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EFFECTIVENESS OF YSCNA MODIFIED CULTURE MEDIUM FOR LUXURIOUS GROWTH OF *XANTHOMONAS ORYZAE* PV. *ORYZAE* ASSOCIATED WITH RICE PLANTS

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A B S T R A C T

In vitro studies were carried out for the isolation, identification, biochemical, cultural and physiological characterization of *Xanthomonas oryzae* pv. *oryzae.* Biochemical and diagnostic tests proved *X. oryzae* pv. *oryzae* as a gram negative bacterium that showed luxurious growth on a new modified yeast sucrose calcium carbonate nutrient agar (YSCNA) medium with maximum average recovery of (83.50). The bacterium produced bright yellow, circular and slightly raised bacterial colonies on tested media compared to previously tested media used for the isolation and multiplication. The pathogen showed best growth at 30°C and pH of 7.0 with mean (94.50) and (103.75) bacterial colonies.

Keywords: YSCNA medium, Biochemical, Cultural, Physiological, Bacterial leaf blight.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* causes bacterial leaf blight (BLB) of rice throughout the rice cultivating areas of the world (Mew, 1987). The pathogen has been detected and characterized by the polymerase chain reaction (PCR) from rice plants (Swings *et al*., 1990). The pathogen is transmitted by irrigation water and rain splashes in the field; survives in weeds of *Leersia haxandra*, and disease control demands timely application of bactericides and weedicides to decrease inoculum source and planting of healthy young rice plants (Naqvi *et al*., 2015). Since the description of *X. oryzae* pv. *oryzae* as the causal agent of BLB, several studies focusing on the disease epidemiology, plant pathogen interaction and its characterization have been conducted so far for the management of the disease (Khan *et al*., 2009). Although, PCR is mostly used for the detection of pathogens in plants but PCR is not capable to answer questions that express quantification of bacterial cells in plants (Lopes *et al*., 2000). The traditional isolation and culturing on agar plates not only allows the pathogen quantification but also for its cultural, physiological and biochemical studies. Nutrient agar is a general purpose medium used for the isolation and culturing of microbes in question. During current study, a nutritionally complex culture medium was developed containing well defined chemical components in very small amounts for the isolation and culturing of *X. oryzae* pv. *oryzae*. During the observations of bacterial colonies grown in petri plates, we performed modifications in the routinely used culture media and a new modified medium YSCNA was developed. The final version yeast sucrose calcium carbonate nutrient agar medium was found most effective than the rest of the medias in question regarding the bacterial colonies, rapid growth and for the pathological studies. Present study was undertaken under following objectives (1) to evaluate various media for their efficacy to support the growth of *X. oryzae* pv. *oryzae*, (2) to perform some biochemical tests on isolated strains of the bacterium in question, (3) to conduct cultural and physiological studies for the isolated bacterium under controlled conditions.

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MATERIAL AND METHODS

Study site: Present study was conducted at Phytobacteriology and Biochemical analysis Lab, Department of Plant Pathology, Bahauddin Zakariya University, Multan.

BACTERIAL ISOLATION AND PRESERVATION

Rice plants leaves showing the characteristic symptoms of bacterial leaf blight (yellow to white water soaked stripes with wavy margins at the edges and on the leaf blade)

were examined on visual basis and collected from the experimental farm of University, kept in plastic bags and brought to laboratory. For isolation, rice leaves were disinfected with 10% ethanol, washed thrice with sterilized water and placed on sterilized petri plates containing Nutrient Agar medium (Bio Basic Inc. Canada) followed by incubation at 28±2°C for 24hrs. Bright yellow, circular and viscous colonies of the bacterium (*X. oryzae* pv. *oryzae*) appeared in petri plates and re-cultured on fresh NA plates (Dye *et al*., 1974). The pathogen was preserved in 40% glycerol solution in sterilized distilled water for the recovery of 16S rDNA in vials at -20°C.

