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Use of agricultural wastes for xanthan production by *Xanthomonas campestris*

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Four different acid-hydrolyzed wastes, from melon, watermelon, cucumber and tomato were compared for xanthan production. Growth of *Xanthomonas campestris*, xanthan biosynthesis, kinetics and chemical composition were investigated. Both growth and xanthan production were dependent on the acid hydrolysate concentrations and available nitrogen. Melon acid hydrolyzed waste was the best substrate for xanthan production. Exopolysaccharide obtained throughout this study was compared to commercial xanthan, showing a very similar chemical composition. Acid hydrolyzed wastes are proposed as a new carbon source for xanthan production.

Keywords: xanthan; agricultural wastes; *Xanthomonas campestris*

Introduction

Xanthan is an extracellular heteropolysaccharide produced by *Xanthomonas campestris*. The structure of xanthan is a pentasaccharide repeat unit, in which alternate glucose residues carry trisaccharide side chains composed of d-mannose and d-glucuronic acid. Internal mannosyl residues are substituted with an acetyl group while external mannosyl residues have pyruvyl substituents linked as cyclic ketals. Content of those organic acids in xanthan isolates is affected by the carbon source and the oxygen and nitrogen available in culture media. These changes in composition, mainly the extent of acetylation, affect properties of xanthan solutions [17].

Because of its unique rheological behaviour, xanthan is one of the major microbial polysaccharides actually employed in many industrial processes. Solutions of xanthan are highly pseudoplastic and show very good suspending properties [12]. The polysaccharide is used as suspending, stabilizing, thickening and emulsifying agent, for food and non-food industrial applications [19].

Xanthan production is usually performed in glucose- or sucrose-containing, nitrogen- or sulphate-limited media [20]. One of the greatest factors limiting the use of xanthan in large-scale fermentation processes is the cost of production when compared to similar polymers from algae or plants. There are two areas where savings could be made in this respect: the cost of feedstocks used for polymer production and the down-stream processing. Some attempts have been made to use cheaper substrates such as citrus waste [1], whey [6,7], corn steep liquor [13], molasses and glucose syrup [4] and olive oil waste waters [10].

This work is concerned with the possibility of using agricultural wastes as lower-cost alternative substrates for xanthan production. These wastes are abundantly produced in southeastern Spain (Almería area), where greenhouse cultures provide a constant supply of these residues during the year (more than 450 000 t are produced yearly). Thus, the objective of this study was to compare different agricultural wastes as substrates for *X. campestris* growth and xanthan production, and their effect on xanthan composition.

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Materials and methods

Microorganism

Xanthomonas campestris NRRL B-1459 and *Xanthomonas campestris* NRRL B-1459 S4-LII were obtained from the US Department of Agriculture (Peoria, IL, USA), and used throughout this study. The strains were maintained on yeast malt (YM) agar (Difco, Detroit, MI, USA) slants stored at 4°C and subcultured weekly.

Media

Acid hydrolysates of sun-dried wastes (AHW) of either tomato (*Lycopersicum esculentum*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus*) plants were used as substrates. Different media were prepared by addition of each hydrolysate to a basal mineral solution (in grams per liter of water: KH_2PO_4, 5.0; Na_2CO_3, 0.5; Na_2SO_4, 0.114; $MgCl_2 \cdot 6H_2O$, 0.163; $ZnCl_2$, 0.0067; $CaCl_2 \cdot 2H_2O$, 0.012; $FeCl_3 \cdot 6H_2O$, 0.0014; H_3BO_3, 0.006), to provide final carbohydrate concentrations of 0.1, 0.2, 0.4, 1 and 3.5 g L^{-1}. When necessary 1 g L^{-1} of NH_4Cl was also added.

Acid hydrolysates were obtained by mixing 10% (w/v) chopped wastes with 1.5% (v/v) sulphuric acid in 2-L pyrex glass flasks (final volume 1 L). Mixtures were autoclaved at 121°C for 2 h. Waste hydrolysates were filtered through paper and the pH of the filtrates was adjusted to 6.6–7 with $Ca(OH)_2$ and filtered again, to remove the precipitate, prior to preparation of the culture media. To adjust final carbohydrate concentration in the media, total carbohydrate content was determined in the hydrolysates.

