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Isolation, Biochemical Characterization and Production of Biopolymers by Phytopathogenic Species of *Xanthomonas* from the Plants of Rutaceae Family

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Plants of rutaceae family, such as *Citrus medica* (citron, bijaura), *Limonia acidissima* (stone fruit, elephant apple) and *Citrus limon* (lemon) are well known for their nutritive and medicinal values. They were analyzed for the presence of citrus cankers on their various body parts such as leaves, stems and fruits. Isolated species of *Xanthomonas* were subjected to biochemical characterization for their identification. Different media were used for the cultivation of these organisms as well as production of biolpolymer from them under laboratory conditions. Isolated species were subjected to cross infect these three members of rutaceae family to investigate their host range. Different sugars, such as glucose and sucrose were tested for optimization of xanthan production. Biopolymers from *Xanthomonas* spp. have got wide spread biotechnological applications.

Key Words: Biopolymers, Xanthan, Rutaceae, Phytopathogenic species, Citrus canker.

INTRODUCTION

Plants of rutaceae family have been long facing the problem of cankers caused on leaves, fruits and stems by phytopathogenic species of *Xanthomonas*, which affects the productivity of the crop in terms of agricultural produce of the citrus fruits. There has been a continuous search for high quality microbial polysaccharides and a great variety of microorganisms have been tested in this regard.

The diseased plants are characterized by the occurrence of conspicuous raised necrotic lesions that develop on leaves, twigs and fruits. Lesions can be detected by drawing the fingers over the surface of infected tissues. On leaves, first appearance is as oily looking, 2-10 mm circular spots, usually on the abaxial surface (reflecting stomatal entry following rain dispersal). Lesions are often similarly sized. Later, both epidermal surfaces may become ruptured by tissue hyperplasia induced by the pathogen. On leaves, stems, thorns and fruit, circular lesions become raised and blister-like, growing into white or yellow spongy pustules. These pustules then darken and thicken into a light tan to brown corky canker, which is rough to the touch. Often a water-soaked margin develops around the necrotic tissue and is easily viewed with transmitted light. On stems, pustules may coalesce to split the epidermis along the stem length and occasionally girdling of young stems may occur. Older lesions on leaves and fruit tend to have more elevated margins and are at times surrounded by a yellow chlorotic halo (that may disappear as canker lesions age) and a sunken center. Sunken centers are especially noticeable on fruits, but the lesions do not penetrate far into the rind thereby not affecting internal quality. Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop[1].

Some of the biopolymers have been attracting the interest of the researchers for their possible potential to be used in the food, cosmetics, pharmaceuticals and oil industries, where they can be used as possible thickening, stabilizing and emulsifying agents. Therefore, it becomes utmost important to identify and study new production sources for these materials, especially when these biopolymers possess good rheological properties.

EXPERIMENTAL

Isolation of phytopathogenic *Xanthomonas* spp. from citrus canker: Various specimens from the plants of rutaceae family were collected and especially the diseased portions from the leaves, fruits and stems were surface sterilized by using 1 % $HgCl_2$ (1:1000 diluted), oozing of the bacteria from the citrus cankers was observed using freshly collected leaves. Crushed canker juice was used to inoculate potato dextrose broth and potato dextrose agar plates as well. Different members (*Citrus medica*, *Citrus limon*, *Limonia acidissima*) were used for the isolation of phytopathogenic species of *Xanthomonas* by the same method.

Maintenance of the isolates using: Yeast mold agar[2], with the following composition in g L^{-1}:3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 25 g agar.

Study of biochemical properties of the isolates: Two isolates from *Citrus limon* and one isolate from *Citrus medica* were subjected to analysis for their biochemical properties by some commonly used biochemical tests such as hydrolysis of starch, hydrolysis of gelatin, production of hydrogen sulphide, fermentation of some commonly used carbohydrates *e.g.*, glucose, sucrose, maltose, lactose, mannitol, xylose, *etc*.

