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Isolation and Screening of Triclosan Degrading Microorganism

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Abstract—*The Triclosan, a phenolic compound, is an antibacterial and antifungal agent present in the consumer daily need products such as toothpaste, soaps, detergents, toys and surgical cleaning treatments. This phenolic compound is an antimicrobial agent but it also seeks potential adverse effect on the organism and environmental health. Bioaccumulation of triclosan causes high level fatal effect on the aquatic life and this passively effect the human health. The prolonged exposure to this compound can have carcinogenic effect. The major source of triclosan in the environment is the sewage waste. The triclosan present in the water damages the aquatic life. They get accumulated in the body of lower animals and gradually they enter the food chain and also the food web of higher plant and animal. And this way it affects the human and environment. In environment, triclosan can be degraded by microorganisms into different other compound which may include chlorophenols and dioxine. In this present work the ability to degrade triclosan by different microorganism isolated from sewage is being checked. Isolate AS1001 was found to be the efficient degrader of Triclosan and it was also observed that it degraded triclosan more efiiciently in form of immobilized cells.*

Keywords*: microorganism, degradation, triclosan.*

1. INTRODUCTION

Triclosan, a polychlorophenoxy phenol, is a broad spectrum antimicrobial agent which is being used on a large scale in different personal care and healthcare goods for the past 40 years. A large number of products ranging from a small soap to kitchenware and electrical appliance is coated with Triclosan. Triclosan exposure has become so common these days that it has not only shown its presence in environmental samples but has also been found in urine samples (Calafat *et al.* 2008), blood samples(Calafat *et al.* 2008) and even in breast milk (Allmyr *et al.* 2006) of human beings across the globe.

Various physical and chemical methods have been adopted for the removal of Triclosan but there are limitations attached with them. The chemical method is not environment friendly plus the degradation products formed in this method are much more toxic than Triclosan itself. Biodegradation is one such method that can be adopted for the removal of Triclosan from environment. One of the major sources of Triclosan in waterways is sewage sludge. Accumulation of Triclosan in sewage sludge takes place from municipal wastewater systems (Heidler *et al.* 2007). A few microbes have been reported to be capable of degrading Triclosan but the database is limited.

2. MATERIALS AND METHODS

2.1 Isolation of Triclosan degrading micro-organisms

The wastewater sample was collected from Birla Institute of Technology, Mesra, Ranchi. The water sample was serially diluted from 10^{-1} to 10^{-2}. The serially diluted samples were spread on the Triptic soya plate containing 30g/ml Triptic soya agar and 1.5% bacteriological agar using L-shaped spreader under sterilized conditions. The medium was also supplement with 1% filter sterilized Triclosan (dissolved in 95% ethanol). The plates were then incubated at 37^0C for 5-6days. The microbial colonies grew on the plates. The colonies showing halo zones were selected for further use. The colonies were purified by streaking them onto agar plates and slants and were preserved at 4^0C.

All the media components used were procured from HiMedia Laboratories Pvt. Limited, Mumbai, India.

2.2 Screening of microbes degrading Triclosan

A loopful of the isolate was taken and was inoculated in 250ml Erlenmeyer flask containing minimal media containing Na_2HPO_4 33.5g/l, KH_2PO_4 15g/l, NH_4Cl 5g/l, NaCl 2.5g/l, bacteriological agar 1.5% and 2% syringe filtered Triclosan. The flask was then kept in the incubator at 37^0C. Media was withdrawn periodically under sterile conditions in microcentrifuge tubes which was then subjected to centrifugation at 10,000 rpm for 20 minutes. The supernatant obtained was then subjected to spectrophotometric analysis.

All the media components were of analytical grade and were obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India.

Determination of the growth curve of isolate

A starter culture was prepared by inoculating a loopful of the selected microorganism in 100ml Erlenmeyer flask containing

25ml nutrient broth and the flask was incubated at 37^0C for 12 hours. After 12 hours one ml of the culture was taken from the flask and was dispensed in another 250ml Erlenmeyer flask containing 150ml nutrient broth under sterile conditions. The freshly inoculated flask was then incubated at 37^0C. At an interval of 1 hour each, 1 ml. of the sample was drawn and its optical density was measured at 600nm.