***In vitro* studies**

Identification of pathogen through biochemical and diagnostic tests: For gram staining, bacterial culture smear was prepared in the form of a thin film on a glass slide and treated with 0.5% aqueous crystal violet solution (BDH, UK) for 60 seconds, lugol's iodine solution (Sigma Aldrich, USA) for 40 seconds and counter stained with 10% fuchsine solution of safranin (Bio Basic Inc. Canada) for 60 seconds. For potassium hydroxide (KOH) test, bacterial suspension was smeared on glass slide as described by Ryu (1940). The starch hydrolysis test was carried out by making a homogenous mixture of nutrient agar and starch in distilled water, bacterial culture was transferred aseptically into petri plates and incubated at 28±2°C for seven days. After removing the superfluous bacterial growth, the plates were flooded with Lugol's iodine solution (Cowan, 1974). For lecithinase test, fresh egg yolk emulsion (1.5ml) was prepared and dissolved in nutrient agar as mentioned by McClung and Tobae (1947); while for oxidase assay, 48hrs old bacterial culture supplemented with 3% glucose was manipulated on filter paper, soaked with (1% w/v) freshly prepared substrate solution of tetramethyl-p-phenylenediamine dihydrochloride as described by Kovaes (1956). The pathogenic nature of the bacterium was studied on *Oryza sativa* plants under field and glass house conditions for hyper sensitivity reaction (Naqvi *et al*., 2015).

Cultural and physiological studies of *X. oryzae* pv. *oryzae*

Modifications of YSCNA medium: YSCNA (Yeast sucrose calcium carbonate nutrient agar medium) is composed of $CaCO_3$ 20.0g, yeast extract 10.0g, glucose 1.0g, NaCl 5.0g, sucrose 20.0g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.25g, peptone 5.0g, beef extract 3.0g and agar 15g in one liter of distilled water and autoclaved. In contrast, 1 L of the final version of NA (Nutrient agar) contains beef extract 3.0g, peptone 5.0g and agar 15g; for PSA (Peptone sucrose agar) sucrose 20.0g, peptone 5.0g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.25g and agar 12.0g; similarly for P-agar (Peptone agar) NaCl 5.0g, peptone 10.0g, yeast extract 5.0g, glucose 2.0g and agar 15g; for YNA (Yeast nutrient agar) yeast extract 10.0g, peptone 5.0g, beef extract 3.0g and agar 15g; for YDC (Yeast dextrose calcium carbonate) calcium carbonate 20.0g, glucose 20.0g, yeast extract 10.0g, NaCl 2.50g and agar 15g. The final pH was adjusted to 7.4 with 1N sodium hydroxide solution (NaOH) before autoclaving.

Growth characteristics of pathogen on different solid agar media: Various differential agar media *viz*., nutrient agar, peptone sucrose agar, yeast nutrient agar, yeast dextrose calcium carbonate agar, P-agar and a new modified yeast sucrose calcium carbonate nutrient agar medium were used to investigate physiological characteristics of the pathogen. A loopful of 72hrs old culture of bacteria was serially diluted and 500µl of bacterial solution from 10^5 dilutions was added in each petri plate on surface of medium, which was uniformly spread with L shape spreader to obtain well separated bacterial colonies followed by incubation at 28±2°C in an incubator for 72hrs. Observations were recorded for colony size, number, color, shape and appearance.

Effect of various temperature, pH levels and light on growth of the pathogen: The investigations were undertaken to determine the optimum temperature, effect of hydrogen ion concentration and to assess the effect of light, darkness and alternate light on the growth of *X. oryzae* pv. *oryzae*. as described above. Inoculated petri plates were incubated at 0, 10, 20, 30, 40 and 50°C temperature, while nutrient agar medium was adjusted to different pH levels viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 either by adding 1N (NaOH) alkali or by concentrated hydrochloric acid (HCl) followed by incubation 28±2°C for 72hrs. The effect of light/ darkness and in alternate light (16hrs light and 8hrs darkness) was assessed by incubating petri plates at 28±2°C for same period of time in light as mentioned above. Observations were taken for number of colonies in petri plates incubated at different temperatures, pH levels, and in light/ dark, bacterial colonies were counted, recorded and analyzed (Schaad and Stalk, 1988).

