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CHARACTERIZATION AND IDENTIFICATION OF *Xanthomonas* spp. ISOLATED FROM INFECTED BRASSICACEAE AND SELECTION OF POTENTIAL XANTHAN GUM PRODUCER

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ABSTRACT

Xanthomonas spp. synthesize a complex exopolysaccharide called xanthan gum, which has significant commercial value. *Xanthomonas*-infected vegetables exhibiting typical spot symptoms were used in this study. Isolation was done by streaking the sample suspension on the Yeast Malt agar. A colony that resembled *Xanthomonas* spp. was purified before a simple phenotypic test and identified using BIOLOG and 16 rRNA sequencing. The screening was done based on their performance by fermentation in a shake flask, under controlled conditions. The yield and viscosity of xanthan gum produced from each bacterium were compared to *Xanthomonas campestris* PV. *campestris* strain ATCC33913. There were 411 wild types of *Xanthomonas* spp. successfully isolated and ten strains were selected for xanthan gum evaluation. The results showed, the xanthan production (g/L) varied from (1.57 - 8.24) with the yield of xanthan from 0.64 to 4.71 g/g biomass. Strain C206 produced the highest xanthan gum concentration (8.24±0.20 g/L) compared to others and the control strain, ATCC 33913 (2.27±0.10g/L). The highest yield of xanthan 4.71±0.18 (g/g biomass) was produced by strain C298 followed by strain C279 with 3.88±0.04 (g/g biomass). From our investigation, the production and yield of xanthan gum and the viscosity of the polymers were significantly dependent on the bacterial strain. Based on the stable viscosity and yield of xanthan produced, *X. campestris* C279 was selected for further studies on product optimization.

Key words: Food component, heteropolysaccharide, pseudoplastic, viscosity, xanthan gum

INTRODUCTION

One of the most significant bacterial polysaccharides produced industrially is xanthan gum. This extracellular polysaccharide which has great commercial significance (Garcia-Ochoa *et al*., 2000) is produced by microbial fermentation by *Xanthomonas campestris* (Becker *et al*., 1998; Petri, 2015). Members of the Brassicaceae (Cruciferae) family, such as cauliflower, broccoli, and cabbage, are typically affected by black rots caused by the majority of *Xanthomonas* strains, which are phytopathogenic (Dzionek *et al*., 2022). Following infection with these bacteria, the plant will get defoliated, lose weight, and lose quality (KC *et al*., 2007; Sharma & Sharma, 2009). Tomato and cabbage crops around the world, especially in Africa have been severely damaged by *Xanthomonas* infection, which has become a major cause of agricultural destruction in some locations worldwide (Massomo *et al*., 2003; KC *et al*., 2007).

Most *Xanthomonas* strains can produce xanthan gum, whereas *Xanthomonas campestris* pv. *campestris* is the most efficient and most frequently used for the industrial production of xanthan gum (Moreira *et al*., 2001). The US Department of Agriculture's Northern Regional Research Laboratories (NRRL) made the initial discovery of xanthan gum in the 1950s (Garcia-Ochoa *et al*., 2000). The research was initiated on xanthan gum in 1961 when research laboratories of the US Department discovered that *Xanthomonas campestris* found on cabbages was able to produce specialized types of exopolysaccharide with unique rheological properties (Katzbauer, 1998; Dai *et al*., 2019).

Xanthomonas spp. are aerobic Gram-negative rod bacteria belonging to Xanthomonadaceae, from the gamma-proteobacteria. These rod-shaped bacteria were yellow-pigmented that can be cultured at different temperatures, ranging from 25 °C to 35 °C in neutral pH (Psomas *et al*., 2007). *Xanthomonas* are motile with single flagellum, catalase-positive, and oxidase-negative bacteria (Garcia-Ochoa *et al*., 2000). Xanthan gum is a heteropolysaccharide. Its primary structure consists of D-mannosyl, and D-glucuronyl acid residues I a molar ratio of 2:2:1 and variable

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proportions of O-acetyl and pyruvyl residues (Garcia-Ochoa *et al*., 2000).