2.3 Morphological and Biochemical Characterization of the selected microbe

The following tests were performed:

2.3.1 Gram Staining

The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer whereas gram-negative cells have a thin layer of peptidoglycan. The Gram staining uses our different reagents comprising Crystal violet, Gram's iodine, Ethyl alcohol (70%) and Safranin.

A thin uniform bacterial smear was prepared and heat fixed onto a glass slide. It was flood with crystal violet for 1 minute and then after washing with water, the smear was again flood with iodine for 1 minute. It was washed under running water and then with ethyl alcohol. Finally it was counterstained with safranin for 1 minute and observed under microscope at 40X and 100X (immersion oil).

2.3.2 Starch hydrolysis

Starch is a branching polymer composed of glucose molecules that are linked by glycosidic bonds. The degradation of starch into its monomeric unit requires the extracellular enzyme amylase. Starch agar plates are flood with Gram's iodine as in the presence of iodine, starch will react with it thus imparting a blue-black color to the media indicating negative result.

Starch agar was prepared, autoclaved and poured into petriplates. After the solidification of agar, the test organism was streaked onto the plates. The plates were then incubated at 37ºC for 24-48 hours. The plates were then flood with Gram's iodine and were observed for the zone of hydrolysis around the colonies.

2.3.3 Lipid hydrolysis

Lipids are high molecular weight compounds that possess large amount of energy. Lipase is the enzyme responsible for the degradation of lipids such as triglycerides into building blocks glycerol and fatty acids.

Tributyrin agar was prepared, autoclaved and poured into petriplates. After solidification of agar, the test organism was streaked onto the plates. The plates were then incubated at 37ºC for 24-48 hours. The plates were then observed for the zone of hydrolysis around the colonies.

2.3.4 Casein hydrolysis

Casein is a milk protein composed of several amino acid subunits linked together by peptide bonds. Proteins undergo step-by-step degradation into peptones, polypeptides, dipeptides and ultimately into amino acids. These degradation reactions are mediated by extracellular enzyme proteases.

Milk agar was prepared, autoclaved and poured into petriplates. The plates after solidification were streaked with the test organism. They were then incubated at 37ºC for 24-48 hours. The plates were then observed for the zone of hydrolysis around the colonies.

2.3.5 Gelatin hydrolysis

Gelatin is a protein which is produced by the hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Gelatin maintains its gel properties below 25ºC and exists as a solid while at temperatures above 25ºC, it is a liquid.

Nutrient broth supplemented with 12% gelatin was prepared and autoclaved in tubes. It was then inoculated with the test organism and incubated at 37ºC for 24-48 hours. The tubes were then kept in refrigerator for 30 minutes and were then observed for liquefaction of the media.

2.3.6 Oxidase Test

While performing this test simply the test culture was made to streak onto an oxidase soaked wat man filter paper. If color change to purple or blue is evident at 30 seconds in1 minute then the result is positive. To get the accurate results it is important that the test is read by one minute (avoid false negatives and false positives). This laboratory test is based on detecting the production of the enzyme cytochrome oxidase by Gram-negative bacteria. It is a hallmark test for the *Neiserria*. It is also used to discriminate between aerobic Gram negative organisms like *Pseudomonas aeruginosa* and other *Enterobacteriaciae*.

2.3.7 Glucose fermentation test

Organisms use different carbohydrates depending upon their enzymatic complement. Some organisms are capable of metabolizing sugars anaerobically such as glucose whereas others use aerobic pathways. In fermentation, substrates undergo anaerobic digestion and produce an acid that may or may not be accompanied by gases.

Phenol red broth containing glucose as a carbon source was prepared and autoclaved in test tubes containing inverted durham tubes. The test organism was inoculated into the broth and was incubated at 37ºC for 24-48 hours. The tubes were then observed for the change in colour and presence of gas bubbles.

2.3.8Nitrate Reduction Test:

Nitrate reduction, it can be determined by growing the organisms in a nitrate broth medium. The medium is basically a nutrient broth consisted of 0.1% potassium nitrate (KNO_3) as the nitrate substrate. Along with that the medium is made into a semisolid medium by adding 1% agar in it. The semi solidity helps the diffusion of oxygen into the medium, that is why for nitrate reduction it favours the anaerobic requirement.

