Xanthan Yield and Conversion Efficiency of Pre-treated Rice Husk, Sweet Potato and Cassava Flours from *Xanthomonas campestris* Fermentation

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Abstract: *Flours from cassava, sweet potato and rice husk were fermented in shake flask culture by Xanthomonas campestris and yielded various quantities of xanthan gum. Fermentation of 5 g/100 ml of each substrate showed that cassava flour yielded highest quantity of xanthan (2.3 g), followed by sweet potato flour (2.0 g), while the rice husk flour recorded the lowest yield (1.2 g). The conversion efficiency of cassava to xanthan decreased with increase in substrate concentration. At a concentration of 0.5 g/100 ml cassava flour, there was 80% conversion efficiency into xanthan as against 32 % conversion efficiency from 2.5 g/100 ml cassava flour. Nevertheless, xanthan gum yield increased with increase in substrate concentration. Acid hydrolysis of cassava, sweet potato and rice husk flours prior to fermentation increased xanthan yield. Xanthan gum from hydrolysed cassava flour was finer and whiter than that from non-hydrolysed flour. Generally, xanthan yield from rice husk flour increased with increase in the heating period during hydrochloric acid hydrolysis. Cell free extracts of X. campestris fermentation broth contained extracellular enzymes with amylolytic, cellulolytic and cyanidase activities*

Keywords: *Cassava, potato, rice husk, xanthomonas, xanthan gum.*

Introduction

Xanthan is a hetero-polysaccharide produced by several pathovars of *Xanthomonas campestris* and by other species of *Xanthomonas.* It has a primary structure consisting of repeating sugar units, comprising β-D-glucose units linked at the 1 and 4 positions and tri-saccharide side chains of D-glucuronic acid unit between two D-mannose units linked at the O-3 position of every other glucose residue in the main chain with a molar ratio of 2.8:2.0:2.0. Xanthan gum is widely used as stabilizer in food industries, cosmetics and medicine (1). Xanthan can be produced from the fermentation of glucose, sucrose or lactose by *Xanthomonas* species. Xanthan can also be produced from carbohydrate sources such as, sugar molasses, cassava starch and crude glycerin (2), (3), (4). The polysaccharide can be precipitated from the growth medium with isopropyl alcohol or ethanol, then dried and ground into cream to off-white fine powder, and packaged (1).

Cassava tuber of the' bitter' variety contains total cyanide in the peel (650ppm)and pulp (310 ppm)that is toxic to humans. However, the 'sweet' cassava variety has lower total cyanide content in the peel (200 ppm) and pulp (38 ppm) (5). Cassava tuber also contains the enzyme, linamarase which hydrolyses its cyanogenic glucosides (linamarine, lotasustralin) to release sugar and hydrogen cyanide (HCN), as well as glucosidases which breakdown starch and disaccharides to glucose (6). Sweet potato tuber contains a peculiar β-amylase (a tetramer) comprising 4 identical units instead of a monomer, that hydrolyses the α-1, 4-glucan linkages of the non-reducing end of a polysaccharide to release successive maltose units (7). Higher level of beta-glucosidase is present in the pulp of sweet potato than in the peel (5). Rice husks are generated annually from rice processing in tons as waste product. Rice residues (straw and hulls) from harvest and processing comprise cellulose (32–47%), hemicelluloses (19–27%) and lignin (5–24%) (8).

Producing xanthan gum from glucose and sucrose is economically not viable because of the relatively high cost of utilizing the substrates (1). It is therefore necessary to examine the potential of alternative considerably cheap plant sources and waste products for xanthan production. In many states of Nigeria, 'mountains' of rice husks waste away and enormous quantities of cassava and sweet potatoes are produced annually. Using plant materials for xanthan gum production requires the ability of *Xanthomonas* species to grow and utilize the components of the plant sources in a culture medium. Hence, the need to establish the ability of the *Xanthomonas campestris* used in this work to express extracellular enzymes capable of hydrolysing cellulose, starch and cyanogenic glucosides present in the substrates. This work therefore, examines cell free extract of *Xanthomonas campestris* for extracellular enzymes capable of breaking down starch, cellulose and cyanogenic glucosides of the substrates, compares the xanthan

yield from bitter cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*) and rice husks flours from *X. campestris* fermentation, as well as determine the conversion efficiency of each substrate into xanthan.

