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Biochemical Characterization of Indole Producing Bacteria: A Brief Protocol

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SUMMARY

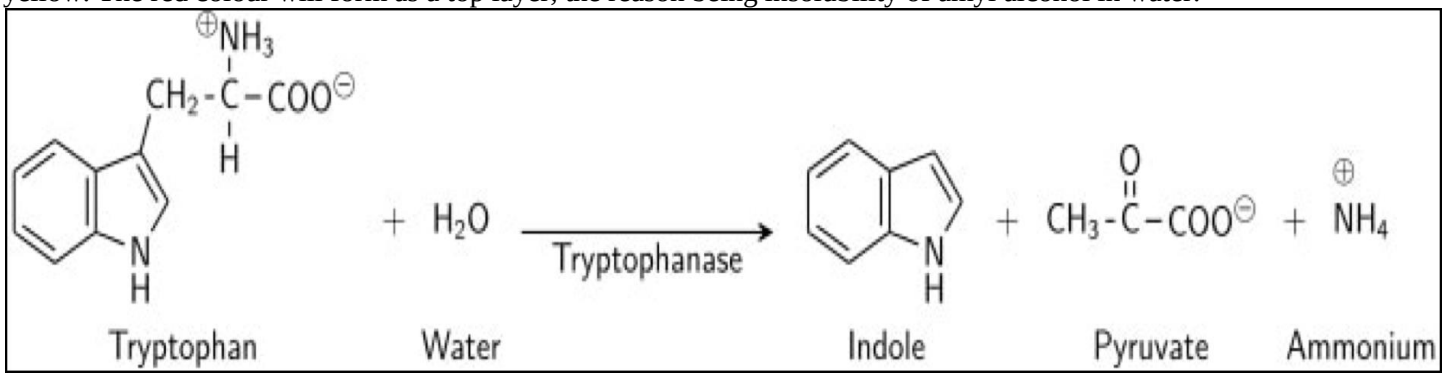
Indole, an aromatic compound produced as a metabolite of Tryptophan amino acid by certain bacteria, when they are grown in tryptophan broth media. A biochemical test, known as indole test, is performed for bacteria to determine their ability to convert tryptophan into indole. Reductive deamination of tryptophan produces indole while the indole pyruvic acid is produced as an intermediate molecule. This reaction is catalyzed by Tryptophanase with the removal of the amine (-NH2) group of tryptophan. The end products produced as a result of the reaction are indole, ammonium (NH4+), pyruvic acid and energy, and as a coenzyme Pyridoxal phosphate is essential. The presence of Indole is detected by adding Kovac's reagent or Ehrlich's reagent. After adding the reagent, within a few seconds, a red colour layer on the top of the medium is formed, which indicates indole positive.

INTRODUCTION

Kitasato (1989) discovered that *Escherichia coli* and *Klebsiella* species (*Aerobacter aerogenes*) can be distinguished based on their ability to produce indole. The indole-positive *E. coli* are still distinguished from *Enterobacter* and *Klebsiella* (indole-negative), by this test. The Indole production test is a part of IMViC procedures i.e. a battery of tests used in the laboratory for the identification of the Coliform group of Bacteria. Among those tests, bacteria's ability to degrade tryptophan is examined by an indole test. The bacteria expressing the tryptophanase enzymes can carry out deamination and hydrolysis of Tryptophan. The detection of indole production depends upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) in acidic conditions. Resindole, a red dye is produced as a result of the reaction.

Principle and Procedure:

The Indole production test is based on the principle of utilization of Tryptophan (an essential amino acid) by the bacteria. Bacteria, which cause deamination and hydrolysis of Tryptophan with the help of Tryptophanase, results in formation of Indole, pyruvic acid and ammonia are Indole production test positive. The indole produced during the reaction is detected by adding Kovac's reagent which produces a Cherry-red reagent layer. Indole, when combined with Kovac's Reagent (containing HCl and p-dimethylaminobenzaldehyde in amyl alcohol), solution turns to cherry red in colour from yellow. The red colour will form as a top layer, the reason being insolubility of amyl alcohol in water.



(**Source:** https://microbiologyinfo.com/indole-test-principle-reagents-procedure-result-interpretation-and-limitations/)

Fig: Deamination of tryptophan by tryptophanase into Indole, Pyruvate and Ammonium.

Procedure-

Tryptophan broth (1%) is pepared by dissolving 10g of tryptophan and 5g of Sodium chloride in 1 L of distilled water. Media was poured in tubes @ 4 ml per tube. Sterilization of media is done through autoclave at 15 psi, at 121°C for 15 min. For indole assay Tryptophan broth is inoculated with test bacteria and incubated for 1-2 days at 35° +/- 2°C. After 2 days of inoculation, 5 drops of Kovac's reagent are added in the tube directly. Shaken the tubes gently after intervals of 10-15 min and allow the tubes to stand to permit the reagent to come to the top. colour change at the top layer is recorded for test positive.

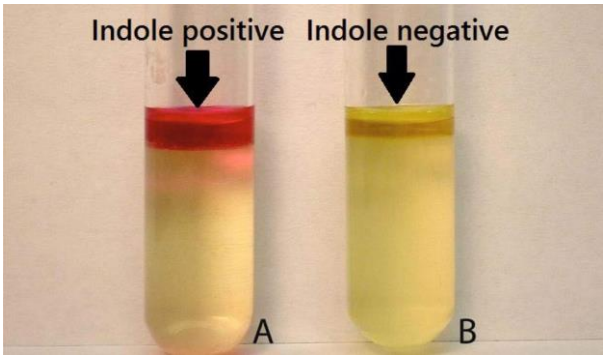
Results: Positive reaction will show a cherry (deep) red colour at the top layer of the media.

