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ASSESSMENT OF ENZYMATIC ACTIVITY OF *XANTHOMONAS CAMPESTRIS PV. VITICOLA* CAUSING BACTERIAL LEAF SPOT OF GRAPES IN COMPARISON TO OTHER PHYTOPATHOGENIC *XANTHOMONAS* SP.

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Abstract–*Xanthomonas campestris pv. viticola* is the causal agent of bacterial leaf spot disease of grapes. This study describes the screening and assay of extracellular enzymes produced by the pathogen namely amylase, cellulase, protease lipase and β-1, 3 glucanase. The enzyme producing capacity of *Xanthomonas campestris pv. viticola* was assessed along with five other *Xanthomons* sp. All the *Xanthomons* sp. were found to produce protease, amylase, cellulase and lipase but their quantity varied. Xcv had shown highest protease, lipase and amylase activity whereas lowest cellulase activity with respect to other *Xanthomons* sp. β -1, 3 glucanase activity was significantly similar in all *Xanthomons* sp.

INTRODUCTION

Grape (*Vitis vinifera*) is an important horticultural crop of importance in the arena of Indian agriculture. Grape has attained industrial significance due to development of wine and raisin production of 2951 Thousand MT (Anonymous, 2019) in India. Grape is a good source of carbohydrates, proteins, and fat. Additionally, the grape contains significant amounts of potassium, vitamin C, and vitamin A and also has a small amount of calcium and phosphorus (Yadav *et al*., 2009). Antimicrobial activity has been reported in several components of grapes including gallic acid, hydroxyl cinnamic acids, flavanols, trans-resveratrol and tannins (Ciafardini *et al*., 2003). Grapes seed extract have also shown anti-viral, anti-carcinogenic and anti-diabetic properties (Kanagarla *et al*., 2012).

Bacterial leaf spot of grapes caused by *Xanthomonas campestris pv. viticola* is an important disease which was noticed for the first time on *Vitis vinifera* cv. Anab-e-shahi at Tirupati (Andhra Pradesh) during 1960 (Nayudu, 1972). The disease is identified by minute water soaked, angular leaf spot which later turns to irregular dark brown to black necrotic region on the leaf surface (Kamble *et al*., 2019).

The pathogenicity of the bacteria depends upon different types of enzymes produced to cleave the heterogenous cell wall of the host which is composed of cellulose, lignin, starch, xylan and pectic substances. Different enzymes like polygalacturonase, cellulase, xylanase and laccase are required to degrade different components of plant cell wall (Cairney *et al*., 1994; Goodenough *et al*., 1991) so as to enable the pathogen to enter the host cells and cause the disease. For degrading plant biomass and tissue establishment, major enzymes required are ligninases, hemicellulases, cellulases, pectinases, xylanases, amylases and proteases (Saparrat *et al*., 2002; Sohail *et al*., 2009). Enzymes released from *Xanthomonas* sp. are potential

enhancers of both the pathogenicity and virulence of the pathogen.

Keeping in view the importance of enzymes, the current study was undertaken to find the quantitative and qualitative activity of different enzymes such as amylase, cellulase, protease, lipase and β-1,3 glucanase of different isolates of *Xanthomonas* sp. with respect to *Xanthomonas campestris pv. viticola*.

MATERIALS AND METHODS

Collection of samples and isolation of pathogen

The diseased samples infected with bacterial leaf spot were collected from Sangli, Maharashtra during November 2017. *Xanthomonas campestris* pv. *viticola* was isolated, purified and stored at -4°C for future use.

