Cellulose production by *Gluconacetobacter kakiaceti* GM5 in two batch process using vinasse as culture media

Möritz Velásquez-Riaño, Nelson Lombana-Sánchez, Andrés Felipe Villa-Restrepo and Erika Pamela Fernández-Calle

ABSTRACT

The present study was conducted to evaluate the cellulose production by *Gluconacetobacter kakiaceti* GM5 by means of two aerobic treatments: the static discontinuous fermentation process (treatment 1) and the discontinuous fermentation process in a rotary shaker (treatment 2). All these experiments were carried out using vinasse as experimental culture media (VM) and were compared with standard media containing glucose at 2% (standard medium (SM)). A sample of each treatment was extracted every 24 h over a period of 168 h. The maximum rates of cellulose produced in treatment 1 using SM added up to 3.63 ± 0.18 g l^{-1}, and to 4.15 ± 0.16 g l^{-1} when VM was used. The amount of cellulose produced in treatment 2 using SM was 2.95 ± 0.09 g l^{-1} (which suggests an increase of 37%), and added up to 1.84 ± 0.07 g l^{-1} when using VM. A better global yield of both treatments in terms of sugar consumption after 168 h was obtained when using VM: 32% in treatment 1, whereas in treatment 2 it was 9%. A 20% decrease on vinasse COD (Chemical Oxygen Demand) values was found to be yet another important advantage of working with this strain.

Key words | byproducts cellulose, fermentation, *Gluconacetobacter kakiaceti* GM5, microorganism, vinasse

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INTRODUCTION

Due to the wide range of applications in the paper wood and textile industries, cellulose is an indispensable raw material (Kato *et al*. 2007). This material is the most important biopolymer produced on Earth in a natural way or by industrial manufacturing (Arioli *et al*. 1998).

The bacterium *Gluconacetobacter xylinus* (formerly *Acetobacter Xylinum*) (Yamada *et al*. 1997) has been used for decades in studies on the cellulose structure (Nguyen *et al*. 2008). Bacterial cellulose (BC) is expected to become a new industrial material because of its unique properties such as mechanical strength, high purity and biodegradability (Sutherland 1998; Kornmann *et al*. 2003). Polysaccharide-producing microorganisms are simple structures capable of constructing a polymer from the raw materials available as well as from secondary raw material sources: beets and cane (molasses, sugar syrup, and sucrose), corn (starch, hydrolyzed starch, glucose syrup, and glucose) or potatoes (starch and starch hydrolyzates) (Kongruang 2008).

The disposal of vinasse, which is the major liquid residue generated by the ethanol industry, represents a major environmental problem (Parnaudeau *et al*. 2008). This black-colored liquid produced at a rate of 10–15 times greater than ethanol itself is a mixture of water and organic and inorganic compounds (Parnaudeau *et al*. 2006), which are the elements that stay behind following different operations performed in the sugar cane production and processing. These hazardous substances bring about a very high BOD (Biological Oxygen Demand) and COD (Chemical Oxygen Demand) in the vinasse, with values rising to as much as 85 and 70 g l^{-1}, respectively (Yesilada 1999), and a pH of 4–5 (Polack *et al*. 1981). Due to its high BOD, vinasse is likely to cause damage to aquatic life, especially when it is discharged in large volumes (Cortez & Brossard-Pérez 1997). A previous study (Velásquez-Riaño & Lombana-Sánchez (2009) proved that producing BC from vinasse is an excellent alternative way to treat organic waste because it can be used in a semi-continuous way. In view of this feature, the present study points out other low cost fermentation processes with the goal of identifying the most suitable one in terms of the global

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yield of BC generated on the basis of sugar consumption using *Gluconacetobacter kakiaceti* GM5.

METHODS

Microorganism

The strain used in this study was isolated from Asian Kargasok tea and was phenotypically characterized as *Gluconacetobacter* sp. GM5 following the *Bergey's Manual of Systematic Bacteriology* (Sievers & Swings 2005). Furthermore, the identity of the bacterial isolate was confirmed by sequencing a 1212 bps rDNA fragment of 16S rRNA, which was carried out at Macrogen, Seoul, Korea. BLAST analysis was obtained from the National Center for Biotechnology Information (NCBI) website at http://www.ncbi.nlm.nih.gov/.

Cellulose determination

The procedure adopted in this study is consistent with Miller's process described by Lapuz *et al.* (1969). The cellulose was observed under 360 nm UV light, and it was compared with the cotton absorbance.

Strain support, Primary Cell Bank (PCB) and Working Cell Bank (WCB)

One hundred vials were prepared by lyophilization and used as PCB and WCB following the protocol described in a previous study (Velásquez-Riaño & Lombana-Sánchez 2009) performing an adjustment to Malik's protocol (1991) (Malik 1991).

Standard growth media and experimental culture medium

The culture medium described by Hestrin & Schramm (1954) was used as standard medium (SM). The composition of H&S medium plus magnesium sulfate included the following components stated in g l^{-1}: glucose 20.00, universal peptone 5.00, yeast extract 5.00, monohydrated citric acid 1.50, anhydride Na_2HPO_4 2.70 and $MgSO_4.7H_2O$ 0.20. Tube glasses of 13×100 mm containing a GY broth were inoculated with a single WCB vial and then cultivated for 120 h at 29 °C. After this, 250 ml conical flasks containing 50 ml of GY broth were inoculated with 5% (v/v) of GY broth (from the above tube glass culture tubes) and then incubated for 22 h at 29 °C. Subsequently, this growth culture was used to inoculate (mother inoculum) all the next essays.