After incubating the cultures we can determine the ability of the isolated microorganism to reduce nitrates to nitrites by adding two reagents; Solution A, which is sulfanilic acid, followed by Solution B, which is α-naphthylamine. Following reduction, the addition of Solution A and Solution B will produce an immediate cherry red colour.

NO_3 —(NITRATE REDUCTAS)→ NO_2^- (red colour on addition of solution of A and B)

Using sterile technique, inoculate the organism into the tube containing medium.Incubate the culture for 24 to 48 hours at 37˚C.By adding 5 drops of Solution A and then five drops of Solution B to the test tube.Now observe the colour change.

All the chemicals and media components used in the biochemical analysis were procured from HiMedia Laboratories Pvt. Limited, Mumbai, India.

3. RESULTS AND DISCUSSIONS

3.1 Isolation of microorganisms

The microbes having ability to degrade Triclosan were isolated on the basis of their capacity to utilize Triclosan as a carbon source. The microbes showing halo zone were selected for further studies.

A total of 180 colonies were isolated on agar plates, all being morphologically similar showed a halo zone around them. There are only a few reports on micro-organisms capable of metabolizing triclosan. Two white rot fungi, *Trametes versicolor* and *Pycnoporus cinnabarinus* (Hundt *et al.* 2000) have been found to be capable of using triclosan and degrading it. Several bacterial species *Sphingomonas* strain Rd1 (Hay *et al.* 2001), *Pseudomonas putida* and *Alcaligenes xylosoxidans* (Meade *et al.* 2001) have also been reported to degrade triclosan. All the bacterial strains reported so far are gram negative in nature.

Table 1: Number of colonies on agar plates

S. No.	Source of isolation	Total no. of colonies obtained	No. of colonies showing halo zone
1.	BIT Mesra sewage water	180	1

Table 2. Gram staining and shape of isolate AS1001

S. No.	Isolate	Gram stain	Shape of the cells
1.	AS1001	Gram negative	Bacilli

Screening of isolate AS1001 for Triclosan degradation

Initially 0.2% Triclosan was added in the minimal media as a sole carbon source. After inoculation, the organism gradually degraded the amount of Triclosan as it incorporated it into its metabolism as a carbon source. The amount of Triclosan degraded was calculated in the form of percentage taking its optical density into account. Maximum degradation of 93.67% was achieved on the 14th day of incubation.

Table 3: Percentage Degradation ofTriclosan.

Days	O.D. 1	O.D. 2	% Degradation of Triclosan
1	0.185	0.195	76.19
2	0.217	0.234	71.74
3	0.149	0.146	81.51
4	0.144	0.149	81.64
5	0.124	0.124	84.46
6	0.124	0.124	84.46
7	0.115	0.115	85.59
8	0.118	0.118	85.21
9	0.110	0.110	86.21
10	0.114	0.105	86.28
11	0.093	0.094	88.28
12	0.096	0.089	88.40
13	0.083	0.073	90.23
14	0.057	0.044	93.67

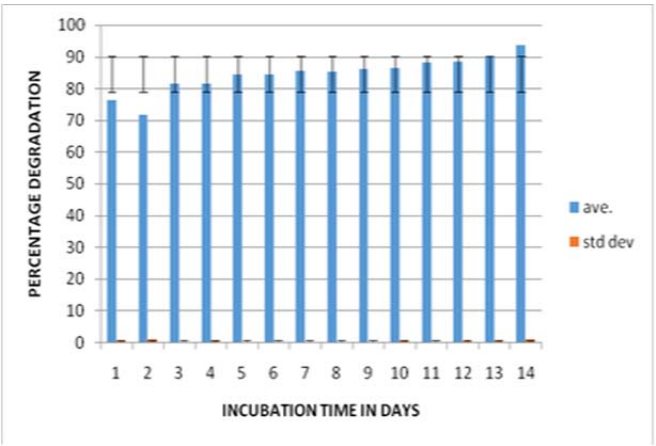

Fig. 1: Bar graph of degradation of Triclosan from the isolated microorganism.