STATISTICAL ANALYSIS

The collected datasets were statistically analyzed subjecting them to analysis of variance (ANOVA) by least significant difference test (LSD) at ($P \leq 0.05$) using SAS

(Statistical analysis system, Version.9.2, SAS Institute, Cary, NC).

RESULTS

Pathogen isolation: The bacterium produced light yellow, raised and circular colonies on the medium (Figure 1).

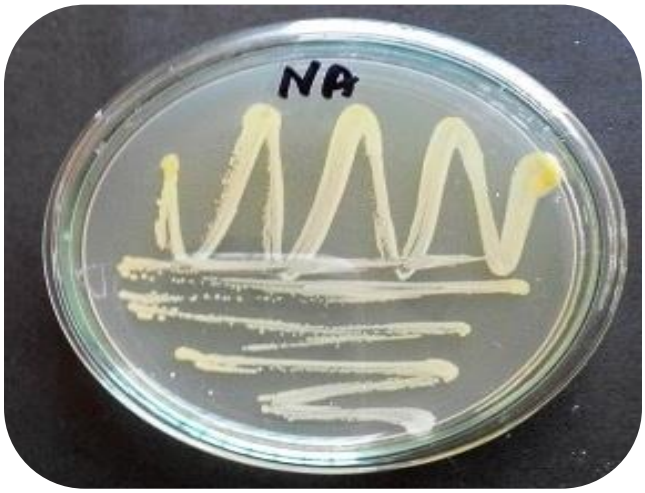

Figure 1. Growth of *Xanthomonas oryzae* pv. *oryzae* on nutrient agar medium.

Biochemical tests and hypersensitivity reaction: A total of five biochemical tests were performed to study the biochemical characteristics of isolated bacterium, results of the gram reaction (gram staining) proved that bacterium was gram negative, rod, produced red color when counter stained with safranin. Potassium hydroxide (KOH) test was performed to confirm gram reaction results, which showed positive reaction and the development of mucoid thread on the glass slide confirmed the bacterium as gram negative. Lecithinase test was also positive resulting in an opaque halo surrounding the colony when grown on the egg yolk agar medium proved the activity of bacterial lecithinases to break down lecithin in egg yolk to insoluble diglycerides. Starch hydrolysis test was also positive that may be possibly due to the fact that the bacterium used starch (a complex carbohydrate made from glucose) as a source of carbon and energy for growth, accomplished by an enzyme i.e. alpha-amylase. Oxidase test was negative due to absence of color development on the filter paper moistened with 1% tetramethyl-p-phenylenediamine dihydrochloride within ten seconds of reaction which identified the bacterium not to produce cytochrome-c-oxidase enzyme of the bacterial electron transport chain. The bacterium showed positive hypersensitive reaction on tobacco leaves (*Nicotiana tobaccum*), which can only be induced by the phyotpathogenic bacteria by the tissue collapse and complete necrosis within 24 to 48 hrs of injection (Table 1).

Table 1. Biochemical and diagnostic tests for *X. oryzae* pv. *oryzae* from rice plant.

Pathogen	Biochemical tests					Diagnostic test
	Gram Staining	KOH[a]	Lecithinase[b]	Starch Hydrolysis	Oxidase	Hypersensitivity reaction
Xanthomonas oryzae pv. *oryzae*	–	+	+	+	–	+

a= Potassium hydroxide test, b= An enzyme found in bacterium which degrade lecithin (a normal component of the egg yolk)

Cultural and physiological studies:

