EXTRACTION AND CHARACTERIZATION OF XANTHANAND XANTHOMONADIN PIGMENT FROM ISOLATED *Xanthomonas spp.* PRESENT IN SPOILED VEGETABLES.

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Abstract

Xanthomonas spp. is a phytopathogen responsible for causing necrotic lesions on the edges of the plant leaves. It infects various susceptible plant species such as cruciferous vegetables. *Xanthomonas* has the capability to produce an exopolysaccharide called Xanthan. When *Xanthomonas spp.* is inoculated in a suitable fermentation media it produces xanthan. Xanthan is used as a natural polymer for various industrial purposes, where it is used as a stabilizer, emulsifier and thickening agent. The research was conducted to selectively isolate *Xanthomonas spp.* from necrotic lesions of spoiled leafy vegetables like cabbage & cauliflower and to produce xanthan. The xanthanwas characterized by various chemicals tests such as Molisch test, Anthrone test, osazone test. The antioxidant capacity of the extracted xanthan was also done using DPPH assay. Xanthomonadin is a yellow coloured pigment which is selectively produced by *Xanthomonas spp*. The Xanthomonadin was extracted from the isolated *Xanthomonas spp.* and its characterization was carried out by absorption spectrum respectively.

Keywords*:-Phytopathogen , Xanthan , Xanthomonadin , Antioxidant*.

Introduction

Microorganisms inhabit various environments in nature. The compounds like enzymes, peptides, polysaccharides, pigments, secondary metabolites etc. either help the organisms to overcome the stressed conditions, or help them grow and survive. By studying the working of these compounds in detail, they can be applied for various purposes for e.g. in pharmaceutical, as additives, colouring agents, textiles, paints, fabrics, etc. Exploring microbes for compounds with various properties will help in suggesting new applications to give a better development in various fields.Xanthomonas species are phytopathogen i.e they do not cause any harm to humans if ingested. All organisms in this genus are plant pathogens. The Xanthomonas pathovars infect a large selection of plants including some of agricultural interest, e.g. cabbage,cauliflower, alfalfa, and beans (cruciferous vegetables)Xanthomonas cells occur as single straight rods, 0.4±0.7 mm wide and 0.7±1.8 mm long. The cells are motile, Gram-negative, and they have a single polar flagellum (1.7±3 mm long) (Prasanna et al,2014).The microorganism is chemiorganotrophic and an obligate aerobe. The bacterium cannot denitrify, and it is catalase-positive and oxidase negative.The colonies are usually yellow, smooth,convex, mucoidal and viscous. Yellow pigments are present in all species of Xanthomonas, but they may be absent especially when strain degradation occurs. The capsular polysaccharide is the Xanthan.

Xanthan is a natural polysaccharide and an important industrial biopolymer. *Xanthomonas* species can produce a polysaccharide named xanthan. The xanthan molecules have a (1,4)-β-d-glucopyranose backbone as in cellulose. The molecular mass of the xanthan molecules is very high ($> 3 \times 10^6$) and the gum dissolves in water to yield highly viscous solutions.Xanthomonadin is pigment produced by the pigmented strains of Xanthomonas for e.g *xanthomonas oryzae*, *xanthomonas campestris*. Xanthomonadin is a brominated aryl-polyene, a yellow pigment, seen in most of Xanthomonads. It offers protection to the organism, against damage caused by visible light in the presence of oxygen called photobiological damage. It can also protect lipids from peroxidation, which may protect the bacterial membrane from oxidative damage. Xanthomonad ins are unique to *Xanthomonas* bacteria and serve as useful chemotaxonomic and diagnostic markers. (Margaritis et al, 1978)

Materials and Methods

Sample collection

Lesioned Cabbage and Cauliflower leaves which showing typical V-shaped brownish or yellowish necrotic leaf edges was collected from different markets.(Prasanna et al,2014)

Surface steriliztion and preparation of inoculum and their enrichment

Out of collected lesioned leaves,selected part of lesioned leaves was cut into small pieces and surface sterilized by using 0.1% of mercuric chloride solution for 0.2 sec, followed by washing with sterile distilled water twice to remove the residual of mercuric chloride.Sterilized lesioned leaves pieces was suspended in to tube containing sterile distilled water, kept standing at room temperature for 10 mins in order to prepare suspension.After preparation of inoculum the enrichment was carried by suspending inoculum in various media broth such as Glucose yeast calcium carbonate broth(GC), Sucrose peptone broth(SP), Wilbrink's broth(WB), Luria Bertani broth(LB) and kept at RT for 1 day on rotary shaker.