Materials and Methods

Sources of Organism and Substrates

Xanthomonas campestris isolated from dung beetle, *Onthophagus hecate* gathered from the Modibbo Adama University of Technology, Yola farm was used. Cassava (*Manihot esculenta*) and sweet potato tubers were purchased from Yola market. Rice husk was obtained from Rumde Jabbe rice mill, Yola. The cassava and potato tubers were washed, peeled, chopped into small pieces, dried and ground into fine powder using mortar and pestle. As well, the rice husks were ground into fine powder using mortar and pestle.

Determination of Starch hydrolysis by Cell-free Extract of *Xanthomonas campestris* Culture

An 18-24 h colony of *Xanthomonas campestris* from nutrient agar was inoculated into 20 ml of sterile soluble starch medium(0.15 % $(NH_4)_2HPO_4$, 0.01 % $MgSO_4.7H_2O$, 0.15 % K_2HPO_4, 10 % (v/w) soluble starch, 0.5 % yeast extract; adjusted to pH 6 with 1M NaOH, and incubated at room temperature(28-30 °C),for 24 h. 5 ml of culture was spun at 2500 rpm for 5 min. 1 ml of the supernatant was transferred into 4 ml soluble starch in a test tube, mixed thoroughly and incubated at30°C for 4 h. Thereafter, 0.5 ml of the digest was transferred into a test tube and 3 drops of Lugol's iodine solution added, mixed and observed. For control experiment, 1 ml sterile soluble starch medium was used to replace the supernatant. Absence of dark-blue colour appearance indicated starch hydrolysis.

Determination of Cellulose Hydrolysis by Cell-free Extract of *Xanthomonas campestris* Culture

Xanthomonas campestris was inoculated into a conical flask containing 20 ml sterile cellulose medium (0.15 % $(NH_4)_2HPO_4$, 0.01 % $MgSO_4.7H_2O$, 0.15 % K_2HPO_4, 10% carboxy-methyl cellulose, 0.5 % yeast extract; adjust to pH 6 with 1M NaOH, and was incubated at room temperature for 24 h. Cell free extract was obtained after spinning 5 ml culture medium at 2500 rpm for 5 min. To 4 ml sterile cellulose medium, 1ml cell free extract was added and incubated at room temperature for 4 h. Thereafter, 0.5 ml of the digest was transferred into a test tube and 3 drops of Gram's iodine solution was added and observed. For control experiment, the supernatant was replaced with 1 ml sterile cellulose medium. Absence of dark-blue colour indicated cellulose hydrolysis (9).

Determination of Tolerable Potassium Cyanide Concentrations by *Xanthomonas campestris*, *Klebsiella pneumonia* and *Escherichia coli*

Xanthomonas campestris was inoculated into 10 ml sterile nutrient broth-KCN medium in test tubes containing different concentrations of potassium cyanide, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1and 0.2 % KCN (w/v). The tubes were incubated at room temperature (28-30 °C), for 24 h. Thereafter, a loopful of *X. campestris* broth from each concentration of nutrient broth-KCN was streaked on nutrient agar plate and incubated at room temperature for 24 h and thereafter, examined for growth. Similarly, *Klebsiella pneumoniae* and *Escherichia coli* were tested for their tolerance of different cyanide concentrations. Appearance of growth on nutrient agar from any concentration signified the ability of the organism to tolerate that cyanide concentration.

Determination of Potassium Cyanide Hydrolysis by Cell-free Extract of *Xanthomonas campestris*

A colony of *Xanthomonas campestris* was inoculated into 20 ml sterile cyanide medium (nutrient broth;0.1 g/100 ml potassium cyanide [KCN]), incubated at room temperature for 24 h. 5 ml of the culture was spun at 2500 rpm for 5 min. The supernatant was transferred into sterile capped tube and stored in a freezer until needed. For cyanide hydrolysis, 1 ml supernatant was transferred into4 ml sterile nutrient broth containing 0.25 g/ml KCN, mixed well and maintained at room temperature for 4 h. Thereafter, the mixture was transferred into 95 ml nutrient agar (i.e. 0.25 % KCN), in 250 ml Erlenmeyer flask and autoclaved at 121 °C and 15 psi for 15 min.