Presence of Xanthomonadin pigment from the isolates: Each of the three isolates was streaked on nutrient agar and potato dextrose agar, incubated at 28 ºC for 48 h. About 2-3 loopful colonies of each bacterial isolate were transferred to 3 mL of methanol in test tubes and were placed in boiling water bath until the pigment was removed. The suspensions were then centrifuged at 13,000 rpm for 15 min to remove the cell debris. The supernatant was decanted and the methanol was allowed to evaporate by keeping the methanol extract in 50-60 ºC-water bath until the optical density of the pigment extract reaches 0.4 at 443 nm. 5 µL of each extract was spotted on a precoated, silica gel plates and a total 20 µL was spotted. The silica gel plates were developed in methanol solvent. The solvent was allowed to move approximately 10 cm and the yellow coloured spots were marked with a pencil while wet. A yellow spot with an average R_f value of 0.45 was taken as positive for the presence of the pigment[3].

Effect of carbohydrates and L-glutamate on the production of xanthan: The initial chemically defined medium was used for the activation of the organisms. It contained the following components (g/L): glucose, 20; KH_2PO_4, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2.0; citric acid, 2.0; H_3BO_3, 0.006; ZnO, 0.006; $FeCl_3 \cdot 6H_2O$, 0.0024; $CaCO_3$, 0.02 and HC1, 0.13 mL/L. The pH was adjusted to 7 with sodium hydroxide before sterilization. Sugars were autoclaved separately.

In formulating the medium for xanthan production and its optimization, 0.3 % pyruvate and 0.5 % succinate were used as organic carbon sources apart from different concentrations of glucose and sucrose used as the main sugar for growth and xanthan production[4]. 0.2 % $(NH_4)_2SO_4$ was the main nitrogen source used in the experiment. Different concentrations of glutamate were checked for optimization of xanthan production starting from 15 mM.

For production and primary analysis of the biopolymer, 250 mL Erlenmeyer flasks were added with 100 mL of the production medium and were incubated on a rotary shaker at 28 ºC temperatures. The production medium was incubated at 28 ºC and analyzed after 96 h.

RESULTS AND DISCUSSION

Two isolates from *Citrus limon* and one isolate from *Citrus medica* and none were obtained from *Limonia acidissima* using PDA medium. Further, these three isolates were analyzed for their biochemical properties.

All the three isolates were checked for their xanthomonadin pigment, characteristic of *Xanthomonas* spp. development of yellow spots at 0.45 R_f value on the silica gel plates is indicative of the presence of xanthomonadin pigment[5].

All the three isolates where tested in a brief attempt to cross infect the selected varieties by using syringe and needle, but attempt to infect *C. medica* with X1 and X2 failed whereas X3 isolated from *C. medica* was successfully used to infect *C. limon*. This clearly indicates that *C. medica* is a highly resistant variety of citrus family, whereas *C. limon* is quite sensitive for all the isolates of *Xanthomonas* (*viz*., X1, X2 and X3). Once injected by a specific volume of isolates, the plants were examined periodically for every 24 h till 7 days.

Xanthan is a Non-Newtonian biopolymer, the rheology of which changes with temperature. At very high temperature and application of shearing forces the viscosity of xanthan gum decreases significantly. Xanthan, an acidic exopolysaccharide consists of pentasaccharide repeat units composed of D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues. Because of its physical properties, it is widely used as a viscosifier, thickener, emulsifier or stabilizer in the food industry.

As mentioned in the flow chart (Fig. 1), at the end of the fermentation, the broth contains xanthan, bacterial cells and many other chemicals. For recovering the xanthan, the cells are usually removed first, either by filtration or centrifugation[6].

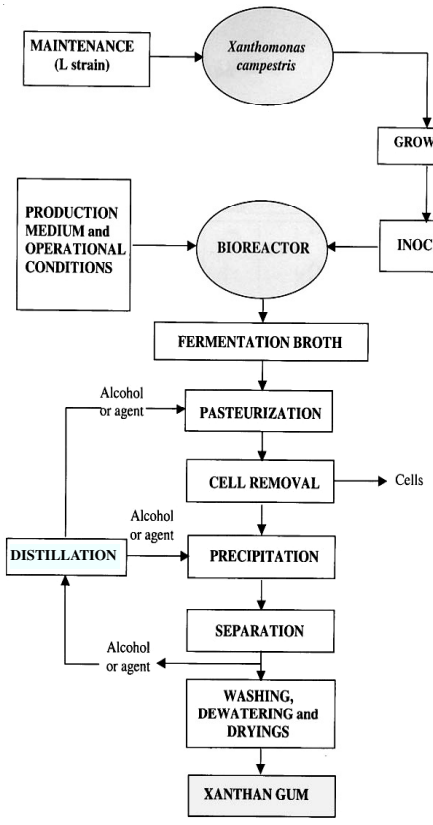

Fig. 1. Outline of xanthan gum production process [Ref. 7]

