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Morphological and Physiological Characterisation of Bacterial Isolates for the Degradation of Malathion

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ABSTRACT

The neurotransmitter acetylcholine builds up at post-synapsis locations and causes neuronal death as a result of malathion's irreversible inactivation of acetylcholine esterase at multiple locations. The gastrointestinal tract, the mucous membranes of the skin, and the lungs are among the many pathways through which malathion is absorbed. The current study examined the morphological and physiological characteristics of bacterial isolates for Malathion degradation.

Keywords: *Malathion, Voges-Proskauer and Citrate, Indole, Methyl-red, Urease, and Catalase tests.*

1. INTRODUCTION

The short-term yields that modern agriculture aims to increase crop growth and output frequently result in ecological deterioration and widespread soil erosion that is unsustainable for humans. Crop yield loss and increased pest risk result from prey and predator imbalances caused by the destruction of agri-ecosystems. The production from a variety of crops is decreased by 28.2 to 40.3% as a result of increasing pests (Oerke, 2006). Consequently, farmers must use pesticides to prevent yield loss in their crops. The majority of insecticides that cause acute toxicity are used in developing countries and belong to the organophosphate, carbamate, and organochlorine families. Organochlorine insecticides are being replaced by organophosphate pesticides. It is a broad-spectrum, non-systemic organophosphorus insecticide used to manage agricultural and domestic pests. The neurotransmitter acetylcholine builds up in post-synaptic locations and causes neuronal death as a result of malathion's irreversible inactivation of acetylcholine esterase at several locations. The gastrointestinal tract, the mucous membranes of the skin, and the lungs are among the many channels by which malathion is absorbed. It has been demonstrated that malathion poisoning affects the immunological, reproductive, and central neurological systems of invertebrates (El-Dib et al., 1996).

2. MATERIALS AND METHODS

Isolation of Malathion degrading Bacteria

In order to isolate malathion-degrading bacteria, the enhanced soil bacterial culture was subculture on MSM media at a 10-3 dilution.

i) A repeated subculturing technique using progressively higher concentrations of malathion in MSM media was employed to get bacterial strains.

ii) In order to create the first subculture, 1 ml of a 10-3 diluted enhanced culture was inoculated into MSM broth supplement, which contained 0.75% glucose as a carbon source and 0.25% malathion. After that, the mixture was kept in an orbital shaking incubator with constant agitation at 240 rpm for 48 hours at 28°C.

iii) The second subculture was created by successively subculturing the first subculture. This was done by inoculating 100 µl of the first subculture's culture broth with MSM broth that had 0.5% glucose and 0.5% malathion, and then incubating the mixture for 48 hours at 240 rpm at 28°C.

iv) To produce the third subculture, 100 µl of the culture broth from the second subculture was inoculated with MSM broth containing 0.75% glucose and 0.25% malathion. After that, the mixture was incubated for 48 hours at 28°C and 240 rpm.

v) To create the fourth subculture, 100 µl of the culture broth from the third subculture was injected with MSM broth that contained 1% malathion. The mixture was then incubated for 48 hours at 240 rpm and 28°C.

vi) Following the dissemination of 100 µl of culture broths from each subculture on the MSM agar medium separately and treatment with 50 mg/L Malathion, the plates were incubated for 24 hours at 37°C.

vii) Following incubation, the morphological traits of the colonies cultivated on each plate were examined, and the majority of the bacterial colonies displayed traits that were repeated.

viii) Using the recurrent streak plate technique, the bacterial strains with distinct morphologies were selected and isolated on Malathion-enriched MSM agar media.

The best isolates were chosen for additional research after the dominating bacterial colonies with various colony characteristics were chosen to test their Malathion potentials.

Morphological characterisation of the bacterial isolates

Using phenotypic analyses such as cell morphology, Gram reactivity, and motility, the isolates of bacteria that degrade malathion were morphologically identified (Holt et al., 1994).