Five phytopathogenic *Xanthomonas* namely *Xanthomonas campestris pv. campestris* (*Xcc*, black rot of crucifers), *Xanthomonas campestris pv. citri* (*Xct*, citrus canker), *Xanthomonas oryzae pv. oryzae* (*Xoo*, bacterial blight of rice), *Xanthomonas campestris pv. punicae* (*Xcp*, bacterial spot of pea), *Xanthomonas axonopodis pv. malvacearum* (*Xam*, bacterial blight of cotton), along with the test bacterium *Xanthomonas campestris pv. viticola* (*Xcv*, bacterial blight of grape) were collected from Plant Pathology Laboratory of ICAR- National Research Centre for Grapes, Pune for the study.

Inoculation of samples

The cultures of six *Xanthomonas* isolates were maintained on nutrient agar (NA) plates. A loopfull culture from 24 hour old culture growing on NA at 28 ± 0.5 °C was used for the studies.

The minimal synthetic medium (MSM) was supplemented with the enzyme substrate as required. Qualitative screening was done on solid medium which was spot inoculated and incubated at 28 ± 0.5 °C.

Quantitative estimation was done in liquid medium in tubes. 24 hour old loopful culture was added in tubes containing 10 ml of the production medium for each enzyme. Tubes were incubated at 30 °C for 3 day in shaking water bath at 150 rpm. Three replicates for each isolate were maintained.

Qualitative screening

Different strains of *Xanthomonas* were screened for amylase, cellulase, protease, lipase as follow:

Cellulase

Cellulase was screened according to Islam and Roy (2018) by growing the culture on cellulase basal medium and incubated at 30 °C. After 48 hrs of incubation, the plates were flooded with 0.1% aqueous Congo red and destained with 1M sodium chloride for 15 min. Clear zone formed surrounding the colony indicated cellulase activity.

Amylase

As per Shanmugasundaram (2015) a culture was grown on starch agar media (Himedia) which was prepared as per the manufacturer's instructions and incubated at 30 °C. After 48 hours incubation the plates were flooded with iodine solution and clear zone of hydrolysis around the colonies indicated positive result.

Protease

According to Alnahdi (2012), bacterial culture was spot inoculated on skimmed milk agar medium prepared by dissolving 1.5g of skim milk powder and 2.0 g agar in 100 ml of distilled water and incubated at 30 °C. After 48 hours incubation the plates were observed for clear zone. Clear zone around the colony was taken as an indication of protease production by the isolate.

Lipase

Bacterial culture was spot innoculated on Tributyrin agar media and incubated at 30 °C as described by Kumar *et al.*, 2012. After 48 hours incubation the plates were observed for clear zone. Clear zone around the colony was taken as indication of lipase production by the isolate.

Quantitative screening

Cellulase

Enzyme production was carried out in cellulase basal broth as per the protocol of Islam and Roy (2018) with minor modification. After incubation, cells were removed from the culture broth by centrifugation at 12,000×g for 20 min. Clear supernatant were stored at -20 °C until estimation.

The Carboxymethylcellulase activity was measured by mixing 0.1ml of supernatant with 0.1ml of 1.0% (w/v) Carboxymethylcellulose in 10mM sodium phosphate buffer, pH 6.5 at 50 °C for 20 min. The reaction was stopped by adding 3,5dinitrosalicylic acid (DNS) reagent. The mixture was boiled for 10 min in boiling water bath and then

cooled in ice. Distilled water (1ml) was added in it for dilution. Optical density was taken at 550 nm in UV- spectrophotometer. The Carboxymethyl cellulase activity was measured by using a calibration curve for glucose.

2. β-1, 3 glucanase

The minimal synthetic media (MSM) supplemented with 0.5% laminarin, adjusted to pH at 7.0 used for analysis of β-1, 3glucanase production as per the protocol of Park *et al.* (2012). A 10 ml aliquot of the medium was dispensed in tubes and autoclaved at 121.5 °C for 15 min. After incubation, the culture was filtered and the filtrate was centrifuged at 4 °C for 10min at 5000×g to obtain the supernatant for testing enzyme activity. β-1,3 glucanase was assayed by using 500 µl of 0.5% laminarin in 50 mM acetate buffer (pH=4.8) with 200 µl of supernatant at 45 °C for 30 min. After 30 min 1ml of DNS reagent was added which was diluted with 2 ml of distilled water. Optical density was taken at 540nm in UV-spectrophotometer. The amount of reducing sugar released was calculated from standard curve for glucose. The amount of β-1, 3 glucanase activity was calculated from standard curve for glucose.

