is formed, yielding a false negative result. This nitrite destruction is evident in quite a few bacteria, particularly some *Salmonella* and *Pseudomonas* spp. and in *Brucella suis*.
- Do not use an excess of zinc; if too much Zn is added, the large amount of hydrogen gas produced may reduce the nitrite (formed from unreduced nitrate) to ammonia (NH<sub>3</sub>) that could result in a false negative reaction or just a fleeting color reaction.

NOVOBIOCIN DISC TEST

- Objective: To test an organism's susceptibility to the antibiotic novobiocin.

- Test procedure

1. Streak a BHI plate using a sterile cotton swab. Turn the plate 90 degrees and re-streak with the same swab
2. Using a pair of alcohol flamed forceps, aseptically place a novobiocin disc in the center of the plate. Apply gentle pressure to disc so it adheres to the surface of the agar but try not to press too much to embed the disc into the agar.
3. Incubate the inverted plate 48 hours at your organism's optimum growth temperature.

- Interpretation

Sensitive (S): No growth around disc; clear zone around disc.

Resistant (R): Growth not inhibited; growth around disc.

OPTOCHIN DISC TEST

- Objective: To test an organism's susceptibility to the chemical, optochin. Optochin susceptibility tests the fragility of the bacterial cell membrane. This test is mainly used to differentiate between *Streptococcus pneumoniae* (sensitive) and other *Streptococcus* species (resistant)
- Test procedure
  1. Pick a single pure colony with a sterile swab to inoculate a SBA plate. Streak the entire blood agar plate with the swab. Turn plate 90 degrees and re-streak with the same swab. Blood agar plate must be used for optochin testing since all species of *Streptococcus* are fastidious organisms and require extra enrichment for growth.
  2. With alcohol flamed forceps, aseptically remove an optochin disc and apply to the center of the plate. Gently apply pressure to disc so that it adheres to the surface of the plate but do not press disc down into the medium.
  3. Invert plate and incubate for 48 hours at your organism's optimum growth temperature.
- Interpretation
  - Sensitive (S): A distinct zone of inhibition (5 to 30 mm) with a clear-cut margin around disc.
  - Resistant (R): Growth not inhibited around disc.
- Precautions
  - Occasionally, a few scattered optochin resistant colonies of *S. pneumoniae* may be observed in a wide zone of inhibition.
  - Occasionally an alpha-*Streptococcus* spp. may exhibit a very small zone (1 to 2 mm) of inhibition. *S. pneumoniae* exhibits a zone of inhibition at least 5 mm or greater in diameter.

OXIDASE TEST

- Objective: To determine the presence of the oxidase enzymes (e.g. cytochrome c oxidase).
- Test procedure and Interpretation
  1. Grow the culture on a BHI plate for 48 hours. Up to 7 day old cultures are fine.
  2. Warm the plate to 20-37°C. Pick a good amount of the test organism with a sterile swab and rub onto the reaction area of a DrySlide card. If the organism is oxidase positive, a purple color will develop on the slide within 20 seconds. The slide is saturated with Kovacs' oxidase reagent (1% N, N, N', N' tetra-methyl-p-phenylene diamine dihydrochloride). Oxidase negative colonies do not change the color of the slide in 20 seconds, or if they do, it would be after 20 seconds and thus negative.

NOTE: Four tests can be performed on a single card. You may pass the unused portion of the card to a fellow student or store the card in its pouch away from light for up to a week.

- Precautions
  - Most Gram-positive bacteria and all *Enterobacteriaceae* are oxidase negative.
  - Do not attempt to perform an oxidase test on any colonies growing on media containing glucose, as glucose fermentation will inhibit oxidase enzyme activity, and result in possible false negatives. Oxidase test on Gram-negative rods should be performed only on colonies from non-selective and/or non-differential media to ensure valid results.
  - The culture should not be older than a week, unless the species is a slow-grower. False results may be obtained if the culture is old.
  - The oxidase reagent quickly auto-oxidizes (by free oxygen in the air) and loses its sensitivity. The reagent should be discarded if any precipitate forms. Avoid undue exposure of the reagent to light. The reagent must be made up fresh each week.
  - Time period for color development must be adhered to since a purple-black color may develop later due to auto-oxidation of reagent and/or a weak positive oxidase organism containing a small quantity of cytochrome c oxidase.