Gram staining Technique: The basic principle of gram staining is the bacterial cell wall's capacity to retain the crystal violet dye following solvent treatment. Gram-positive bacteria have more peptidoglycan, whereas gram-negative species have more lipids. (NCBI, Nishant Tripathi et al, 2022). The procedure is:

i) The glass slides were cleaned and sterilised, and each bacterial isolate was applied separately and heat fixed.

ii) Following heat fixing, the bacterial smear was treated with two drops of crystal violet for 30 seconds, followed by a 10-second rinse with distilled water.

iii) Two drops of Gram iodine were then added to the smear, and it was left for a minute. The crystal violet was again removed with distilled water.

iv) 95% ethanol was then applied to the smear, and the ethanol was again eliminated by washing.

v) Lastly, distilled water was used to rinse the smear after it had been stained for 30 seconds with the counterstain safranin.

vi) To assess the Gram reaction, the slides were examined under a microscope.

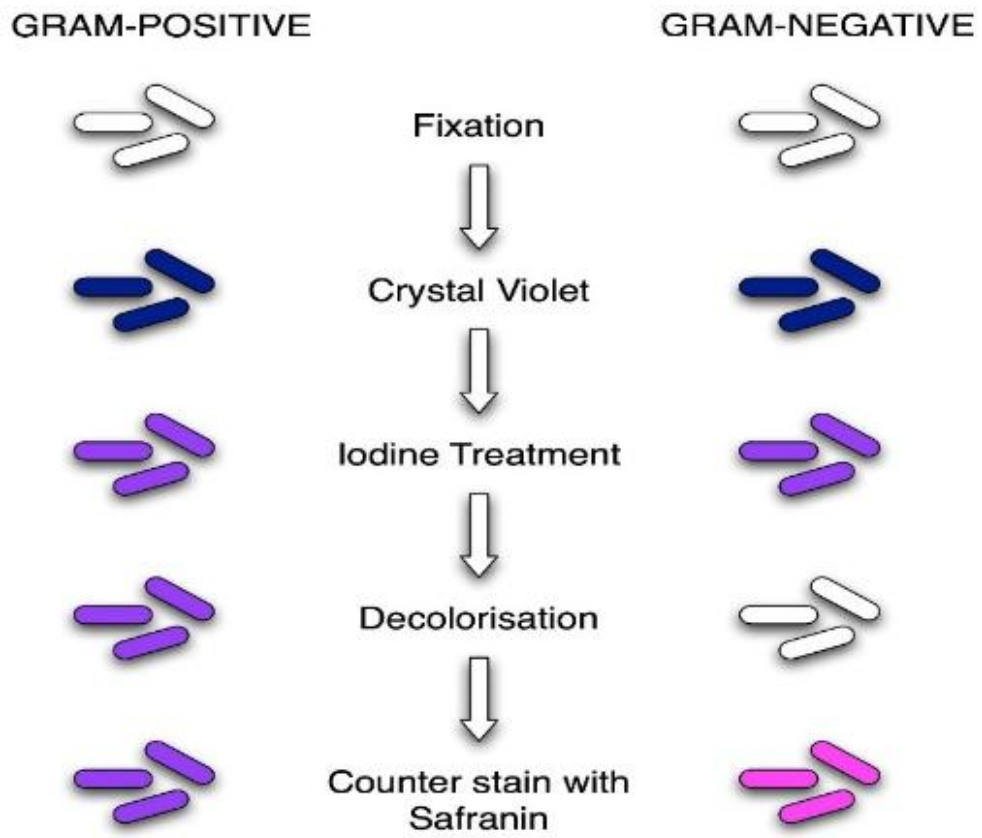

Figure 3.4 Principle of Gram Staining

Physiological characterisation of Bacterial isolates

To evaluate the physiological traits of the bacterial isolates, the IMVic (Ind0le, Methyl-red, Voges-Proskauer, and Citrate) test for the sugar, Catalase, and Urease were employed.

Indole test (Harley and Prescott, 2002): The Indole Test evaluates an organism's capacity to convert the amino acid tryptophan into indole. The following is how the Indole test was conducted:

i) Aseptically inoculate a small quantity of 24-hour-old cultures of isolated bacterial strains in a sterile tube filled with tryptone broth, then incubate for 24 to 48 hours at 37°C.

ii) *Tryptone Broth:* A nutrition broth with 1% tryptone in distilled water that is used to identify coliforms that produce indole. After incubation, immediately add five drops of Kovac's reagent to the culture tube.

iii) *Kovac's reagent:* Concentrated hydrochloric acid, para-dimethylaminobenzaldehyde (DMAB), and isoamyl alcohol make up the biochemical reagent.

iv) The culture is Indole positive (within seconds) if the top of the media appears pink or crimson red, while it is Indole negative (within seconds) if it appears yellow or cloudy.