Protease

Enzyme production was carried out in mineral salt medium as stated by Alnahdi (2012). After incubation, cells were removed from the culture broth by centrifugation at 5000 rpm for 20 min at 4 °C to obtain the cell free supernatant (CFS) to be used for further assay. The protease activity was estimated by the method described by Beg *et al.* (2003). 1 ml of cell free supernatant (CFS) was added to 1 ml of 1% (w/v) casein solution in glycine-sodiun hydroxide buffer of pH 10.5 and incubated for 10 min at 60 °C. The reaction was stopped by adding of 4 ml of 5% trichloroacetic acid. The reaction mixture was centrifuged at 3000 rpm for 10 min and to 1mL of the supernatant 5 ml of 0.4M sodium carbonate was added followed by addition of 0.5ml Folins – ciocalteu reagent. The amount of tyrosine released was determined by using UV – VIS spectrophotometer at 660nm.

Amylase

The six *Xanthomonas* isolates were grown in nutrient broth liquid media containing 1% yeast extract, 0.5% Peptone, 0.5% sodium chloride (w/v) at pH 6.0. The supernatant of the culture was obtained after centrifugation at 10,000 rpm for 10min at 4°C which was used to determine amylase activity. Amylase activity was determined by the procedure of Bernfeld (1955) using soluble starch as a substrate. The reaction mixture containing 200 µl of 1% substrate (w/v) in 0.1M phosphate buffer (pH-7.0) and 150 µl of enzyme solution was incubated for 30 min at 37 °C. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min and cooling at room temperature. To the solution 8 ml of de-ionized water was added. Absorbance of each solution containing the brown reducing product was measured at 489 nm in a UV-spectrophotometer. The amount of amylase released was calculated from standard curve for glucose

Lipase assay

Lipase activity was determined by the procedure of Mahadik *et al.* (2002) and was measured by mixing 0.1ml of enzyme solution with 0.9 ml of p-nitrophenylpalmitate (PNPP) containing emulsion. The solution was incubated for 30 min at 37 °C. The reaction was stopped by adding 1M sodium carbonate. Optical density was taken at 410 nm in UV- spectrophotometer. The lipase activity was measured by using a calibration curve for PNPP.

Determination of protein content

Total soluble protein content of each of the 5 enzyme samples was determined by the Lowry's method (1951) using crystalline bovine serum albumin as standard. Enzyme activity has been expressed in International Units (U) viz., the amount of the enzyme that liberated 1 µmol of substrate equivalent per minute under the assay conditions. Values given are mean ± standard error (SE), derived from the triplicate samples. In all cases the UV-spectrophotometer used was of Thermo Spectronic Helios Alpha.

Statistical analysis

The collected data was statistically analyzed and subjected to analysis of variance (ANOVA) by using statistical analysis software package (SAS, 2002).

RESULTS

Qualitative analysis of enzymes

By measuring the colony diameter and zone of clearance obtained after incubation, it was found that highest cellulase enzyme production was

exhibited by *Xcv* wheras other manifested negative response (Fig.1A). The intermediate protease enzyme production was observed in *Xcv, Xct* and *Xcp* whereas *Xam* had highest clearance zone. *Xoo* did not show any zone of clearance (Fig.1B). Amylase production was screened positive through large zone of clearance by all *Xanthomonas* sp. except *Xcp*. *Xcv* showed large zone of clearance whereas *Xoo, Xcc, Xam* and *Xct* manifested an intermediate response for amylase enzyme (Fig.1C). *Xcv* and *Xoo* exhibited large zone of clearance for lipase production. *Xam* and *Xcp* showed intermediate zone whereas *Xct* and *Xcc* did not show zone for lipase enzyme, signifying a negative response (Fig.1D).