The molten nutrient-KCN agar was poured in Petri dishes and allowed to solidify. *Xanthomonas campestris* was inoculated into two plates and *Klebsiella pneumoniae* into another two plates. *X. campestris* plates were incubated at room temperature while *K. pneumoniae* plates were incubated at 37 °C for 48 h. For control experiment, the supernatant was replaced with 1 ml of sterile nutrient broth. Growth of *K. pneumoniae* in the test experiment and lack of growth in the control experiment signified the breakdown of the cyanide by the cell-free extract.

Production of Xanthan from Various Substrates; Bitter Cassava Flour, Rice Husk flour and Sweet Potato Flour

The xanthan production medium contained 5 g of the appropriate substrate (i.e. rice husk, cassava or sweet potato flour) and involved alternating periods of agitation and batch fermentations for 31 h. Essentially, an 18 h colony of *Xanthomonas campestris* from nutrient agar plate was inoculated into100 ml sterile xanthan fermentation medium (Yeast extract [0.5 %], substrate [5 %], citrate [0.2 %], glutamate [0.2 %], $MgSO_4.7H_2O$ [0.1 %], K_2HPO_4 [0.15%], adjusted to pH 6.0 with 1 M NaOH). The culture was incubated, shaking at 390 rpm and room temperature for 7.5 h, then left to stand (not shaking) for 16 hours, and subsequently shaken again for 7.5 h at 390 rpm. Thereafter, the broth culture was spun at 2,500 rpm for 10 min. The supernatant was transferred into Erlenmeyer flask and twice its volume of absolute ethanol was added

to precipitate xanthan (10).The precipitate was transferred to a watch-glass and dried at room temperature.

Calculation of conversion efficiency = w_2/w_1*100

Where w_1= weight of substrate in grams

w_2 = weight of xanthan in grams.

Acid Hydrolysis of Substrates and Xanthan Production

Five grams of either sweet potato flour, rice husk flour or cassava flour (as carbon source) were added to 100 ml of xanthan fermentation medium (Yeast extract [0.5 %], substrate [5 %], citrate [0.2 %], glutamate [0.2 %], $MgSO_4.7H_2O$ [0.1 %], K_2HPO_4 [0.15%], adjusted to pH 6.0 with 1 M NaOH), then 10 ml concentrated HCl was added and properly mixed. The mixture was boiled for 10 min. Thereafter, it was cooled to ambient temperature and adjusted to pH 6, using 2M NaOH, then sterilized at 121 °C for 15 min at 15 psi and finally cooled to room temperature. A colony of *X. campestris* from nutrient agar plate was inoculated into the sterilized medium. Fermentation and xanthan precipitation proceeded as described for non-hydrolysed substrates.

Acid Hydrolysis of Rice Husk Flour at Different Heat Treatment Periods and Xanthan Production

Six samples, each consisting of 5g rice husk flour in 100 ml of xanthan fermentation medium were prepared. To each sample, 10 ml concentrated HCl was added and properly mixed. The mixtures were heated for different periods, viz., 10, 20, 30, 40, 50 and 60 min. Thereafter, each was cooled to ambient temperature and adjusted to pH 6, using 2M NaOH and sterilized at 121 °C for 15 min at 15 psi. To each sample, a colony of *Xanthomonas campestris* was inoculated, the fermentation and xanthan precipitation were done as described previously for non-hydrolysed substrates.

Results

Growth at various concentrations of potassium cyanide agar medium

Examination of bacterial species for potassium cyanide tolerance showed growth in KCN concentrations up to 0.09 g/100 ml for *Klebsiella pneumoniae* while *Xanthomonas campestris and Escherichia coli* survived higher concentrations up to0.2 g/100 ml (Table 1).

Table 1 Growth of bacteria species in nutrient agar containing varying concentrations of potassium cyanide (KCN)

Og.	Potassium cyanide concentrations (g/100 ml)									
	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.20
X. c	+	+	+	+	+	+	+	+	+	+
K. p	+	+	+	+	+	+	+	+	-	-
E. c	+	+	+	+	+	+	+	+	+	+

Key: +, growth; -, no growth.

Og: Organism, *X.c* : *Xanthomonas campestris, K. p: Klebsiella pneumoniae E.c: Escherichia coli.*

Extracellular Amylase, Cellulase and Cyanidase/Cyanide Hydratase Activities in cell-free Extract

Cell-free extract of *X. campestris* grown in starch medium, hydrolysed starch as demonstrated by the disappearance of dark-blue coloration after iodine application, thus indicating amylases activity. As well, cell-free extract of *X. campestris* grown in cellulose medium, showed cellulose hydrolysis as demonstrated by the disappearance of dark-blue colour after the addition of iodine to the hydrolysate, indicating cellulase activity. *Klebsiella pneumoniae* and *X. campestris* grew in KCN-nutrient agar medium containing the cell-free extract treated 0.25 g/100 ml KCN, thereby indicating cyanide degradation. There was no growth in the control experiment.