Commercially, due to its superior properties, xanthan was widely used as a thickener, stabilizer, and emulsifier in different areas of industries, both food and non-food industries (Faria *et al*., 2010). It is reported that 30 000 tons of xanthan gum were produced annually worldwide, corresponding to $408 million of the market values (Silva *et al*., 2009), and approximately 5 million kg per year were used in the oil industry as a drilling fluid viscosifier (Yoo & Harcum, 1999).

Xanthan gum was largely employed in the pharmaceutical, cosmetic, paper, and oil sectors as gelling agents, flocculants, or viscosity-controlling agents (Becker *et al*., 1998). One of the most important unique properties of xanthan gum is it has a pseudoplastic characteristic, which is high viscosity at low shear that help to enhance the sensory qualities in the food products (Katzbauer, 1998). Besides, this unique polysaccharide also has high stability in a broad range of pH and temperature, and high solubility (Sharma *et al*., 2006). Xanthan gum is a non-Newtonian fluid, thus its viscosity is nearly unaffected by any temperatures, starting from the freezing point, up to the boiling point of pure water (Garcia-Ochoa *et al*., 2000). It is completely soluble in both hot and cold water, making it capable of providing high viscosity to the solution even at low concentrations. Used as a stabilizer for emulsions and suspension, a small concentration of xanthan gum is enough to confer the required properties without affecting the final food product (Sharma *et al*., 2006; Palaniraj & Jayaraman, 2011). Another important property of xanthan gum is resistance to enzymatic degradation by many enzymes such as cellulase, protease, amylase, and pectinase (Garcia-Ochoa *et al*., 2000).

Most of the previous studies reported that the yield and properties of xanthan gum are dependent on the strain used (Soudi *et al*., 2011; Demirci *et al*., 2017). Thus, isolation and identification of novel strains of *Xanthomonas* spp. are important to improve xanthan yield and its rheological quality (Torres & Galindo, 1997). The majority of the literature on the production of xanthan gum mostly used strains from a collection of cultures, mainly ATCC 33913 and their derivatives, and reports dealing with the isolation and characterization of new novel strains are very few. Torrestiana *et al*. (1990), Torres and Galindo (1997) and Kassim (2011) reported the isolation of novel strains of *Xanthomonas* which have great potential in xanthan production.

This article reported the production and properties of xanthan gum produced by a novel strain of locally isolated *Xanthomonas* spp. The work involved the isolation, screening, and identification of potential isolates and xanthan gum characterization.

MATERIALS AND METHODS

Samples collection

Samples of *Xanthomonas*-infected *Brassicas* that displayed a typical spot symptom were collected at random. The characteristic spot sign of *Xanthomonas* infection was taken from two main sampling sites; vegetable growing fields in Cameron Highlands, Pahang, Malaysia, and in the local retail market around Kuala Lumpur, Malaysia. Samples of diseased cabbages, cauliflower, and lettuces were collected from both sampling sites. Infected *Brassica* usually shows yellow necrotic lesion characteristics. They were randomly collected, placed in sampling bags, and brought to the lab immediately for further processing.

Isolation of bacteria

For each of the samples, leaf tissue segments were cut into small pieces from the lesion margins on the symptomatic plant leaves. Then, the tissue segment was teased in drops of 0.85% (w/v) of NaCl solution and left to stand in a laminar flow cabinet for 10 to 15 min. The resulting suspensions were streaked onto Yeast Malt (YM) agar and incubated for 72 h at 30 °C. Plates were inspected for the bacterial colony that resembled characteristics of *Xanthomonas* spp. which have pale to strong yellow, convex, mucoid colonies. The colonies were purified by re-streaking onto another YM agar containing (g/L); glucose (10.0), malt extract (3.0), yeast extract (3.0), and peptone (5.0). The pure bacterial strains were maintained on YM agar slants at 4 °C and subcultured every 14 days to maintain strain viability. The isolates were also maintained in 20% (v/v) glycerol for long-term storage.