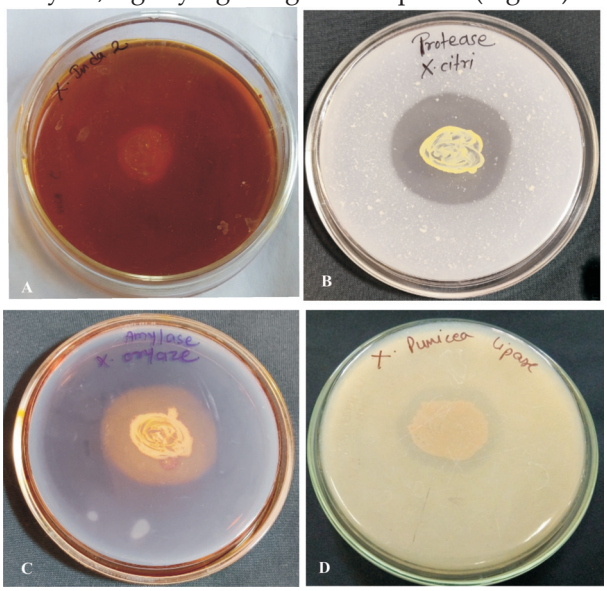


Fig. 1. Zone of clearance around *Xanthomonas* colonies indicates enzyme production. (A) Cellulase (B) Protease (C) Amylase (D) Lipase

Quantitative analysis of enzymes

Protease

The highest protease activity of 31.85U/ml/min was observed in *Xcv* followed by 30.57U/ml/min in *Xam*whereas lowest protease activity of 12.33U/ml/min was observed in *Xcc.* Protease activity of 16.37, 15.34 and 15.09U/ml/min was shown by *Xoo*, *Xcp* and *Xct* respectively (Table 1).

Cellulase

Xcv showed lowest cellulase activity (12.32U/ml/min) as compared to *Xct* which had highest cellulase activity (32.93U/ml/min). *Xoo, Xam*, *Xcc* and *Xcp* showed enzyme activity of 27.78, 23.34, 21.83 and 19.19U/ml/min respectively (Table 1).

Amylase

Xcv showed higher amylase enzyme (0.59U/ml/min) followed by *Xcc* and *Xcp* (0.58U/ml/min). *Xoo*, *Xam* and *Xct* showed lower amylase activity (0.57U/ml/min) (Table 1).

Lipase

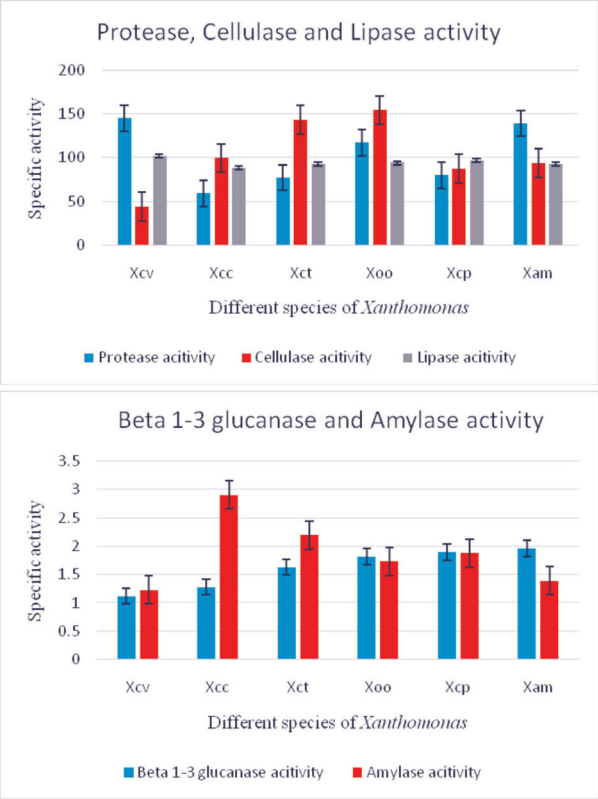
Highest lipase activity was observed in *Xcv* (2.14U/ml/min) followed by *Xcp* (2.13U/ml/min) whereas *Xcc* showed lowest lipase activity (1.94U/ml/min). However no significant difference was observed in lipase activity of *Xam* and *Xct* (2.04U/ml/min) (Table 1).