Methyl Red test (Harley and Prescott, 2002): An organism's ability to carry out varied acid fermentation and produce stable acids as end products is assessed using the Methyl Red Test.

i) In a sterile tube filled with methyl red Voges-Proskauer (MR-VP) broth, aseptically inoculate a small quantity of 24-hour-old cultures of isolated bacterial strains. Then, incubate for six days at 37°C.

ii) *MR-VP Broth*: To differentiate the coli-aerogenes group, this kind of glucose phosphate broth—also referred to as buffered glucose broth—is used. 7.0 g/l of buffered peptone, 5.0 g/l of potassium phosphate, and 5.0 g/l of dextrose.

iii) The culture tube should be immediately filled with four to five drops of methyl red indicator after incubation.

iv) If the media's colour changes to red, the culture is methyl red positive; if it stays the same colour, the culture is methyl red negative.

Voges-Proskauer test (Harley and Prescott, 2002): The Voges-Proskauer test shows if an organism can ferment glucose and produce acetylmethylcarbinol.

i) Use a sterile tube filled with methyl red Voges-Proskauer (MR-VP) broth to aseptically inoculate a small quantity of 24-hour-old cultures of isolated bacterial strains. Then, incubate for six days at 28°C.

ii) Immediately after incubation, pour 0.6 ml of Barritt's solution (4% KOH in ethanol and 5% α-naphthol) into the culture tube.

iii) When the media's colour changes to cherry red, it signifies a Voges-Proskauer positive culture; when it stays the same or turns yellow-brown, it signifies a Voges-Proskauer negative culture.

Citrate Utilisation Test (Harley and Prescott, 2002): This test assesses an organism's ability to use sodium citrate as its only carbon source.

i) Aseptically inoculate a tiny quantity of 24-hour-old cultures of isolated bacterial strains onto Simmons citrate agar slants, then incubate for six days at 28°C.

Simmons Citrate Agar: Ingredients for this agar medium include: fifteen grams per litre of agar, two grams per litre of ammonium dihydrogen phosphate, two grams per litre of trisodium citrate, 0.8 grams per litre of disodium ammonium phosphate, 0.08 grams per litre of bromothymol blue, 0.2 grams per litre of magnesium sulphate heptahydrate, and five grams per litre of sodium chloride.

ii) Examine the media's colour change following incubation.

iii) A culture that is citrate positive is indicated by media that turns a deep blue colour; a culture that is citrate negative is indicated by media that stays green or stays the same colour.

Catalase test (Harley and Prescott, 2002): The enzyme catalase, which converts hydrogen peroxide into oxygen and water, is detected by the catalase test.

i) Aseptically inoculate six days at 28°C with a tiny quantity of 24-hour-old cultures of isolated bacterial strains on Bushnell Hass agar slants.

ii) *Bushnell Haas Agar***:** Magnesium sulphate 0.200 g/l, calcium chloride 0.020 g/l, ammonium nitrate 1.000 g/l, ferric chloride 0.050 g/l, monopotassium phosphate 1.000 g/l, dipotassium phosphate 1.000 g/l, and agar 20.000 g/l are among the contents.

iii) After incubation, add a few ml of 3% H_2O_2 solution directly to the culture tube.

iv) The culture is catalase positive if there are many gas bubbles on the medium; catalase negative cultures do not develop gas bubbles.

Urease test (Harley and Prescott, 2002): The urease enzyme, which breaks down urea into carbon dioxide and ammonia, is detected by the urease test.

i) Aseptically inoculate a tiny quantity of 24-hour-old cultures of isolated bacterial strains in a sterile tube filled with urea broth, then incubate for six days at 28°C.

ii) *Urea Broth:* It contains the following: 20.0g of urea, 9.1g of potassium dihydrogen phosphate, 9.5g of di-sodium hydrogen phosphate, 0.1g of yeast extract, and 0.01 phenol red.

iii) Observe how the medium's colour changes after incubation.

iv) If the media colour changes to a vivid magenta or brilliant pink, the culture is urease positive; if the media colour changes to a different colour, the culture is urease negative.