Identification of bacterial isolates

Phenotypic identification was done according to morphological, physiological, and biochemical tests as described previously (KC *et al*., 2007; Soudi *et al*., 2011). Genotypic identification of the presumptive colonies which were closely related to *Xanthomonas* spp. were further identified by using Biolog Gen III Microarray and 16S rRNA sequencing. Total genomic DNA was isolated from the cultured cell by using Yeastern Biotech co. genomic DNA Mini Kit according to manufacturer's instructions. The quality of the genomic DNA was evaluated using a Nano drops Spectrophotometer (Thermo Scientific). The Polymerase-Chain-Reaction (PCR) amplification of the gene segment was performed by using forward primer; 27F (5'-AGAGTTTGATCCTGGCTCAG-3') primer, and reverse primer; 1522R (5'-AAGGAGGTGATCCARCCGCA-3') primer. A total volume of 25 µL PCR reaction mixture contained 1 µL of DNA, 1 µL of each primer, and 9.5 µL UTaq polymerase (MO BIO, USA). The amplification program consisted of an initial denaturation (95 °C

for 5 min), 25 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 15 s, and extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 5 min. 3µL of PCR products were run on 1.7% (w/v) TAE agarose gel at 100 V for 60 min. DNA elution was extracted from specific bands of DNA from agarose gels for 16S rRNA sequencing.

Selection of potential strain for xanthan production

Strains of *Xanthomonas* spp. that had been identified were screened based on their growth, biopolymer production, and viscosity. This experiment was conducted in the batch fermentation method as described by Salah *et al*. (2010).

Inoculation and culture media

For each isolate, a loopful of pure colonies on the YM agar plate was transferred to a 250 mL conical flask containing 50 mL of Yeast Malt Media (YM). The cultivation mixture was then incubated at 30 °C, 24 h, and 200 rpm. Subsequently, 10% (w/v) of these cultures were transferred into 250 mL, containing 90 mL of production medium which contained (g/L): glucose (30.0), yeast extract (3.0), K_2HPO_4 (2.0), and $MgSO_4.H_2O$ (0.1). The flask was incubated under similar culture conditions for 72 hr. A typed strain of *X. campestris* pv. *campestris* (ATCC 33913) was used as the reference strain in all of the experiments.

Analytical methods

Biomass estimation

The biomass concentration was determined using the cell dry weight method. Culture broth samples were centrifuged at 13 000 rpm for 30 min at 4 °C. The pellet was collected as cell biomass and was dried in an oven at 60 °C until a constant weight was obtained.

Recovery and determination of xanthan concentration

Supernatants from cultures were collected and mixed with 3 times the volume of isopropanol. The precipitate recovered after centrifugation at 10 000 rpm for 15 min was dried in an oven at 60 °C until a constant weight was obtained.

Viscosity determination

The viscosity of 3% (w/v) aqueous solution of xanthan synthesized by each strain was measured at different shear rates (5, 10, 30, 60, 80, 100) rpm with a Brookfield viscometer (Model LV, Brookfield Engineering Inc., USA) with spindle no. 41.

RESULTS AND DISCUSSION

Bacterial isolation and identification

A total of 411 bacteria were isolated from the samples. Out of these, 52 were selected based on the biochemical features which gave positive reactions on KOH reaction, catalase test, bile esculin, starch hydrolysis, and citrate utilization, while negative reactions on oxidase test, indole test, and nitrate reduction (Vos *et al*., 2001) as well as the characteristic yellow and mucoid appearance of *Xanthomonas* spp. colonies on YM agar (Table 1). Thirteen of those strains which had biochemical features of *Xanthomonas* spp. were subjected to a BIOLOG test. Finally, 10 strains were selected for further studies after they were confirmed as *Xanthomonas* spp. strains using 16S rRNA sequencing. Identities of the strains and the NCBI accession numbers of their closest matches on the database are presented in Table 2.

Xanthan gum production

The 10 strains whose identities had been confirmed by 16S rRNA sequencing were subsequently used in xanthan production experiments. They were compared based on cell biomass, concentration, yield, and viscosity of xanthan produced (Table 2). The results showed that all the strains grew adequately and xanthan production varied widely among the strains. The concentration and yield of xanthan among the studied strains ranged from 1.57 to 8.24 g/L and 0.64 to 4.71 g/g respectively. The highest concentration of xanthan (8.24 g/L) was produced by strain C206 while strain C298 had the highest yield (4.71 g/g). Interestingly, all the strains, except C272, gave higher xanthan production and yield than the ATCC strain under the conditions applied in this study. This observation indicated all strains isolated are promising for improving xanthan production. The maximum xanthan production recorded from the strains in this study was also higher than that reported for ATCC 33913 (7.0 g/L) that was cultivated on the Luria Bertani medium (Psomas *et al*., 2007). The trend among the strains investigated in this study shows that the concentration and yield of xanthan produced during fermentation are strain-dependent. A similar

Table 1. Biochemical features of *Xanthomonas* spp.