Further purification may include precipitation using water-miscible non-solvents (isopropanol, ethanol, acetone), addition of certain salts and pH adjustments[6]. The FDA regulations for food grade xanthan gum prescribe the use of isopropanol for precipitation. After precipitation, the product is mechanically dewatered and dried. The dried product is milled and packed into containers with a low permeability to water.

The xanthan gum was recovered by centrifuging the fermentation broth at 15,000 × g for 25 min to remove the biomass. The remaining biopolymer was recovered by adding 99.5 % ethanol (3 volumes), the precipitates were retained on a Whattman filter (No. 1). The wet precipitate was dried at 40 ºC. The dried xanthan gum was weighed and the average result expressed in grams of xanthan per liter of medium[8] or it can also be expressed as the amount of xanthan produced per kilogram of sugar utilized.

From the experimental design used to check the effect of sucrose and glucose on the production of xanthan, it is evident that at higher concentrations (> 20 g/L) glucose rarely affects the production of xanthan whereas sucrose (at 30 g/L concentrations) shows exponential linearity upto 40 g/L concentration. Thus, it is concluded that glucose is the preffered source of carbon for accelerated growth of the organism (Fig. 2), whereas sucrose favours the synthesis of biopolymer[4].

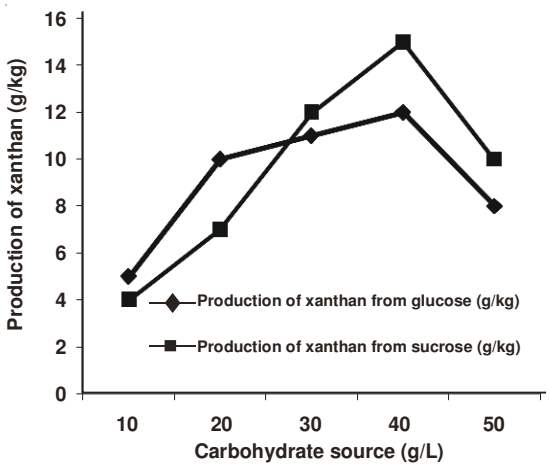

Fig. 2. Comparative analysis of different C sources on the production of xanthan

However, addition of small amounts of organic acids such as pyruvic acid, succinic acid to the production medium also favours the synthesis of biopolymer[4]. This is supported by the fact that in the actual medium 0.3 % of pyruvate and 0.5 % of succinate were used as organic C sources apart from the use of sucrose or glucose.

Presence of L-glutamate in the medium (> 20 mM) has exerted positive effect on the synthesis of biopolymer upto 40 mM. Increase in the concentration of L-glutamate beyond 40 mM has shown a drastic decrease in the formation of biopolymer (Fig. 3).

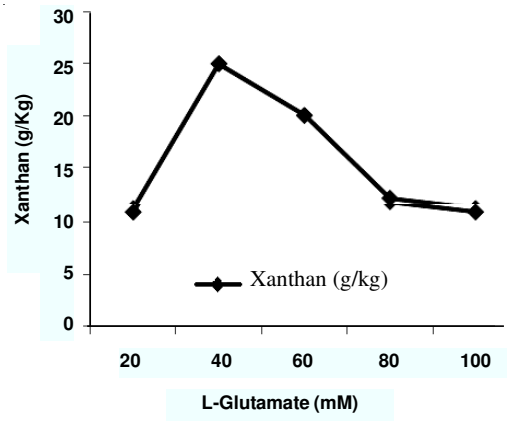

Fig. 3. Effect of L-glutamate on the production of xanthan

Amino acid, such as L-glutamate serves the role of a better N-source apart from the $(NH_4)_2SO_4$ used in the actual production medium. The yields obtained with L-glutamate are two folds as compared to a medium without its presence.

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