3. RESULTS AND DISCUSSIONS

Morphological characterisation of the bacterial isolates

Gram staining revealed the following morphological characteristics of the bacterial isolates that degrade malathion:

Gram staining

Among the fifteen isolates, 11 isolates (KSCM-01, KSCM-02, KSCM-05, KSCM-06, KSCM-07, KSCM-08, KSCM-10, KSCM-11, KSCM-13, KSCM-14 and KSCM-15) demonstrated a favorable response to Gram staining, whereas the other four (KSCM-03, KSCM-04, KSCM-09 and KSCM-12) were negative for Gram staining.

Physiological characterisation of bacterial isolates

Indole Test

Among the fifteen isolates, 7 isolates (KSCM-04, KSCM-05, KSCM-06, KSCM-11, KSCM- 12, KSCM-14 and KSCM-15) were found to be positive for Indole. The eight isolates that remain (KSCM-01, KSCM-02, KSCM-03, KSCM-07, KSCM-08, KSCM-09, KSCM-10 and KSCM-13) were Indole negative.

Methyl Red Test

Among the fifteen isolates, 7 isolates (KSCM-01, KSCM-04, KSCM-07, KSCM-08, KSCM-12, KSCM-14 and KSCM-15) show positive results, while the other eight isolates showed negative results (KSCM-02, KSCM-03, KSCM-05, KSCM-06, KSCM-09, KSCM-10, KSCM-11 and KSCM-13).

Voges–Proskauer (VP) Test

Among the fifteen isolates, 6 isolates (KSCM-04, KSCM-06, KSCM-08, KSCM-12, KSCM-14 and KSCM-15) showed positive and remaining 9 isolates are negative (KSCM-01, KSCM-02, KSCM-03, KSCM-05, KSCM-07, KSCM-09, KSCM-10, KSCM-11 and KSCM-13).

Citrate Utilisation Test

Among the fifteen isolates, 7 isolates (KSCM-04, KSCM-05, KSCM-06, KSCM-09, KSCM-10, KSCM-11 and KSCM-14) were found to be citrate-using, while the other 8 isolates tested negative (KSCM-01, KSCM-02, KSCM-03, KSCM-07, KSCM-8, KSCM-12, KSCM-13 and KSCM-15).

Catalase Test

Among the fifteen isolates, 9 isolates (KSCM-01, KSCM-02, KSCM-03, KSCM-06, KSCM-08, KSCM-10, KSCM-12, KSCM-13 and KSCM-15) contains catalase enzyme whereas remaining 6 isolates (KSCM-04, KSCM-05, KSCM-07, KSCM-09, KSCM-11 and KSCM-14) lacks Catalase.

Urease Test

Among the fifteen isolates, 8 isolates (KSCM-01, KSCM-02, KSCM-03, KSCM-07, KSCM-10, KSCM-11, KSCM-12 and KSCM-15) contains Urease enzyme and remaining 7 isolates (KSCM-04, KSCM-05, KSCM-06, KSCM-08, KSCM-09, KSCM-13 and KSCM-14) not producing Urease.

Isolates	Shape	Grams Stain	Indole	MR	VP	Citrate	H2S	Catalase

KSCM-01	**Rod**	**Positive**	-	+	-	-	-	+
KSCM-02	**Rod**	**Positive**	-	-	-	-	-	+
KSCM-03	**Rod**	**Negative**	-	-	-	-	-	+
KSCM-04	**Rod**	**Negative**	+	+	+	+	-	-
KSCM-05	**Cocci**	**Positive**	+	-	-	+	-	-
KSCM-06	**Cocci**	**Positive**	+	-	+	+	+	+
KSCM-07	**Cocci**	**Positive**	-	+	-	-	-	-
KSCM-08	**Rod**	**Positive**	-	+	+	-	-	+
KSCM-09	**Rod**	**Negative**	-	-	-	+	-	-
KSCM-10	**Cocci**	**Positive**	-	-	-	+	-	+
KSCM-11	**Cocci**	**Positive**	+	-	-	+	-	-
KSCM-12	**Rod**	**Negative**	+	+	+	-	-	+
KSCM-13	**Cocci**	**Positive**	-	-	-	-	+	+
KSCM-14	**Cocci**	**Positive**	+	+	+	+	-	-
KSCM-15	**Rod**	**Positive**	+	+	+	-	-	+

Morphological and Physiological characterization of the fifteen bacterial isolates

The indole test assesses how well an organism can digest the amino acid tryptophan. Indole is produced by bacteria cultivated in tryptophan-rich media, suggesting that the bacteria may degrade tryptophan. It is used in the IMViC assays, a series of tests intended to differentiate between Enterobacteriaceae species. The red dye rosindole, which is used to identify indole in the media, is created when indole and p-Dimethyl amino benzaldehyde (DMAB) react chemically in an acidic environment (Winn et al., 2006).