Culture conditions

Inocula were prepared in YM broth (Difco). *X. campestris* cells were incubated in this medium at 30°C under continuous agitation (120 rpm) for 18 h, harvested by centrifug-

ation and washed twice in sterile distilled water. One-millilitre volumes of the suspensions (OD 0.5 at 540 nm) were inoculated into 250-ml Erlenmeyer flasks containing 25 ml of mineral medium supplemented with the corresponding AHW. All media, once inoculated were incubated at 30°C on a rotatory shaker (120 rpm) for 5 days. Experiments were performed in triplicate.

Analytical methods

Biomass determination: For biomass estimation, cultures were harvested by centrifugation (14 000 × *g*), washed twice with sterile distilled water and evaporated to dryness in preweighed vials at 105°C overnight.

Carbohydrate content: Once the cultures were centrifuged (14 000 × *g*), 10-ml volumes of supernantant fluids were analysed for their total carbohydrate content, using the anthrone reagent, which was prepared by dissolving 200 mg of anthrone (Sigma Chemical Co, St Louis, MO USA) in 5 ml absolute ethanol, and this solution was made up to 100 ml with 75% sulphuric acid (v/v) [8]. This method was also used to measure carbohydrate content in acid-hydrolyzed wastes and samples of xanthan solution.

One-millilitre samples were placed into thin-walled glass tubes and cooled in ice-water. Five millilitres of a cooled solution of anthrone reagent were added, swirling the tube in the ice-water during this addition. The tubes were allowed to stand a few min, and mixtures were transferred to a boiling water-bath. After exactly 10 min, samples were returned to the ice-bath, and when cool the green colour was measured at a wavelength of 625 nm. Carbohydrates give a characteristic green colour on heating with anthrone in sulphuric acid solution. The colour is due to the condensation with anthrone of furfural derivatives formed from the sugars in hot acid [8]. Distilled water was used as blank. Pure glucose solutions (20–100 μg ml^{-1}) were used as standard.

Xanthan separation and analysis: Exopolysaccharides were extracted from the culture supernatant, with two volumes of cold isopropanol by vigorous shaking. After 10 min, precipitated exopolysaccharides were filtered onto predried and preweighed GF/A Whatman filter discs (Whatman International, Springfield Mill, Kent, UK), and washed with 50 ml of isopropanol/water (3:1 v/v). The filter disc plus precipitate was dried at 60°C for 24 h. Filters were reweighed and the concentration of exopolysaccharide in the culture broth was calculated.

Dried samples of raw xanthan obtained as described before, were analysed. Total carbohydrate content was determined using the anthrone reagent [8]. Pyruvate content was determined enzymatically, using lactate dehydrogenase (type II, Sigma Chemical Co), after hydrolysis of 0.2–0.4 g dried xanthan in 50 ml of 1 M HCl for 3 h, and neutralisation with $BaCO_3$ [16]. Acetyl content was determined as follows: 200 μl of xanthan solution (1% w/v) were added to 400 μl of a 1:1 mixture of 2 M hydroxylamine HCl and 3.5 M NaOH. After standing for 2 min at room temperature, 200 μl of 5.65 M HCl and 200 μl of 0.37 M $FeCl_3.6H_2O$ in 0.1 M HCl, were added. The brown reddish colour which developed was measured at 540 nm. Solutions of 0.04 M acetyl choline HCl in 0.001 M sodium acetate (pH 4.5) ranging from 10–80 μg ml^{-1} were used as standard [9]. Uronic acids were determined in 0.2 ml of 0.1% xanthan solutions, by addition of 1.2 ml of 0.0125 M sodium tetraborate in concentrated H_2SO_4. The mixture was chilled in an ice bath for 5 min, homogenized, heated at 100°C in a boiling bath and chilled again. Then, 20 μl of 8.8 mM 3-phenylphenol in 125 mM NaOH, were added and optical density was measured at 520 nm. Pure glucuronic acid solutions (100–400 μg ml^{-1}) were used as standard [2]. Elemental analysis was performed in a Leco CNHS-923 analyser. Chemical analysis was also performed with samples of commercial xanthan (Sigma Chemical Co), for comparison.