Examples- *Escherichia coli, Aeromonas hydrophila, Bacillus alvei, Edwardsiella* sp., *Flavobacterium* sp., *Aeromonas punctata, Plesiomonas shigelloides, Pasteurella multocida, Pasteurella pneumotropica, Klebsiella oxytoca, Haemophilus influenzae, Enterococcus faecalis, Vibrio* sp. and *Proteus* sp., but not *P. mirabilis* and *P. penneri*.

Absence of deep red colour is Indole negative.

Examples:

Most of the species of *Bacillus, Haemophilus* and most *Klebsiella* sp. are indole negative. Some other bacteria include, *Pseudomonas* sp., *Actinobacillus* spp., *Aeromonas salmonicida, Alcaligenes* sp., *Bordetella* sp., *Enterobacter* sp., *Proteus mirabilis, Lactobacillus* spp., *Neisseria* sp., *Pasteurella haemolytica, P. penneri, Salmonella* sp., *Serratia* sp., and *Yersinia* sp., are also indole negative.



(**Source:** https://microbiologyinfo.com/indole-test-principle-reagents-procedure-result-interpretation-and-limitations/)

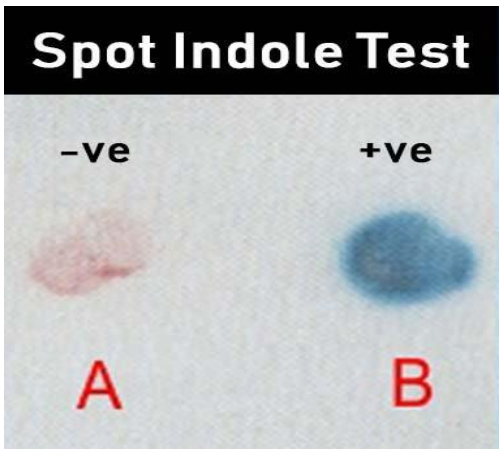
Fig: Results of indole test: (A) Indole positive, (B) Indole negative

Media and Composition:

The media required for the Indole production test must contain Tryptophan, and one such media is Tryptophan broth. For tryptone broth, 10g of Tryptone along with 5g of Sodium chloride are dissolved in 1 litter of sterile water. 4 ml of the prepared broth is dispensed in each tube. Cap the tube and autoclave it at 121^{o}C under 15 psi pressure for 15 minutes, followed by storing the tube at 4 to 10°C in the refrigerator. Now, dissolve DMAB in alcohol and gently heat the solution to get the aldehyde in it. After this, in the mixture of aldehyde and alcohol, slowly add the acid. The solution prepared must be pale yellow in colour. It is stable for short duration only, and stored in a refrigerator in a glass bottle of brown colour. Kovács reagent is available commercially are Amyl or isoamyl alcohol (150.0 ml), *p*-dimethylaminobenzaldehyde (10.0 g), concentrated HCl (50.0 ml)

Procedure of Rapid Spot Test:

The production of indole can also be detected with the help of Rapid spot test. For the spot test, 5% *p*-dimethylaminobenzaldehyde or 1% *p*-dimethylaminocinnamaldehyde is prepared in 10% (v/v) concentrated HCl is used as a reagent. Initially reagent moist filter paper is taken on which one loop-full of 18-24-hour old full grown colony were spread off. Colour change of sample spreaded area is observed. Within 20 seconds, the development of blue colour indicates the presence of indole whereas no colour or the appearance of a slightly pink colour on the filter paper indicates negative results.



(**Source:** https://microbenotes.com/spot-indole)

Fig: Indole positive (left) and control (right).

Significance of Indole production test:

Indole production test is important in the identification of members of Enterobacteriaceae and certain anaerobic species. Sometimes it is helpful in differentiation of species within a genus. For example, differentiation of *Klebsiella oxytoca* (indole positive) from *Klebsiella pneumoniae* (indole negative). Similarly, *Proteus vulgaris* can be differentiated from *Proteus mirabilis* and *P. penneri* where the former one is indole positive, and the later two are indole negative.

Precautions to be taken during test:

- Use fresh tryptophan broth for best results. Do not inoculate tryptophan broth tubes for more than 48 hours as after 48 hours' indole itself may be attacked and further degraded. If it happens, the indole will eventually disappear and we may get a misleading negative test result.
- Acid production may inhibit indole production due to a change in pH, hence peptone media with glucose should not be used.
- Anaerobes, particularly *Clostridium* species, form indole but can rapidly break it down as it is produced; therefore, false negative reactions may occur, hence incubation period must be given consideration. Indole production decreases with the decrease in oxygen tension. Hence, the cultures we want to test must be incubated aerobically.

CONCLUSION

To get closer to the identification of bacteria, various biochemical tests are available. The test of microbial biochemistry reduces the cost and time needed for their identification. The accuracy of identifying unknown samples is also increased with the knowledge of microbial metabolism. One of the biochemical tests that analyze the enzymatic profile of bacteria is the indole test. It distinguishes bacteria based on the ability of the test bacterium to utilize tryptophan amino acid. It differentiates the coliform bacteria from other Enterobacteriaceae members.

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