β-1,3 glucanase

Xcv showed β-1,3 glucanase activity of 0.65U/ml/min. However, no significant difference was

Table 1. Enzyme activity of different isolates of Xanthomonas sp.

Sr no.		Xcv	Xcc	Xct	Xoo	Xcp	Xam
1	Protease acitivity (U/ml/min)	31.85	12.33	15.34	16.37	15.09	30.57
	Total protein (mg/l)	0.22	0.21	0.20	0.14	0.19	0.22
	Specific activity	144.79	58.71	76.72	116.92	79.42	138.95
2	Beta 1-3 glucanase acitivity (U/ml/min)	0.65	0.63	0.67	0.62	0.61	0.61
	Total protein (mg/l)	0.58	0.49	0.41	0.34	0.32	0.31
	Specific activity	1.12	1.28	1.63	1.82	1.90	1.96
3	Cellulase acitivity (U/ml/min)	12.32	21.83	32.93	27.78	19.19	24.34
	Total protein (mg/l)	0.28	0.22	0.23	0.18	0.22	0.26
	Specific activity	44.00	99.23	143.17	154.33	87.22	93.61
4	Lipase acitivity (U/ml/min)	2.14	1.94	2.04	1.97	2.13	2.04
	Total protein (mg/l)	0.021	0.022	0.022	0.021	0.022	0.022
	Specific activity	101.90	88.18	92.72	93.80	96.81	92.72
5	Amylase acitivity (U/ml/min)	0.59	0.58	0.57	0.57	0.58	0.57
	Total protein (mg/l)	0.48	0.20	0.26	0.33	0.31	0.41
	Specific activity	1.23	2.90	2.19	1.73	1.87	1.39



Graph 1. Comparative analysis of specific activity between different *Xanthomonas* sp.

observed between different *Xanthomonas* sp. (Table 1).

DISCUSSION

Plant pathogen produces an array of enzymes capable of attacking plant cell components. Each enzyme is coded by a different gene (Goodman *et al.*, 1986) for maximum pathogenic potential. It is important for invading bacteria to colonize tissue rapidly and reach high population levels before growth (Eve Bi'lling, 1987). Gram negative plant pathogenic bacteria secrete a wide range of plant cell wall degrading enzymes such as cellulases, amylases, lipases, proteases and beta 1-3 glucanases, as a part of virulence (Kang *et al.*, 1994; Jha *et al.*, 2005). In the present study the production of cellulase, amylase, protease, lipases and beta 1-3 glucanase were studied with different phytopathogenic *Xanthomonas*. *Xanthomonas sp.* employ a suite of virulence factors to colonize plant tissues, including adhesins, cell wall-degrading enzymes, extracellular polysaccharide and protein secretion systems (Büttner and Bonas, 2010).

Severin *et al.* (1985) reported that *Erwinia carotovora pv. carotovora*, *E. c. ssp. atroseptica*, *Erwinia chrysanthemi* ssp. *chrysanthemi* and *Xanthomonas campestris pv. pelargonii* (the causal pathogens of soft-rot of potato, dahlia and pelargonium, respectively) were able to produce pectinase and cellulase enzymes. Xylanolytic and cellulolytic enzymes were produced by various*Xanthomonas axonopodis pv. punicae* strain (Amat *et al.*, 2014). Goto *et al.* (1959) confirmed the production of cellulase in all *Xanthomonas* spp., *E. carotovora*, *E. milletiae*, *Corynebacterium sepedonicum*. Current study also shows production of cellulase enzyme in all the *Xanthomonas* sp. Comparatively low cellulase activity was observed in *Xanthomonas pv viticola* than the other *Xanthomonas* sp.