Results

Preliminary studies were performed with two bacterial strains (*X. campestris* NRRL B-1459 and *X. campestris* NRRL B-1459 S4-LII) grown in YM solid medium containing 10–90% (w/v) AHW of either melon, tomato, watermelon or cucumber. After 48 h incubation both strains grew well in all media, however *X. campestris* NRRL B-1459 S4-LII colonies were larger and showed a more mucoid aspect and this strain was selected for further experiments. Also, in initial experiments the incubation time for optimum recovery of exopolysaccharides in *X. campestris* cultures was investigated. Thus, culture media samples were removed at intervals and quantitative extraction of xanthan was made. Maximum polysaccharide recovery was obtained in 5-day-old cultures (data not shown), so this incubation period was selected for biomass determination and xanthan extraction and quantification.

When *X. campestris* was grown in media with AHW of melon, tomato, watermelon and cucumber as sole carbon and energy source at concentrations of 0.1, 0.2, 0.4, 1.0 and 3.5 g L^{-1} (as total carbohydrate content), higher concentrations of AHW in culture media yielded higher values of biomass production (Figure 1). Thus, microbial growth was highly correlated to AHW concentration for all plant residues. Growth was also strongly influenced by the addition of a nitrogen source to culture media. The nitrogen effect was more relevant at low concentrations of AHW. Generally, growth levels were higher in media supplemented with 0.3% NH_4CL when compared to those obtained in unamended media, with the exception of cucumber AHW media, in which biomass production was larger in the absence of added nitrogen source (Figure 1). Melon and tomato residues only allowed growth of the microorganism at AHW concentrations above 1 g L^{-1}, in cultures without a supplemental nitrogen source.

Xanthan production was investigated under the same conditions as biomass. According to results presented in Figure 2, polysaccharide production was highly influenced by the addition of an exogenous nitrogen source. This was observed mainly in media supplemented with tomato, watermelon, and cucumber residues, in which almost no xanthan production was achieved in the absence of an added nitrogen source. Melon AHW supported xanthan production in both nitrogen amended and unamended media,

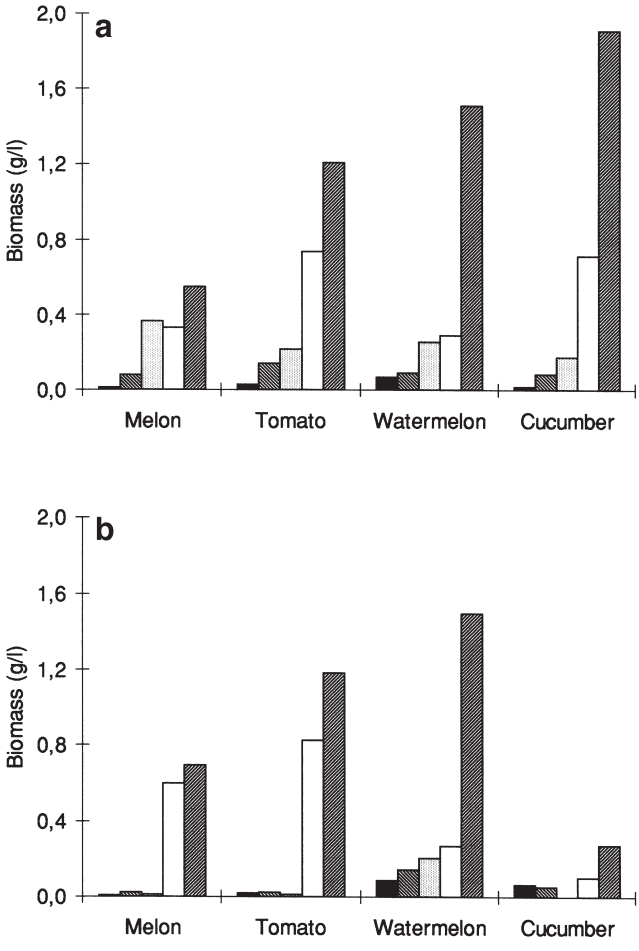

Figure 1 Biomass (g L^{-1}) of *Xanthomonas campestris* cultured in acid-hydrolyzed residues of melon, tomato, watermelon and cucumber, at concentrations of 0.1 (■), 0.2 (▧), 0.4 (▩), 1.0 (□) and 3.5 (▨) g L^{-1} (adjusted as total carbohydrate content), either in the presence (a) or absence (b) of ammonium chloride (0.1% w/v).