When the pH falls below 4.4, the pH indicator Methyl Red (MR) turns red. The methyl red test evaluates an organism's capacity to generate and sustain glucose fermentation as a byproduct. Through fermentation, many bacterial species can more easily produce acids from glucose that are higher than the bacterial system's capacity to act as a buffer. Lactic acid, acetic acid, succinic acid, and formic acid are among the organic acids that are commonly produced by glucose during mixed acid fermentation. The pH of the phosphate buffer in the media will drop to less than 4.4 due to the acid buildup that occurs during mixed acid fermentation. Methyl red, a pH indicator, turns red in culture tubes when the pH falls below 4.4. Methyl red turning yellow indicates that mixed acid fermentation is not being used and that the pH is higher than 6.0.

The Voges-Proskauer test is used to identify organisms that produce acetoin, a precursor of 2,3 Butanediol, through the Butylene Glycol pathway. After bacterial culture has been inoculated into the MR-VP broth via the butylene glycol route, acetoin is transformed into diacetyl by adding potassium hydroxide (KOH) and VP reagents. Then, diacetyl and oxygen mix

to create a red hue. The culture's colour will change from reddish-brown to pink when acetoin is present. A culture will turn yellow instead of brownish-green if acetoin is not present.

Citrate test can be used to determine whether a microbe can use citrate as its only carbon source. The alkaline pH of the media is caused by the production of sodium bicarbonate (NaHCO3) and ammonia (NH3) from sodium citrate and ammonium salts. Citrate is used by aerobic bacteria because they have a full Tricarboxylic acid cycle and only require a citrate adsorption mechanism. Bacteria produce the enzyme citrate, which produces acetate and oxaloacetate, which gives the culture tubes their blue hue. Anaerobic bacteria like Escherichia coli and Klebsiella pneumoniae catabolize citrate (Bott, 1997) and lactic acid bacteria (Drider et al., 2004) uses citrate lyase, an enzyme that breaks down citrate into acetate and oxaloacetate (Schneider et al., 2000).

Catalase Animal, plant, and bacterial cells contain the enzyme catalase, which can change hydrogen peroxide into oxygen and water. Active catalases are vital elements of the cellular defense system that shield cells from oxidative damage. (Dantas et al., 2008). There are three known kinds of bacterial catalases: Bifunctional catalase peroxidases, heme-containing catalases, and non-heme-containing catalases (Chelikani et al., 2004). Almost all bacterial species, including Escherichia coli (Claiborne and Fridovich 1979) and Bacillus subtilis (Loewen and Switala, 1987) comprises a range of catalases. Different studies have indicated that halophiles and other extremophiles have unique catalases (Brown-Peterson and Salin, 1993), Thermophiles (Kagawa et al., 1999) and Psychrophiles (Lorentzen et al., 2006).

The enzyme urease, which is found in all plants, different types of algae, fungus, and bacteria, helps to hydrolyze urea into negatively charged carbonate and positively charged ammonia (Omoregie et al., 2017). Earlier research demonstrated that urease can be extracted from various bacterial species, including Bacillus (Dejong et al., 2006), extracted from Yersinia, Citrobacter, Enterobacter, Pseudomonas, and Serratia (Alizadeh et al., 2014). Additionally, a number of aerobic bacterial taxa, such as Morganella, Proteus, Providencia, Pseudomonas, Sarcina, Serratia, Streptococcus, Morganella, Lactobacillus, Fusobacterium, Clostridium, Enterobacter, and Ureaplasma, produced the urease enzyme, which efficiently broke down urea in the soil (Phang et al., 2018). There are two types of urease produced by soil microorganisms. It is located either extracellularly, emitted from cells, or intracellularly, directly linked to ureolytic bacteria. (Skujip, 1976)

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