Verma (1986), reported that all pathogenic strains of *Xcm* produce appreciable amount of extracellular proteases. Multiple proteases with different patterns of peptide bond cleavage had also been reported in *Xcm* (Gholson *et al.*, 1989). Xu and Gonzalez (1989) reported that an extracellular protease isolated from *Xanthomonas campestris pv. oryzae* might play an active role in leaf blight of rice. Tang (1989), demonstrated that for normal pathogenicity to plant *Xcc* produced protease enzyme. Also, two extracellular proteases which have been purified from *Xcc*, the causal agent of black rot disease of cruciferous plants, were suggested to have a role in black rot pathogenesis (Dow *et al.*, 1990). In the current investigation protease activity was observed in all *Xanthomonas* sp. However, *Xcv* showed highest protease activity followed by *Xam*. This manifested the importance of protease enzyme in pathogenesis which is similar to the study reported by Keen *et al.* (1969), where in *Pseudomonas lachrymans* produced caseinolytic protease during pathogenesis in cucumber. Increased protease activity was also observed in tobacco tissue infected by *Agrobacterium tumefaciens*. Reddy *et al.* (1969), reported that protease of *Xanthomonas alfalfae* played a important role during pathogenesis

A direct influence on the plant–pathogen interaction was shown for endoglucanase and polygalacturonases from *Xcc* and for lipase/esterase, cellulase, endoglucanase and xylanase from *Xoo* (Rajeshwari *et al.*, 2005; Hu *et al.*, 2007; Jha *et al.*, 2007). Qiurun *et al.* (2016), purified the lipase from *Xoo* YB103 (LipXO) and reported the specific activity of 373.9 U/mg. In present study lower lipase specific activity (92.64U/mg) of *Xoo* was observed. However, highest lipase specific activity was

observed in *Xcp* (97.03U/mg) followed by *Xcv* (96.69U/mg). Sole *et al*. (2021), reported seventeen proteins including proteases, xylanases, and the lipase XCV0536 (also referred to as LipA), which were previously identified as a virulence factor of *X. campestris pv. vesicatoria.* Aparna *et al.* (2019), reported LipA as a cell wall-degrading enzyme with a carbohydrate-binding domain essential to the protein's virulence function from *Xoo*.

Extracellular enzymes such as xylanase, endoglucanase, amylase and protease, contributed individually or collectively to the virulence of *Xanthomonas* (Chatterjee *et al*., 2003; Hu *et al*., 2007). Screening the transposon-based mutant library of *Xcc* revealed that a mutation of XAC0798, which encoded an α-amylase, affected virulence and induced an enhanced hypersensitive-like response (Laia *et al*., 2009). Tang *et al.* (2020) reported that amyA*Xcc* is responsible for the extracellular amylase activity of *Xcc*, and indicated that extracellular amylase played an important role in *Xcc* virulence. In present study all *Xanthomonas* has produced amylase. High enzyme activity was observed in *Xcv* followed by others. Beta 1-3 glucanase activity was similar in all the *Xanthomonas* sp including *Xcv*.

It was observed that enzyme activity of cellulase, lipase and protease were significantly higher than the amylase and beta 1-3 glucanases in all the *Xanthomonas sp.* It was also deduced that *Xcv* had showed highest protease, lipase and amylase activity with respect to other *Xanthomonas* sp. Further purification and molecular studies on cell wall degrading enzymes in different plant-pathogenic bacteria can be done.

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