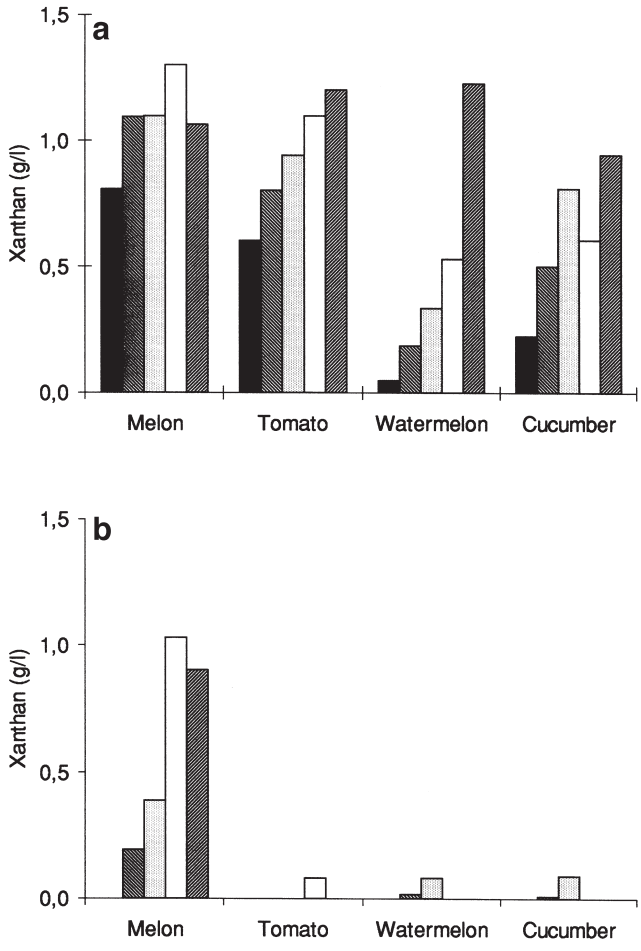

Figure 2 Xanthan production (g L^{-1}) by *Xanthomonas campestris* cultured in acid hydrolyzed residues of melon, tomato, watermelon and cucumber, at concentrations of 0.1 (■), 0.2 (▧), 0.4 (▩), 1.0 (□) and 3.5 (▨) g L^{-1} (adjusted as total carbohydrate content), either in the presence (a) or absence (b) of ammonium chloride (0.1% w/v).

although polysaccharide concentrations were higher with NH_4Cl (Figure 2). As indicated for biomass, xanthan production was also correlated to AHW concentration. Thus, xanthan yields increased as AHW concentration rose. This pattern was not exhibited in melon AHW media, in which xanthan production increased to concentrations of 1 g L^{-1} AHW. At this concentration, maximum amounts of polysaccharide (approximately 1.3 g L^{-1}) were obtained. Moreover, xanthan production was highest at any melon AHW concentration when compared to other plant residues (Figure 2). On the basis of these results, melon AHW at concentrations of 1 g L^{-1} supplemented with 0.1% NH_4Cl was the medium selected for further experiments.

Data presented in Figure 3 show that both biomass and polysaccharide production increased as total carbohydrate content in the media decreased. Maximum levels of biomass were reached at 48 h, when some 40% of the carbon source had been depleted. Similar or slightly smaller amounts of biomass were maintained in 5-day-old cultures. However, xanthan production increased constantly until 96–120 h, when highest polymer concentrations were obtained. At that point, 10% of the carbon source remained undegraded.

