ISOLATION, IDENTIFICATION AND IN-VITRO ANTIBIOTIC SENSITIVITY PATTERN OF LOCAL ISOLATES OF *XANTHOMONAS* FOLLOWED BY PRODUCTION OF XANTHAN GUM USING AGRO-INDUSTRIAL WASTE

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Abstract: Xanthan are water soluble exopolysaccharides produced by *Xanthomonas* species. These *Xanthmonas* species were isolated from local infected samples i.e. cabbage leaf (Black rot) by *Xanthomonas campestris* and lemon (Citrus Canker) by *Xanthomonas axonopodis* and were confirmed on the basis of biochemical testing and Gram staining followed by its antibiotic sensitivity testing which gave zone of inhibition more than 20mm for some antibiotics like Chloramphenicol. These polysaccharides have much common applications and normally produced in submerged fermentation by using different carbon sources i.e. usage of different agro-industrial wastes. Xanthan is an ecofriendly and natural gum produced by using waste as fermentation substrate which can be alternative to other synthetic gums as well as traditional gums like agar, carrageenan, locust bean gum, gluten, guar gum which are obtained from aquatic algae (seaweeds) and plants hence disturbing natural environment. An attempt to synthesize Xanthan from agro-industrial wastes by submerged fermentation yielded a dry weight of 0.422gm/50mL of potato peels and 0.388gm/50mL of chickoo peel extract by *Xanthomonas axonopodis* whereas 0.558gm/50mL of potato peels and 0.398gm/50mL of chikoo peel extract by *Xanthomonas campestris* and the YDC standard gave 0.027gm/50mL and 0.043gm/50mL for *Xanthomonas axonopodis* and *Xanthomonas campestris* respectively. The work emphasizes the possibility of using agricultural waste as lower cost alternative substrates for Xanthan production which is widely used food additive and can be alternative for an agar

Index Terms - *X. axonopodis,* citrus canker, *X. campestris,* black rot, antibiotic sensitivity tests

1. INTRODUCTION

Xanthomonas are a group of around 27 bacterial species that cause disease in around 400 different plants. These include important food crops such as rice, soyabean and tomato. The species *Xanthomonas oryzae* causes the devastating disease rice bacterial blight which results in the loss of up to 50% of rice plants. These huge crop losses threaten the food supply for those who depend heavily on rice as a staple foodstuff. *Xanthomonas* bacteria grow best at around 30°C. They cause huge problems in areas with a warm, wet climate. Bacteria enter plant leaves through breathing pores (stomata) and water releasing pores (hydathodes) in the leaf surface. Machinery and insects damage plants creating wound entry points for *Xanthomonas* bacteria. Once inside the plant, the bacteria reproduce and move around using the plant's water transportation system (xylem). Most *Xanthomonas* species produce a sticky, glue-like substance called xanthan, which blocks water transportation causing plants to wilt and eventually collapse. *Xanthomonas* bacteria don't enter living cells, but feed on nutrients released from plant cells as they become leaky and start to die. Symptoms vary according the *Xanthomonas* species, here are some examples: Citrus plants (e.g. oranges and lemons): *Xanthomonas axonopodis* causes areas of plant tissue to die and form cankers, leading to the loss of leaves and fruit and damage to overall plant health. Brassica plants (e.g. cabbage, broccoli, cauliflower and sprouts): *Xanthomonas campestris* causes black rot disease, where leaves become yellow and sticky, then wilt and die. Rice: *Xanthomonas oryzae* causes pale green, water-soaked streaks to form on leaf tips and veins (T.C.Yang et al., 2002). When lesions join together, the whole leaf can turn white and die. Eventually bacteria can spread to the whole plant, causing it to wilt and even die. Onions: *Xanthomonas axonopodis* infection causes bacterial blight. Black spots form on leaves, reducing area available photosynthesis and so less sugar is produced. Plants become stunted and produce smaller onion bulbs.

The bacterial genus *Xanthomonas* contains organisms that resemble Pseudomonas (small, motile, Gram-negative rods), are aerobic, produce yellow pigments termed Xanthomonadins. Streaked colonies of *Xanthomonas* produced copious extracellular slime- a complex polysaccharide composed of more than one type of sugar (a heteropolymer). About 20,000 tonnes of xanthan are produced industrially from *Xanthomonas campestris* each year. The gum itself is colourless. The bacteria have a yellow pigment in the wall, but it is extractable only with organic solvents so it does not interfere with the commercial processing of xanthan (R. Vidhyalakshmi et al., 2012). Several other microorganisms like Rhizobium and Chromobacterium species produce extracellular slimes, but with different properties that may not be as useful as those of *Xanthomonas*. Such properties are not just confined to bacteria. For example, the common leaf-surface fungus Aureobasidium pullulans is grown commercially on starch and produces the exopolysaccharide pullulan. This polymer has unique film-forming and adhesive properties that make it useful for producing a film-wrap for foods.