Xanthan production from flours of rice husk, sweet potato and cassava

Cassava yielded the highest quantity of xanthan (2 g), from 5 g of flour while, the rice husk yielded the least. Similarly the conversion efficiency was highest with cassava flour (40 %), and the lowest from rice husk (16 %) (Table 2).

Table 2 Variations in Xanthan yield and conversion efficiency from various substrates

Substrates	Substrate concentration (g/100 ml)	Xanthan yield (g)	Conversion efficiency (%)
R.F	5	0.8	16
SP.F	5	1.2	24
C.F	5	2.0	40

Key: C.F Cassava flour, S.P.F: Sweet potato flour R.F: Rice husk flour

Variation in Conversion Efficiency with Concentration of Different Substrates

The quantity of xanthan produced increased with the concentration of cassava flour but the conversion efficiency increased with decrease in the concentration of cassava flour. Similar observation was also made with the rice husk. However, the conversion efficiency is higher with cassava than rice husk (see Table 3)

Table 3 Variations in conversion efficiency and xanthan yield with different substrate concentrations

Substrate	Substrate concentration (g/100 ml)	Xanthan yield (g)	Conversion efficiency (%)
C.F	0.5	0.4	80
	1.0	0.7	70
	1.5	1.0	66
	2.0	1.2	60
	2.5	1.4	56
	5.0	2.1	42
R.F	2.5	0.6	24
	5.0	0.8	16

Key: C.F Cassava flour, R.F: Rice husk flour

Variation in xanthan yield from different hydrolyzed and non-hydrolyzed substrates

Xanthan yields from hydrolysed cassava, sweet potato and rice husk were higher than those from non-hydrolysed substrates. The yields from hydrolysed and non- hydrolysed cassava were higher than that of sweet potato, which was greater than that of rice husk (see Table 4)

Table 4 Variations in xanthan yield from hydrolysed and non-hydrolysed substrate

Substrate Flour	Substrate concentration (% v/w)	Xanthan yield (g)	
		Non-hydrolysed	Hydrolysed
C.F	5	2.1	2.3
S.P.F	5	1.8	2.0
R.F	5	0.8	1.0

Key: C.F Cassava flour, S.P.F: Sweet potato flour R.F: Rice husk flour

Variation in xanthan yield and conversion efficiency of acid hydrolysed rice husk flour for different periods

Xanthan yield and conversion efficiency from rice husk increased with increased in acid hydrolysing period up to 50 min and thereafter, remained unchanged. The highest xanthan yield (1.7 g) and conversion efficiency of 34 % was observed after 50 min while the least xanthan (1.0 g) and conversion efficiency of 20 % was observed after 10 min of acid hydrolysis (see Table 5).

Table 5 Variation in xanthan yield and conversion efficiency of acid hydrolyzed rice husk flour at different boiling periods

Boiling time (min)	Rice husk (g/100ml)	Xanthan yield (g)	Conversion efficiency (%)
10	5	1.0	20
20	5	1.4	28
30	5	1.5	30
40	5	1.6	32
50	5	1.7	34
60	5	1.7	34

Discussion

The growth of *X. campestris*, *E. coli* and *K. pneumoniae* in KCN-nutrient agar medium showed their ability to breakdown hydrocyanic or prussic acid (HCN), and suggests the presence of cyanidase and or cyanide hydratase enzymes. KCN has been shown to react with water and produce HCN and KOH. Cyanidase and/or cyanide hydratase are known to degrade hydrocyanic acid (dissolved hydrogen cyanide in water) or HCN to form formate and ammonia (11), (12). However, *K. pneumonia* was unable to grow in KCN concentration greater than 0.09 g/100 ml. It was not clear why this was the case. Nevertheless, when the cell-free extract of *X. campestris* broth culture hydrolysed relatively high KCN concentration (0.25 g/100 m/), *K. pneumoniae* grew in the hydrolysate-nutrient agar medium (Table 1), also suggesting the presence of extracellular cyanidase and/or cyanide hydratase activity in the cell-free extract. Invariably, *X. Campestris* will be able to grow and utilize nutrients in a medium containing 'bitter' cassava pulp containing cyanogenic glucosides which are hydrolysed by endogenous β-glucosidase of bitter cassava to yield toxic HCN (13), (14) (15). As well, the hydrolyses of starch and cellulose by cell free extract of *X. campestris* broth culture revealed also the presence of extracellular amylase and cellulase enzymes respectively.