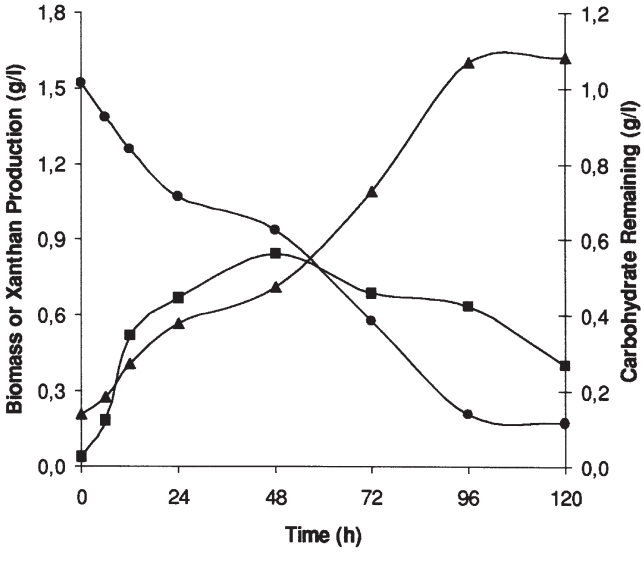

Figure 3 Biomass (■), xanthan production (▲) and carbohydrate consumption (●) by *Xanthomonas campestris* cultured in media containing 1 g L^{-1} acid-hydrolyzed residues of melon, supplemented with 0.1% ammonium chloride.

The composition of raw xanthan obtained from AHW of melon media supplemented with 0.1% NH_4Cl, was compared to that of commercial xanthan. Results presented in Figure 4, showed that both polymers shared a very similar chemical composition, although values for total carbohydrate, uronic acids, acetyl and pyruvyl contents, were slightly higher in commercial xanthan. Raw xanthan contained 38.16% C, 0.62% N, 0.35% S and 5.35% H.

Discussion

Two strains of *X. campestris* were tested regarding their ability to grow with AHW as sole carbon and energy source. From data obtained in these preliminary experiments, it was observed that both strains grew well on the different AHW tested, however, one of them (*X. campestris* NRRL B-1459 S4-LII) showed a very mucoid colonial aspect, and since a relation between colonial phenotype and exopolysaccharide production has been reported [3,15], this strain was used in further experiments.

Both cell growth and xanthan production are clearly influenced by the type and initial concentration of AHW as well as by the presence of an exogenous nitrogen source (Figures 1 and 2). Thus, differences observed in biomass and xanthan production by *X. campestris* in the presence of various AHW, could be primarily ascribed to the different composition of AHW.

The efficiency of substrate utilization would depend not only on the composition but also on the concentration of the substrate. Thus, as shown in Figures 1 and 2, important differences in biomass and xanthan production could be observed in media supplemented with different concentrations of the same AHW. Generally, an increase in AHW concentration causes higher yields in biomass and xanthan synthesis. However, the extent of such increase differs among wastes. This may be due to a different availability of sugars released during hydrolysis. Increased waste concentrations showed a positive effect on bacterial growth when cultured on tomato, watermelon and cucumber AHW (quantitatively in this order). This positive effect was not so evident when *X. campestris* was cultured on melon AHW. In these media, growth was affected little by increases in waste concentrations and xanthan yield did not reach maximum production levels at the highest AHW concentration tested. Similar results have been reported by Bilanovic *et al* [1] who observed higher substrate utilization in most diluted media composed of citrus wastes.

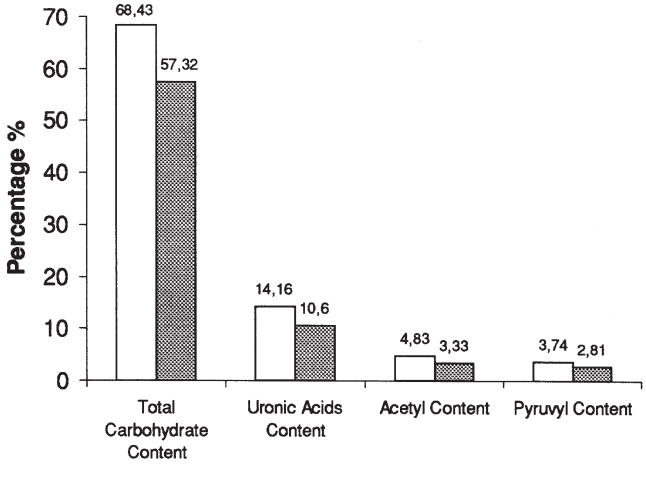

Figure 4 Chemical composition of commercial xanthan (□) and raw xanthan obtained throughout this study (▨).