Characteristic	Results
Gram staining	Negative
Cell morphology	Rod
Cell arrangement	Single, pair
KOH reaction	+
Oxidase test	-
Catalase test	+
Bile esculin	+
Indole test	-
Nitrate reduction	-
Starch hydrolysis	+
Citrate utilisation	+
Motility	+

Source: Bergey's Manual

Table 2. Identification of selected bacterial strains isolated from infected Brassica samples and its xanthan gum production after 72 h of cultivation

Isolates	BIOLOG Identification	16S rRNA sequencing	NCBI Accession no.	Biomass (g/L)	Xanthan production (g/L)	Xanthan yield (g/g biomass)
C85	*Acenetobacter johnsonii*	NA	NA	NA	NA	NA
C194	ND	*Xanthomonas arbaricola pv pruni*	KJ156334.1	2.96 ± 0.02	5.87 ± 0.14	1.97 ± 0.05
C205	*Xanthomonas campestris pv campestris*	*Xanthomonas campestris pv campestris*	KM458095.1	1.37 ± 0.20	4.34 ± 0.06	3.16 ± 0.03
C206	*Xanthomonas campestris pv campestris*	*Xanthomonas campestris pv campestris*	KF270091.1	3.47 ± 0.03	8.24 ± 0.20	2.38 ± 0.07
C253	*Xanthomonas campestris pv raphani*	*Xanthomonas campestris*	KM252981.1	1.83 ± 0.10	4.32 ± 0.05	2.36 ± 0.03
C254	*Xanthomonas campestris*	*Xanthomonas campestris pv campestris*	NR119219.1	2.60 ± 0.16	5.73 ± 0.12	2.20 ± 0.02
C256	*Stenotrophomonas maltophilia*	ND	NA	NA	NA	NA
C272	*Xanthomonas campestris pv raphani*	*Xanthomonas gardneri*	KM463765.1	2.47 ± 0.09	1.57 ± 0.06	0.64 ± 0.01
C279	*Xanthomonas campestris pv campestris*	*Xanthomonas campestris pv campestris*	NR074936.1	1.97 ± 0.08	7.62 ± 0.21	3.88 ± 0.04
C295	*Xanthomonas campestris pv campestris*	*Xanthomonas campestris pv phaseoli*	NR104856.1	2.57 ± 0.06	6.46 ± 0.23	2.52 ± 0.04
C298	*Xanthomonas campestris pv campestris*	*Xanthomonas campestris*	NR074936.1	1.18 ± 0.02	5.57 ± 0.24	4.71 ± 0.18
C316	*Microbacterium maritypicum*	ND	NA	NA	NA	NA
C309	*Xanthomonas campestris*	*Xanthomonas campestris*	NR074936.1	2.84 ± 0.06	5.39 ± 0.24	1.90 ± 0.05
ATCC	*Xanthomonas campestris*	NA	ATCC 33913	1.47 ± 0.05	2.27 ± 0.10	1.54 ± 0.05

ND: Not Detected
NA: Not Applicable

observation was reported by Borges and Vendruscolo (2008) in their evaluation of xanthan production by 30 strains of *X. campestris* pv. *pruni* that were isolated from the leaves of plum and peach. Thus, the selection of an effective producer strain should be prioritized in xanthan production.