Serial dilution and spread plate count

Serial dilution of enriched culture was carried out by preparing dilutions ranging from ($10^{-1}$to 10^{-8}). These dilutions were then subjected to spread plate count.(Radunović et al, 2012)

Identification of isolates

Identification of the isolates was done with reference to cultural, morphological, and biochemical characteristics. 16 isolatesof *Xanthomonas* was identified and nomenclatured as (X1-X16).(Bergey's Manual of bacteriology, 2012)

Extraction of xanthan from identified *Xanthomonas spp*

24hrs old LB and GC broth grown isolate was grown in Production broth, and incubated on shaker for 96 hrs. Cells were pelleted by centrifugation at 10,000 rpm for 10 mins. Supernatant was collected and twice volume of acetone added and kept for 10 mins at room temperature till the precipitate was observed.Theprecipitate collected by centrifugation at 10,000 rpm for 10mins at 4ºC. Drying of precipitate was done in pre-weighed petriplate, overnight at 60°C and the yield was estimated by weighing again. (Moosavi-Nasab et al, 2008)

Characterization of extracted xanthan

A. Molisch test

In 2ml of Xp-10 xanthan solution ,1 drop Molisch reagent and 1-2ml conc. Sulphuric acid was added from sides gently.Distilled water was used as negative control and 1% glucose solution as positive control.Presence of purple ring at the interface indicates positive test.

B. Anthrone test

In 1ml Xp-10 xanthan solution,4ml of Anthrone reagent was added. On heating in boiling water bath for 10mins, observe the colour change. Distilled wateris taken as negative control and 1% glucose solution as positive control. Bluish green colour indicates positive test.

C. Osazone test for presence of Mannose

3ml of hydrolysed Extracted xanthan solution was added to 3ml of phenylhydrazine reagent, mixed and kept for boiling till precipitate formed. The time required for white or yellow-pale orange precipitate formation was recorded. Few drops of precipitatewasobserved microscopically for structure of crystals present.

Antioxidant activity by DPPH assay

The antioxidant activity of the extracted xanthan was done using DPPH assay in which various dilutions of the extracted crude xanthan were made i.e of 1:2,1:5 and 1:10 same dilutions were carried out for standard for comparative analysis.

Production and Extraction of X-10 xanthomonadin

X10 was selected for pigment extraction as it showing characterstic yellow colour colonies. X10 was grown on LB agar plate for 48 hrs. The growth was scrapped and suspended in adequate amount of methanol in tightly capped tubes. Incubation was done at room temperature by shaking gently at regular time intervals, till the methanol turned yellow and growth turned white. Cells were pelleted by centrifugation at 14,000 × g for 15 mins till clear supernatant was obtained. Clear supernatant was the methanolic extract which contain Xanthomonadin pigment. (Soudi et al, 2011)

Characterization of extracted xanthomonadin using U.V spectrophotometer

The methanolic extract was studied for absorption spectra at wavelength range from 400-600 nm using of U.V. spectrophotometer. The Lambda (λ) max was determined by plotting graph of absorbance versus range of wavelengths. In most members of *Xanthomonas* species, the methanolic extract has a major absorption maximum at 445 or 441nm. (Soudi et al, 2011)

Results

Characterization of isolate X6

For Isolation the spread plate count was performed and the following results were obtained.The isolated strains was characterized on the bases of its cultural, morphological and biochemical characteristics by referring to Bergey's manual of systematic bacteriology .

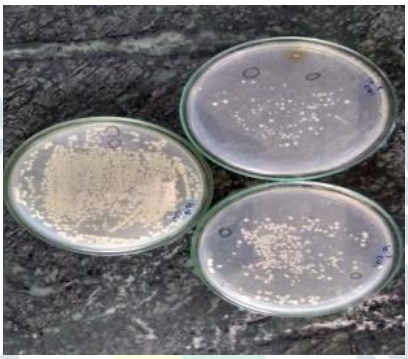

Fig 1: Isolation on Luria Bertani plates.

A.Cultural characteristics

The isolated strains were 16 from which 8 strains were isolated from cabbage and the other 8 were isolated from cauliflower and were named as X1 to X8 for cabbage and from X9 to X16 for cauliflower. Howeverall the 16 isolates showed typical mucoidal and yellow coloured colonies.

B.Morphological characteristics

On Gram staining few isolates, which were X2, X5, X6, X10, X13, X15 and X16 showed their morphological characteristics to be Gram negative, while the rest were showing characteristics of being Gram positive.

C.Biochemical characterisation

table 1: Biochemical characteristics for all 16 isolates.

Test	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	X 11	X 12	X 13	X 14	X 15	X 16	std
Sugar fermentation test																	
Glucose	+	+	+	-	-	-	+	-	-	+	-	-	-	-	+	+	V
Lactose	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	V
Mannose	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	V
Salt broth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N
IMViC																	
Indole	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	V
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N
V.P	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N
Citrate	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	P
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N

Keys: V = variable, P and [+] = positive, N and [-] = negative, V.P = Vogus Proskauer.

From all these isolates X6 was chosen for xanthan production and X10 for extraction of Xanthomonadin as it showed typical yellow colour pigment.

Characterization of X6 xanthan

A. Yield of X6 xanthan

The polysaccharide extracted from the isolate produced by the fermentation was orange-yellow in colour on drying. The yield observed for xanthan was found to be 0.96g/50ml of the fermentation medium.