The clearest differences among wastes used throughout this study could be observed by comparing the effect of nitrogen supplementation (Figures 1 and 2). Low values obtained in growth and xanthan production in tomato, watermelon and cucumber AHW without such supplementation, demonstrated a lack of or very low levels of available nitrogen in those substrates. Only melon AHW seemed to have a nitrogen content great enough to support both growth and xanthan production, mainly at higher concentrations. These differences were less evident when an external nitrogen source was added to the media. Thus, the addition of a nitrogen source is necessary for polymer production when cellulosic wastes are used as substrates. A similar conclusion has been obtained for exopolysaccharide synthesis by *Enterobacter* from these kinds of wastes [11]. Generally, exopolysaccharide production is favoured by nitrogen limitation [20]. On the contrary, the results presented here showed that nitrogen supplementation enhanced xanthan yields (Figures 1 and 2). It is well known that cell growth and xanthan formation need different nutrient requirements, xanthan biosynthesis being favoured by a high concentration of carbon, while there must be enough nitrogen to support growth [14]. In our experiments, nitrogen-unamended media yielded both lower biomass levels and xanthan production.

Kinetic behaviour of xanthan production in melon AHW, was mainly restricted by the uncontrolled variation in the proportion of the growth-limiting nutrient in such a complex medium. With adequate oxygen and carbon supplies, xanthan synthesis occurs throughout the time course, during both exponential and stationary phases [18]. As shown in Figure 3, more than 50% of xanthan was obtained after growth ended. Most batch assays are stopped after 5 days of culture, since at this time growth usually ceases [20]. Our comparative nutritional assays to select waste, concentration and nitrogen supplementation were carried out at this time. In fact, kinetic studies performed in the optimal medium selected, demonstrated high xanthan yields at this time. However, even when growth decreased continuous xanthan production was observed. Higher xanthan values could probably be achieved in longer culture periods since xanthan yield did not reach stabilisation or decrease after 5 days of culture (Figure 3). At this time carbohydrate remaining in the media would be too low to support xanthan production, so other carbon compounds present in the acid hydrolysate could probably be used.

Xanthan composition is affected by the carbon source, the oxygen availability and the time of recovery from culture [20]. Changes in culture conditions mainly influence acetyl and pyruvyl contents, although molecular mass can also be affected [3,20]. Since culture media composition and culture conditions influence not only quantity but also quality of xanthan, it is important to define the exopolysaccharide composition obtained in different media. Thus, a comparison with commercial xanthan was made in this study. Data presented in Figure 4 show slightly lower

chemical component values in raw xanthan than those obtained in the commercial product, probably due to the fact that raw xanthan was not purified. Exopolysaccharides obtained from AHW have a brown colour that can derive from contaminants of culture media which coprecipitated. The most likely contaminants found in xanthan preparations are charged polymers such as proteins and nucleic acids and water bound to the hygroscopic xanthan molecule [20]. Elemental analyses also reflect this contamination. Slight amounts of nitrogen were obtained (about 0.62%) which were probably derived from cell surface and extracellular proteins such as cellulases [5] secreted by *X. campestris*. Both the presence of sulphur (0.35%) and the lower levels of total carbon in raw xanthan, when compared to the theoretical values of xanthan containing one pyruvyl and two acetyl residues per two repeating units (43% carbon) [20], could be also ascribed to contaminants.

The results presented here suggest that AHW could serve as a carbon source readily available for xanthan biosynthesis production. Nevertheless, an optimization of culture media should be made to enhance exopolysaccharide production. Xanthan yield reached *ca* 1.6 g L^{-1} in the best condition (AHW of melon at 1 g L^{-1} of carbohydrate with nitrogen supplementation). This amount is very low in comparison with levels cited from other inexpensive substrates namely, 12 g L^{-1} from citrus waste [1], 14 g L^{-1} from whey [13] or 7.71 g L^{-1} from olive oil waste waters [10].

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