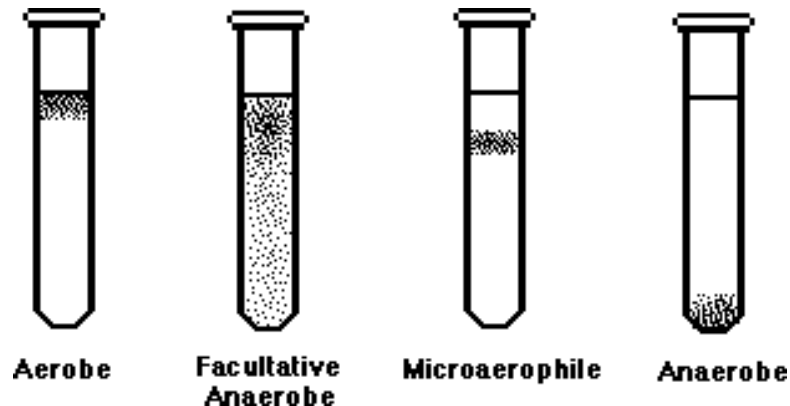
- As an alternative to Kovacs' reagent, one may use a few drops of a 1:1 mixture of 1%  $\alpha$ -naphthol in 95% ethanol and freshly prepared 1% aqueous dimethyl-*p*-phenylenediamine oxalate.

**OXYGEN REQUIREMENT TEST (Thioglycollate Test)**

- Objective: To determine the organism's oxygen requirement.
- Test procedure
  1. Inoculate 5 ml of BHI broth with your unknown organism and incubate overnight. We have found that broth cultures provide much more accurate results than using inoculum from a plate. However, if you are inoculating from a plate, make sure you use a very light inoculum.
  2. Obtain a thioglycollate tube and make sure that it does not have more than 20% of the medium in pink color. This may happen due to oxidation of the top layer of the medium. To restore anaerobic conditions, such a tube should be placed in boiling water for 10 minutes and then cooled to room temperature. If you do not see any pink color against a white background, the tube is good to use.
  3. Use a sterile narrow thin needle (rather than a thick one), insert into your culture broth and slowly stab a thioglycollate tube to the bottom. Carefully remove the needle along the same stab line. Do not shake the tube or move the needle around, or you will introduce extra oxygen into the medium. The needle should reach all the way to the bottom of the tube.
  4. Incubate the tube at 30°C (without any regard to the optimum temperature requirement of your species) for 24 hours before reading the tube.
- Interpretation
  - Aerobe: band of growth on the top of the tube. Some species have a tendency to grow very rapidly in thioglycollate tube so that the growth covers a rather thick band from the top and extends to the line of stab where there is oxygen available (brought in by the needle). So it is best to look at the bottom 1-cm of the tube and if it is clear with no growth whatsoever, then you can be sure that you have an aerobe.
  - Microaerophile: band of no growth at the top, then a band of growth extending a short distance down preceded by no growth to the bottom. The bottom 3-cm of the tube should be clear of any growth.
  - Facultative Anaerobe: growth can occur either throughout the tube or can begin at some point below the surface and extend all the way to the bottom, even in the 1-cm bottom of the tube.

-- Anaerobe: growth only at the bottom fifth of the tube.

The following diagrams show ideal examples:



#### PENICILLIN DISC TEST

- Objective: To test the organism's susceptibility to antibiotic penicillin.
- Test procedure and Interpretation: See the Optochin Disc Test.

#### SALT TOLERANCE TEST

- Objective: To test organism's ability to tolerate various osmotic concentrations.
- Test procedure
  1. Use a sterile loop or needle to inoculate broth tubes with different salt concentrations.
  2. Incubate at the optimum temperature for 48-96 hours.
- Interpretation

Positive = growth; negative = no growth

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