Out of six different media tested for the growth of *X. oryzae* pv. *oryzae*, a new modified YSCNA medium significantly showed tremendous growth of the pathogen as obvious by the maximum recovery of colonies of the bacteria (83.50×10^5cfu/ml). NA was 2nd best supporting medium with (78.75×10^5cfu/ml) bacterial colonies followed by YDCA medium with (74.56×10^5cfu/ml) recovery of bacterial colonies. Moderate growth of bacterium was observed on P-Agar and YNA with (69.25×10^5cfu/ml) and (62.50×10^5cfu/ml) bacterial colonies respectively. Significantly least number of bacterial colonies (42.75×10^5cfu/ml) was counted on PSA as pathogen grew scantily on the medium. Present study showed that modified yeast sucrose calcium carbonate nutrient agar medium (YSCNA), the best medium which was amended with Sucrose, glucose, peptone and calcium carbonate as the carbon sources. Colony diameter of *X. oryzae* pv. *oryzae* on various solid agar based media were recorded in the range of 1.0-to-5.2mm, maximum diameter of the colony 3.60mm was observed on modified YSCNA medium followed by 3.40mm on NA while minimum diameter of colony was measured in P-agar (2.60mm). Colonies of bacterium *X. oryzae* pv. *oryzae* were mostly circular, flattened, slightly raised and glistening with light yellow to bright yellow color on all tested media (Table 2).

Table 2. Cultural characteristics and colonial morphology of *X. oryzae* pv. *oryzae* on various solid agar media.

Media	Colonies of bacterium (10^5cfu/ml)	Size of the colony range (mm)	Average size of the colony (mm)	Colony characters		
				Color	Shape	Appearance
NA	78.75 ± 1.02 ab(8.97)[a]	2.0-5.0	3.40	Bright yellow to yellow	Circular	Flattened
PSA	42.75 ± 1.50 e(6.71)	1.0-5.0	3.00	Light yellow to yellow	Irregular	Slimy, Glistening
YNA	62.50 ± 2.65 d(7.39)	1.0-4.0	3.10	Light yellow	Irregular/ Circular	Flattened, Slimy, Glistening
YDCA	74.50 ± 2.22 bc(8.47)	1.0-4.0	2.80	Bright yellow	Circular	Slightly raised, Flattened
P-Agar	69.25 ± 7.97 c(7.97)	1.0-4.0	2.60	Light yellow to yellow	Circular/ Irregular	Flattened, Glistening
YSCNA (Modified)	83.50 ± 1.09 a(9.47)	2.2-5.2	3.60	Bright yellow	Circular	Flattened, Slightly Raised
*LSD	5.87	---	---	---	---	---

[a] Figures in the parentheses are √ x+1 transformed values, *LSD= Least significant difference

Effect of temperature, pH levels and light: Number of colonies of test pathogen increased when pH level increased from 4.00 to 7.00 and decreased with the further increase in the pH level. Bacterial colonies recorded at pH 7.00 were significantly greater (94.50×10^5cfu/ml) as compared to other pH levels. Least number of bacterial colonies was recorded at pH level of 4.00 and 9.00 with (10.50×10^5cfu/ml) and (16.75×10^5cfu/ml), while the pathogen failed to nurture at extreme pH levels of 10.00 and 3.00 (Table 4). Pathogen growth on various temperature levels showed that maximum number of colonies (103.75×10^5cfu/ml) were obtained at 30°C and the least number of colonies (12.75×10^5cfu/ml) were recorded at 40°C whereas the pathogen failed to grow at extreme temperature levels of 0°C, 10°C and 50°C (Table 4). Findings of light and darkness effect on colony growth of bacterium showed that maximum number of colonies (87.75×10^5cfu/ml) were observed in darkness followed by (76.75×10^5cfu/ml) colonies recorded in alternate light i.e., (16hrs light and 8hrs dark) while, relatively lesser number of colonies were calculated in petri plates being kept in light all the time (Table 3).