Gum is the common term for hydro colloidal gels-polysaccharides that have an affinity for water and exhibit binding properties with water and other organic/inorganic materials. Traditionally, they have been derived from a wide variety of plants. Chemically gums are carbohydrate polymers or polysaccharides (however, gelatin is a protein). Polysaccharides are present in all life forms. They have a number of unique chemical and physical properties. They serve as structural material to the plant kingdom, as energy reserves, adhesives and also information-transfer agents. Microbial polysaccharides are composed of regular repeating units of simple sugars like glucose, mannose, fructose, etc. These polysaccharides are sometimes termed as slime or exopolysaccharides. Dextran, discovered in early 1940s, was the first microbial polysaccharide to be commercialized. The second microbial polysaccharide commercialized was xanthan. Xanthan gum is a natural polysaccharide and an important industrial biopolymer. It was discovered in 1963 at Northern Regional Research Center (now called The National Center for Agricultural Utilization Research) of the United States Department of Agriculture (USDA) (Margaritis and Zajic, 1978). The polysaccharide B-1459, or xanthan gum, produced by the bacterium *Xanthomonas campestris* NRRL B-1459 was extensively studied because of its properties that would allow it to supplement other known natural and synthetic water-soluble gums. Substantial commercial production began in early 1964. The toxicological and safety properties of xanthan gum for food and pharmaceutical applications have been extensively researched. Xanthan is non-toxic and does not inhibit growth. It is non-sensitizing and does not cause skin or eye irritation. . On this basis, xanthan has been approved by the United States Food and Drug Administration (FDA) for use in food additive without any specific quantity limitations (Kennedy and Bradshaw, 1984). In the United States, the only available xanthan gum is food grade. It is relatively expensive due to glucose or sucrose being used as the sole carbon source and the very stringent purity standards of the Food and Drug Administration for foods. For food-grade xanthan gum, up to 50% of the production costs are related to downstream purification steps, many of which would not be necessary for non-food applications. Another cost reduction could be achieved by using less expensive substrates, such as waste agricultural products like potato peels and fruit peels (Aarthy Palaniraj, Vijayakumar Jayaraman, 2011).

2. MATERIALS AND METHODS

2.1. PLACE OF STUDY

The present study was carried out in the Biotechnology laboratory, Department of Biotechnology, B.K. Birla college, Kalyan.

2.2 COLLECTION OF SAMPLES

The total of 5 infected lemon samples and cabbage leaves were collected from local market. At each time of collection, precaution was taken to minimize cross contamination of samples and they were carried in clean plastic bags. After washing, samples were transferred to plates containing sterile distilled water and kept at room temperature.

2.3 ISOLATION

The infected parts of lemon by Citrus canker and Cabbage leaves by Black rot were cut into pieces and placed on YDC (Yeast Dextrose Carbonate Agar) agar plates. YDC was prepared by dissolving yeast extract 5.0 gm, $CaCO_3$ 10.0 gm, D-glucose 10.0 gm and agar 8.5 gm in 500 ml of distilled water. It was gently heated until all the components ate properly dissolved. The pH was adjusted to 6.4-6.8 using 0.1N NaOH or 0.1M HCL as needed. It was properly sterilizes by autoclaving at 121^0C, 15 psi for 15-30 minutes. The plates were incubated for 3-4 days at room temperature i.e. 28^0C. The suspected colonies were again transferred onto YDC medium and incubated for well isolated colonies.

2.4 IDENTIFICATION OF THE ISOLATES

2.4.1 Colony morphology:

The *X. axonopodis* and *X. campestris* grown on YDC were yellow and mucoid. They were yellow, convex and mucoid and differed in colony size (1-3 mm).

2.4.2 Gram staining**:**

Gram staining was performed to differentiate bacterial species into gram-positive and gram-negative, based on the Gram reaction, size, shape, arrangement.

2.4.3 Biochemical testing:

Biochemical tests (Indole Test, MR-VP Test, Citrate Utilization Test, Triple Sugar Iron Test) were done. Some more test were also carried out.

Catalase test:

A loopful of 48 hours old slant growth of the bacterium was smeared on a slide and was covered with few drops of hydrogen peroxide (H_2O_2).