Viscosity

Based on their apparent viscosity, xanthan gum produced by all strains showed pseudoplastic behavior (Figure 1), as expected for xanthan aqueous solution (Katzbauer, 1998; Moreira *et al*., 2001; Borges & Vendruscolo, 2008). The apparent viscosity decreased with an increase in shear rate. This important characteristic helps in enhance the sensory qualities of a food product. Among the analyzed strain, xanthan synthesized by strain C272 presented the highest apparent viscosity (592.8 mPa.s), while xanthan produced by strain C205 produced the lowest apparent viscosity (98.85 mPa.s) at 5 rpm (10 s^{-1}) shear rate, at 25 °C. Our results agreed with previous findings of others. Borges and Vendruscolo (2008) determined the viscosity of xanthan synthesized by 30 strains of *Xanthomonas campestris* pv. *pruni* presented apparent viscosity between 1.3 to 12.7 mPa.s in the same shear rate. Generally, viscosity and xanthan gum production were inversely proportional. The viscosity of fermentation broths has been used widely as an indicator for xanthan production, but these may not necessarily reflect the quality of the polymer produced. A recent finding by Moreira *et al*. (2001) showed that the high viscosity of xanthan gum might be due to a high concentration of low-quality polymer. The novel isolates of *Xanthomonas campestris* pv. *campestris* (strain C279) was chosen for further studies based on the optimization of physicochemical parameters for xanthan gum production. This strain showed high and stable viscosity at the low shear rate and was feasible for an intermediate yield of xanthan production.

CONCLUSION

These 411 wild types of *Xanthomonas* spp. were successfully isolated from symptomatic *Brassica* samples. Strain C206 produced the highest xanthan gum concentration but C298 gave the highest yield of xanthan (g/g biomass). Strains and culture conditions had a substantial impact on the synthesis of xanthan gum and the viscosity of the polymers. Based on the stable viscosity and yield of xanthan produced, *X. campestris* C279 was selected for further studies on the optimization and revaluation of the culture condition under the lab scale.

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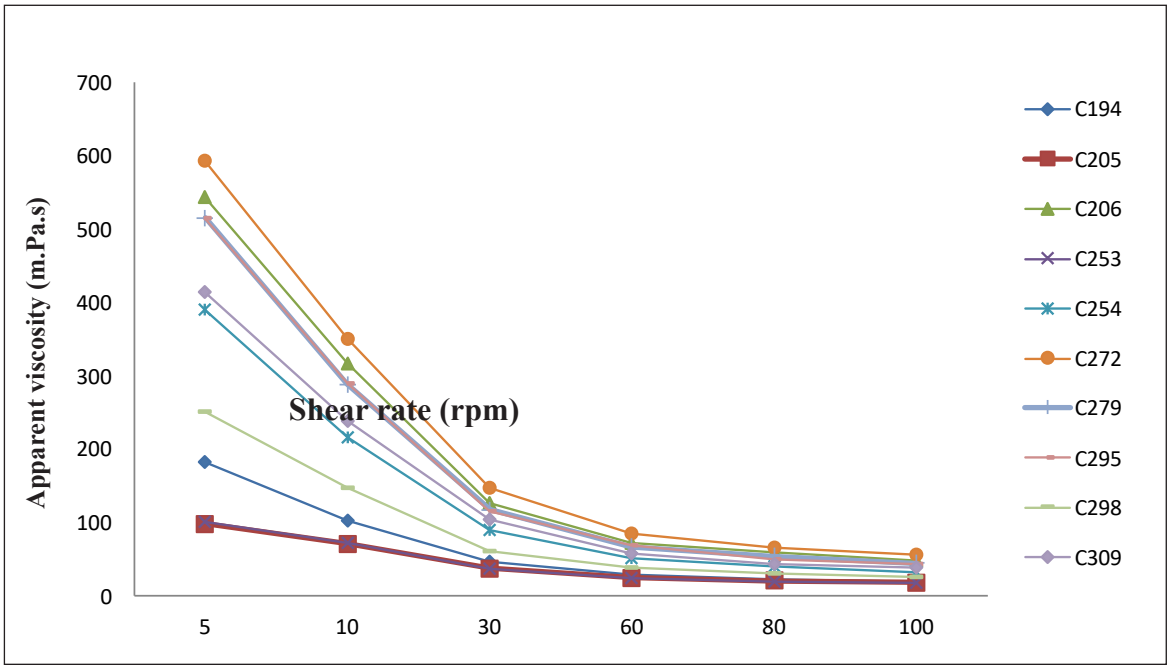

Fig. 1. Apparent viscosity at 25 °C, of the polymer under different shear rates, produced by novel strains *of Xanthomonas* spp.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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