Table 3. Effect of pH, temperature and light/ dark on the growth of *X. oryzae* pv. *Oryzae.*

pH levels	Colonies of bacteria (10^5cfu/ml)	Temperature levels (°C)	Colonies of the bacteria (10^5 cfu/ml)	Light/ Darkness	Colonies of the bacteria (10^5 cfu/ml)
3.00	0.00 ± 0.00 f(1.00)[a]	0.00	0.00 ± 0.00 d(1.00)	Light	70.25 ± 2.61 c(8.51)
4.00	10.50 ± 0.50 e(3.51)	10.00	1.00 ± 0.31 d(1.01)	Dark	87.75 ± 2.67 a(9.45)
5.00	14.25 ± 1.80 de(4.04)	20.00	63.50 ± 2.02 b(7.72)	Alternate light	76.75 ± 3.24 b(8.91)
6.00	40.25 ± 2.05 c(6.37)	30.00	103.75 ± 2.63 a(10.54)	---	---
7.00	94.50 ± 1.74 a(9.98)	40.00	12.75 ± 1.65 c(5.39)	---	---
8.00	66.25 ± 2.24 b(7.91)	50.00	0.00 ± 0.00 d(1.00)	---	---
9.00	16.75 ± 1.93 d(4.24)	---	---	---	---
10.00	0.00 ± 0.00 f(1.00)	---	---	---	---
LSD*	4.69	---	4.58	---	8.22

[a] Figures in the parentheses are √ x+1 transformed values, *LSD= Least significant difference

DISCUSSION

Xanthomonas oryzae pv. *oryzae* was characterized on the basis of biochemical tests and its growth as described by Isaka (1970). Colonies of *X. oryzae* pv. *oryzae* were recovered easily from the infected leaves rather than infected seeds due to the involvement of many other saprophytic bacteria and fungi in infected seeds (Di *et al*. 1991; Suslow *et al*. 1982). Our results are in line with the findings of the Suresh *et al*. (2013) and Khan *et al*. (2000) who also demonstrated the better isolation frequency of the bacterium from diseased leaves rather than any other part of the plant. The reason may be due

to association of saprophytic bacteria and fungi in contaminated seeds which make it impossible to obtain single colony of the bacterium on any synthetic artificial medium. We suggest that limitation of bacterial isolation from the diseased seeds might be possible if specific selective or the semi selective medium be utilized for this purpose rather than the differential medium as was used in our study. The results of gram reaction proved the bacterium as gram negative and KOH test counter signed the gram reaction results as mentioned by Suresh *et al*. (2013). In the present research results revealed that the modified YSCNA medium supported luxurious growth of pathogen as sucrose and glucose were found to be the best carbon sources followed by galactose, fructose, mannose and mannitole while starch, xylose and lactose were regarded as a poor source of carbon (Tanaka 1964; Tsuchiya *et al*. 1982). Our results coincide with Watanabe (1963); Suresh *et al.* (2013) and Rukhsana *et al*. (2012) who found glucose ($C_6H_{12}O_6$) and sucrose ($C_{12}H_{22}O_{11}$) as the most beneficial carbon sources yet we suggest that during medium preparation for bacterial growth weightage should be given to sucrose rather than glucose because surely it will double the carbon supply to bacterium as compared to glucose. The bacterium produced light yellow, circular dome shaped colonies on the medium, yellow color and mucoid colonies is the characteristic of *Xanthomonads* due to the presence of EPS (Extracellular polysaccharides) in sugar containing media (Gerhardt, 1981). Our results support the findings of Rukhsana *et al*. (2012) who reported that infected leaf samples of rice gave bright yellow, circular, viscous and smooth bacterial colonies when plated on YDCA medium. During present study, pH level of 7.0-to-7.5 best supported the growth of the *X. oryzae* pv. *oryzae* as demonstrated by (Cho, 1975; Gomathi, 1991; Thimmegowda, 2006). Dye and Lellliott (1974) reported 25°C to 27°C as optimum temperature required for growth of *X. oryzae* pv. *oryzae.* Our findings will be helpful for plant bacteriologists, microbiologists and agriculturists to study bacteria by utilizing cheaper sources.

CONCLUSION

It may be concluded from the results that *X*. *oryzae* pv. *oryzae* is a phytopathogenic bacterium having best growth on YSCNA medium. The temperature 30ºC and pH 7.0 in dark conditions best supported the recovery of maximum bacterial colonies.

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