Potassium Hydroxide (KOH) Solubility Test:

A loop full of bacteria was aseptically removed from culture plates with tooth pick, placed on glass slide in a drop of KOH (3 %) solution and stirred for ten second using a quick circular motion of hand, then the tooth pick was raised a few centimeter's above the slide and observed for the formation of viscoid strand represent the bacterium as Gram-negative.

Starch Hydrolysis Test :

1% Starch agar plates were prepared and autoclaved. After solidifying, starch agar plates were streaked with loopful culture of the bacterium and incubated for five days at 28^0C. After incubation period was over, the plates were flooded with Lugol's iodine solution and observations were drawn for starch utilization by the bacterium.

Gelatin Liquefaction Test:

This test was performed for the screening of gelatinase enzymes produced by various organisms that catalyze the hydrolysis of gelatin present in medium.

2.5 ANTIBIOTIC SENSITIVITY TESTING

Sensitivity of isolates to different antibacterial agents was determined in-vitro by employing a modified disk diffusion test of the Kirby- Bauer method. It mainly involves diffusion of the agent into the medium surrounding the disc and hence measuring the diameter of zone of inhibition. Sensitivity test was performed for identified *Xanthomonas* strains by using Mueller-Hinton agar and 5 different antibiotic discs. Antiobiotic disk Streptomycin, Vancomycin, Chloramphenicol, Ciprofloxacin and Tetracycline.

2.6 FERMENTATION

Fresh chickoo peels *(Manilkara Zapota)* were collected from juice centres as well as sweet potato peels *(Ipomoea Batatas)* were collected which were part of household waste. 50gm of these were taken for each

isolate and was finally grounded properly using water followed by boiling in order to moisturize the substrate. The softened waste along with the water from the flasks were thoroughly mashed using a mortar and pestle for liquid substrate. Along with addition of some amount of K_2HPO_4 the pH was adjusted to 7.2 throughout the process. 72 hours broth culture of two isolates identified as *Xanthomonas* species were inoculated to the autoclaved substrate and incubated for 6 days at 28^0C. Fermentation was carried out with YDC as standard.

2.7 RECOVERY OF XANTHAN GUM

The biomass was separated from the fermentation broth after a fermentation period of 1 week by a round of centrifugation at 5000 rpm for 5 minutes. This is carried out in a 50 ml polypropylene centrifuge tube. After centrifuging, the supernatant so obtained was transferred into twice the volume of Isopropanol and was stored further at 4°C for 1 hour. Centrifugation was performed once again to recover the precipitated xanthan gum at 10,000 rpm for 10 minutes at 4°C. The supernatant so obtained was discarded and the pellet was allowed to dry at 28°C overnight. The dried Xanthan gum was then weighed and recorded.

3. RESULTS AND DISCUSSIONS

The source of plant disease includes citrus canker and black rot of lemon and cabbage leaf respectively. Among four microorganism isolated from them two were identified as *Xanthomonas.*

Table 1. List of *Xanthomonas* isolated from various sources

SAMPLE	PLANT DISEASE	PROBABLE ISOLATE
X_1	CITRUS CANKER	*X. axonopodis*
X_2	BLACK ROT	*X. campestris*

Table 2. Morphological characterization

Colony Characters	*Xanthomonas campestris*	*Xanthomonas axonopodis*
Size	2-3 mm	1-2 mm
Shape	Circular	Circular
Colour	Yellow	Yellow
Opacity	Opaque	Opaque
Margin	Entire	Entire
Consistency	Mucoid, Sticky	Mucoid, Sticky
Elevation	Convex	Convex
Gram's Nature	Gram Negative	Gram Negative