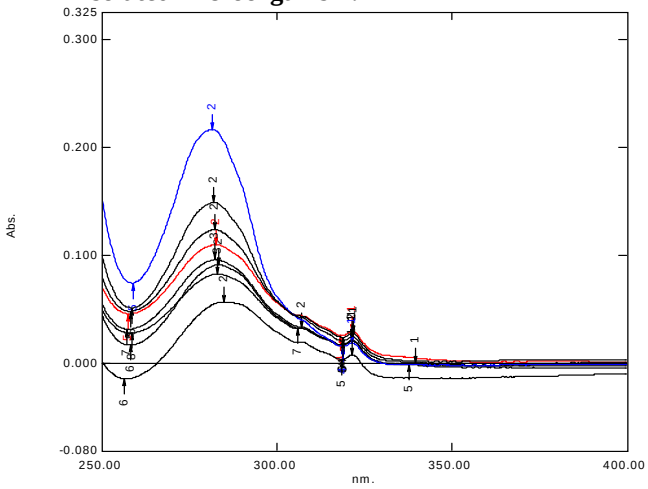

Fig. 2. Overlay of UV-Visible Spectrum showing gradual decrease in concentration of Triclosan.

The UV-Visible spectrum is clearly showing decrease in intensity of the peak over several days of incubation inferring that the amount of Triclosan in the minimal media is gradually decreasing upon incubation.

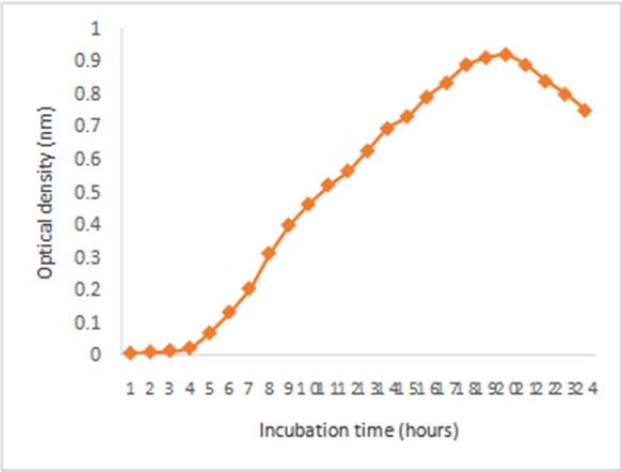

Fig. 3. Growth curve of the isolate AS1001.

The growth curve is very well denoting all the four phases of growth. Initial lag phase is depicted between three to four hours of incubation. The isolate went into its early log phase after four hours of incubation and gradually went up to 18 hours of incubation. Then the isolate went into stationary phase which tapered off after 21 hours of incubation.

3.2 Morphological and biochemical characterization:

A) Morphological Characteristics	Result
1. Slants	Filiform (continuous,thread like growth with smooth edges)
2. Plates • Size • Pigmentation • Shape • Margin • Elevation	 Small/pinpoint Dull white Circular – unbroken, peripheral edge Entire Convex
3. Broth	Uniform fine turbidity

B) Biochemical Characteristics	Result
1. Gram's stain	Gram negative rods
2. Starch hydrolysis	Negative
3. Casein hydrolysis	Positive
4. Lipid hydrolysis	Positive
5. Gelatin hydrolysis	Positive
6. Oxidase test	Positive
7. Glucose fermentation	Negative
8. Nitrate reduction test	Positive

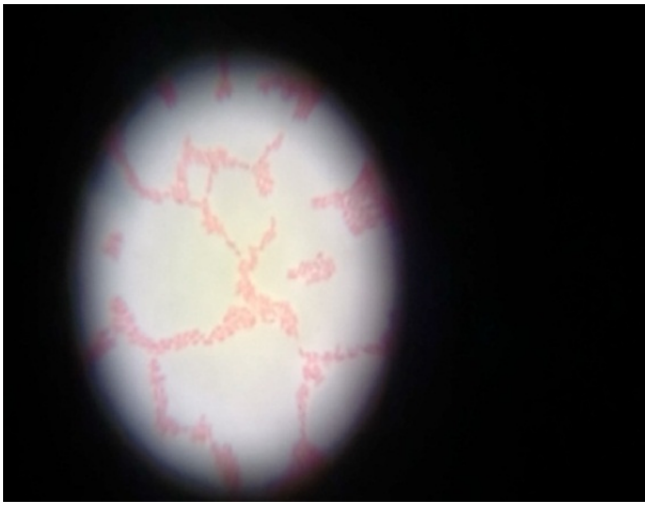

Fig. 4. Gram stained cells of AS1001

4. ACKNOWLEDGEMENT

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