Xanthan gum yield from *X. campestris* fermentation of cassava, sweet potato and rice husk flours showed that cassava flour yielded the highest quantity of xanthan (2.0 g/100 ml), while the least (0.8 g/100 ml) was from rice husk flour with conversion efficiency of 40 % and 16% respectively (Table 2). This may be attributed to the higher carbohydrate content of cassava (16) compared to rice husk which contains mainly hemicellulose, cellulose and lignin (17). It is also likely that the activity of endogenous β-glucosidase and linamarase in bitter cassava hydrolysed cyanogenic glucosides and produced fermentable sugars (15), which may have influenced the amount of xanthan produced.

Similarly, the activity of endogenous β-amylases of *Ipomoea batatas* in hydrolysing sweet potato starch (5) may have impacted on the amount of sugar and the quantity of xanthan produced. It is also conceivable that the higher yield of xanthan from cassava than sweet potato is due to the greater amount of starch and cellulose in cassava and perhaps higher yield of sugar from acid and enzyme hydrolyses than with sweet potato. Xanthan gums produced from cassava and sweet potato in this work are higher than those produced from agro-industrial by-products such as, beet molasses and sugarcane molasses (18), (19). The variations in the quantities of xanthan produced may be due to the nature of substrates and the fermentation process used in this work that includes 'agitation rest period' during the process. It may also be due to the differences in *Xanthomonas campestris* strain used.

Xanthan yield from cassava and rice husk flours increased with the quantity of the substrates (Table 3), however, the conversion efficiency from substrate to xanthan decreased with increase in substrate quantity (Table 3, 4). The difference in quantity of xanthan produced may be attributable to the composition and nature of substrates, such as starch and cellulose in cassava compared to cellulose, hemicellulose and lignin in rice husk (16), (17).

Production of xanthan gum with acid hydrolysed and non-hydrolysed substrates showed higher quantity of xanthan gum and conversion efficiency from the acid hydrolysed substrates than non-hydrolysed substrates (Table 4). This observation agrees with the report of higher xanthan gum yield from gelatinized cassava

starch than from raw cassava starch (20). The increased xanthan yield from acid hydrolysed substrate may be attributable to the effect of HCl hydrolysis on biomass and the quantity and types of sugars available in the hydrolysates (21). Xanthan gum produced in this work from hydrolysed cassava pulp appeared smoother and whiter than that from non-hydrolysed sample, apparently due to substrate modification and hydrolytic action of the acid (21). It has been shown that chemical composition of fermentation medium affects the properties of xanthan (1), (22).

The quantity of xanthan produced from fermentation of rice husk flour with *X. campestris* increased with acid hydrolysis and boiling time from 10 min to 50 min and remained unchanged with further boiling time, indicating that 50 min is the optimal boiling period (Table3). The highest xanthan yield of 1.8 g from 5 g hydrolysed rice husk was obtained after 50 min boiling time (Table 3). The increase in xanthan yield with boiling time may be attributable to the increase in the requisite hydrolysates from acid hydrolysis of rice husk flour for xanthan production. It has also been shown that increase in duration of acid hydrolysis of substrate correlates largely with increase in quantity of reducing sugars produced (23), (24), (21).

Conclusion

In conclusion, *X. campestris* produced extracellular amylolytic and cellulolytic enzymes which apparently enhanced the degradation of starch and cellulose/hemicellulose respectively in the cassava, sweet potato and rice husk, and xanthan yield. In addition, extracellular cyanidase and cyanide hydratase in cassava detoxified HCN from cyanogenic glucosides and produced hydrolysates for *X. campestris* growth and xanthan yield. The conversion efficiency of substrate to xanthan was highest with cassava followed by sweet potato, and decreased with increase in substrate concentration. Also, increase in acid hydrolysis of substrates with boiling time enhanced the quantity of xanthan produced. Further research will focus on optimizing conditions for the hydrolyses of the substrates, particularly rice husk, so as to improve conversion efficiency and xanthan yield

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