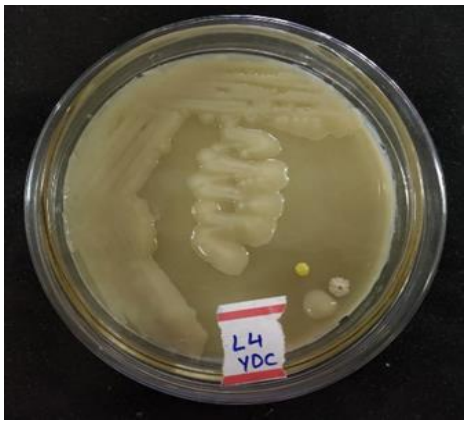

Fig 1: Xanthomonas campestris

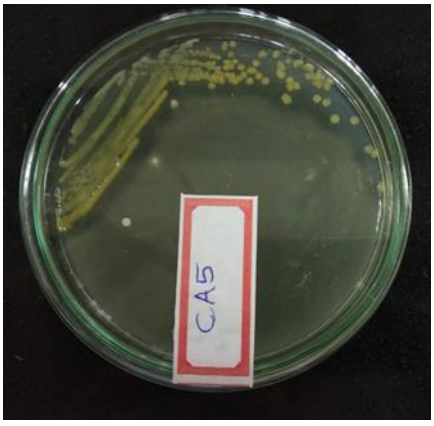

Fig2: Xanthomonas axonopodis

Table 3. Biochemical characterization of isolates

	Indole	Methyl Red	VP	Citrate	TSI	Catalase	Starch hydrolysis	Gelatin liquefaction	KOH
X_1	_	_	_	+	$K^{+/}A^{+/}G^{+/}H_2S^+$	+	+	+	+
X_2	_	_	_	+	$K^{+/}A^{+/}G^{+/}H_2S^+$	+	+	+	+
STANDARD	_	_	_	+	$K^{+/}A^{+/}G^{+/}H_2S^+$	+	+	+	+

The biochemical test done for characterization and the results are listed in above table. These reports clearly indicate or help in identification of *Xanthomonas* strains where they were compared with standard strain of *Xanthomonas* (R. Vidhyalakshmi et al., 2012).

Table 4. Antibiotic Sensitivity Testing

Name of Antibiotics	**Concentration (mg/mL)**	**Zone of inhibition (in mm)**	
		X_1	**X_2**
STREPTOMYCIN	10	20	20
TETRACYCLINE	30	-	-
CHLORAMPHENICOL	30	30	30
VANCOMYCIN	30	-	-
CIPROFLOXACIN	5	40	40

AST was performed using antibiotic discs of Tetracycline, Chloramphenicol, Vancomycin, Ciprofloxacin and Streptomycin. *X. campestris* showing zone of inhibition was found to be sensitive. The isolate was almost resistance to Tetracycline and Vancomycin. It was sensitive to Streptomycin, Chloramphenicol and Ciprofloxacin.

As it was studied that mango and sapodilla peels meet the required industrial level sugar concentration and they are preferred substrates giving about 9.92 gm/L and 2.23 gm/L of xanthan gum respectively (Dr. Biswa Prasun Chatterji et al., 2015). Sweet potato and chikoo peels provide starch and sucrose and substrates for fermentation yielded approximately 0.38 gm/50mL and 0.47 gm/50mL respectively. Hence, the main aim of this work was to emphasize the possibility of using agricultural wastage as lower cost alternative substrate for xanthan production this is widely used food additive as well as an agar alternative due to it's viscous properties.

The amount of Xanthan obtained by fermentation on sweet potato and chickoo peels with various isolated *Xanthomonas* is tabulated in Table 5.

Table 5. Xanthan Gum Obtained (gm/50mL)

Strain	YDC BROTH	CHICKOO PEELS	SWEET POTATO PEELS
Xanthomonas axonopodis	0.027	0.422	0.388
Xanthomonas campestris	0.043	0.558	0.398

X. campestris seems to be the highly yielding isolate as well as chickoo peels can be the suitable carbon substrate alternative.

Fig 3: Xanthan gum produced by *Xanthomonas* strains

4. CONCLUSION

Canker and black rotting are one of the most economically devastating bacterial disease of *Citrus aurantifolia* plant and Cruciferous plants caused by *Xanthomonas axonopodis* and *Xanthomonas campestris* respectively. In the present research work, we performed isolation, biochemical characterization and antibiotic sensitivity tests. We found significant result for both strains by action of antibiotics like Chloramphenicol and Ciprofloxacin. Therefore, pathogen responsible for citrus canker and black rotting were detected. While isolation of these bacteria on YDC agar copious exopolysaccharide slime was observed which is ultimately an economically important Xanthan gum. Despite they are considered as a economically devastating microbial agents leading to spoilage of vegetables and fruits, but only in the terms of external appearance. *Xanthomonas* are safe environmental agents, neither causing any infections or affecting human health. Therefore Xanthan can be termed as an eco-friendly gum which is produced using

agro industrial waste like potato peel, chickoo peels and many more as carbon substrates. This Xanthan can be ideal alternative for other synthetic gums and traditional gums like agar, salai gum which are produced by consuming and disturbing marine as well as natural environment such as algae and plants. This was an attempt to obtain xanthan gum by fermentation using isolated strains from infected samples in association with waste like sweet potato and chickoo peels. Small amount of sticky and highly viscous xanthan gum was obtained after proper centrifugation precipitation of fermentation broth by chilled isopropanol.

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