B.Qualitative test for X6 xanthan as polysaccharide

The polysaccharide extracted from X6 was tested by molisch and anthrone test, and confirmed results for the presence of carbohydrate was seen.

C.Osazone test for the presence of mannose

X6 xanthan was tested by osazone test, it was observed that there was orange precipitate observed within 30 secs at room temperature. The microscopic appearance showed broomstick shaped crystals,specific for mannose due its formation within 1-5 min.this test id used for the detection of mannose residue in any carbohydrates.

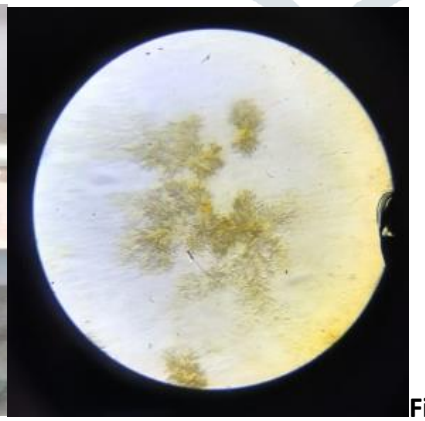

Fig.2:Positive test for Molisch test. Fig.3:Positive test for Anthrone test. Fig.4:Crystals of Mannose observed in Osazone test.

Study of X6 xanthan for its radical scavenging activity.

The radical scavenging activity of xanthan was tested by DPPH assay. The change in colour from purple to yellow was seen where the standard used was ascorbic acid and absorbance was taken in uv spectrophotometer at 517nm.As the concentration of the extracted X6 xanthan was not known it was diluted with D/W in 3 different ratios.

table 2: Radical scavenging activity of x6 xanthan at different dilutions by dpph assay

Sr.no	Diluted X6 xanthan ratios	Absorbance at 517nm	% radical scavenging activity of xanthan
1.	Unknown (1:2)	0.246	43.86%
2.	Unknown (1:5)	0.236	40.77%
3.	Unknown (1:10)	0.189	26.21%

Characterization of X10 xanthomonadin

A.Extraction of xanthomonadin

The pigment X10 xanthomonadin was extracted using methanol as a solvent and the extracted xanthan was present in crude form.

B.Absorption spectra of xanthomonadin

The absorption spectra of methanolic extract of xanthomonadin was studied. The lambda max was found to be 440nm where the absorbance was 0.477 units respectively, as expected for xanthomonadin.

graph 1 : absorption spectra of methanolic extract of X10 xanthomonadin.

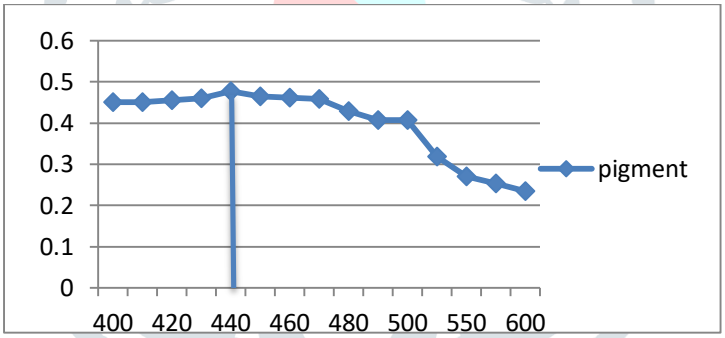

Fig.5:Extracted Xanthomonadin Pigment

Discussion

The extraction and characterization of xanthanand the Xanthomonadin pigment from isolated*Xanthomonas* spp. was carried out.The isolation of *Xanthomonas* was done using glucose yeast calcium carbonate agar(GC) and 16 different isolated spp. were obtained which were nomenclature as X1-X16, which included both spp. isolated from cabbage and cauliflower. The specific characteristics which Xanthomonas should possessis that Gram negative, catalase positive and oxidase negative depending on these characteristics isolates were obtained other biochemicals test were also done such as IMViC which is specifically done for Gram negative organism and sugar fermentation test was done however the results obtained were variable these results were then compared and analysed using Bergey's manual of biotechnology 2010.For xanthan production X6 isolate was used and for pigment production X10 isolate was used which gave characteristic coloured pigment. The yield of extracted xanthan was found to be 18.2gm/L of production media, as compared with other research papers the yield obtained was 6.3gm/L, however the parameters for production were kept similar. The xanthan also has anti-oxidant activity which was detected using DPPH assay which was found to be 43.86% at 1:5 dilution of extracted xanthan.Characterization of xanthan was detected using various qualitative test such as Osazone test which detects the presence of mannose residue which is determined by appearance of broomstick like crystals when seen under microscope, also Molisch test and Anthrone test was done to determine the presence of carbohydrates which gave the results as expected for Xanthan.Characterization of pigment was done using absorption spectra and the lambda max obtained was 440nm however the standard lambda max for xanthomonadin is 441nm.

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