The experimental culture medium employed in this test series was pure vinasse (VM). It was provided by the Cali-based alcochemical enterprise Sucromiles S. A. of Colombia. The vinasse was sterilized in an autoclave for 15 minutes at 121 °C and 15 pounds/inch^2 of pressure and then initial values were determined for pH, residual total sugars and COD in accordance with the procedure of *Standard Methods for Examination of Water and Wastewater* (1998).

Determination of the total residual sugars

Total residual sugars used in the essays realized in connection with this study were established in line with Anthrona's method, described by Gerhardt (1981).

Treatment 1 and 2

Treatment 1 comprised static discontinuous fermentation realized in seven series of tests (one test series for each sample type realized every 24 h). The conical flasks employed throughout the series of tests contained 85 ml of SM or VM, and 15% of mother inoculum. Every test series with SM and VM was incubated at 29 °C for 24, 48, 72, 96, 120, 144 and 168 h to determine cellulose production. Before and after inoculation and in every sample time an aliquot of SM or MV was used to establish its pH, total residuals sugars, COD (only in this treatment) and BC production. The cellulose was removed from any sample type by filtration (using Whatman paper N° 1) and then washed with distillate water and placed in a solution of 50 ml of sodium hypochlorite at 1.5%. After 8 h it was washed again with distillate water and dried in a heater at 105 °C until it reached constant weight. The microbial growth was measured by dry weight; the data were drawn and the growth velocity constant k and the mean duplication time g were determined, taking as a basis one balanced microbial culture and imitating one first order autocatalytic reaction (Stanier *et al.* 1996).

Treatment 2 focused on the discontinuous fermentation that occurred inside a shaker at 29 °C over a period of 168 h at 250 revolutions per minute. This procedure was realized in line with the same protocol as in treatment 1. In this treatment, COD was not determined.

Statistical analysis

All the essays were realized in triplicate and repeated twice. Data were expressed as a mean ± standard deviation (SD), and variation co-efficiency was estimated. The cellulose production change ratio ($g\,l^{-1}$) in SM and VM was determined by *t*-test for two samples assuming unequal variances. Also, the total sugar consumption yields were compared. The other variables, e.g. total residual sugars ($g\,l^{-1}$), pH and COD ($mg\,l^{-1}$) were averaged, too.

RESULTS AND DISCUSSION

All the results obtained in the first screening, which comprised the phenotypic identification of microorganism observing *Bergey's Manual of Systematic Bacteriology* (Sievers & Swings 2005) and the physico-chemical tests done to the biopolymer following Miller's protocol described by Lapuz *et al.* (1969), clearly showed that the strain used in this study belongs to the genus *Gluconacetobacter* and the product formed by his microorganism was cellulose. The 16S rDNA sequences of the bacterial isolate were determined and compared with the NCBI data. BLAST results for 16S rDNA indicated that the bacterial isolate showed 99% homology with *Gluconacetobacter kakiaceti* sp. nov. (Iino *et al.* 2012), formerly *Gluconacetobacter rhaeticus*.

Treatment 1 and 2

Treatment 1 was a discontinuous static fermentation process using SM and VM, which lasted up to 168 h. The maximum average cellulose production in SM reached a value of $3.63 \pm 0.18\,g\,l^{-1}$, which was obtained at the end of fermentation (similar production was obtained after 120 h). An even major production value was obtained when using VM for over 168 h: $4.15 \pm 0.16\,g\,l^{-1}$. Although clearly major yield rate of cellulose obtained with the experimental medium MV (near 12%) was observed, when an analysis of the rate of change in the production of biopolymer throughout the process was realized, a statistically significant difference ($p > 0.05$) between both culture media was not found (Figure 1).

The next essay, which tallies with treatment 2, was a discontinuous fermentation process realized inside a shaker over a period of 168 h at 29 °C and 250 revolutions per minute. In both culture media the growth cultures did not show any pellets and a heterogeneous mass was formed. The cellulose production yield was lower than in the first

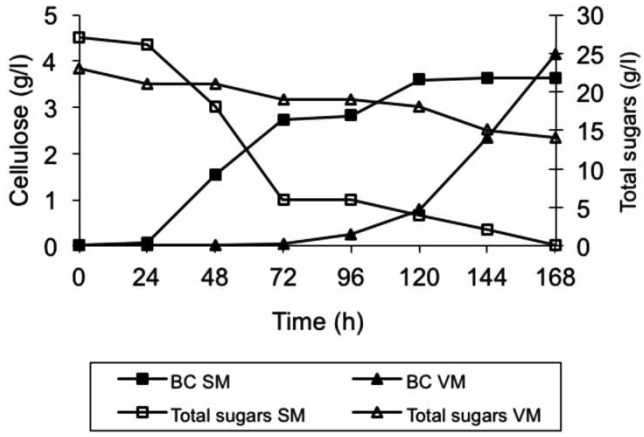

Figure 1 | Bacterial cellulose (BC) production ($g\,l^{-1}$) and total sugars consumption ($g\,l^{-1}$) in a discontinuous static fermentation with SM (Standard Medium) and VM (Experimental Medium) for 168 h (treatment 1).

treatment: the biopolymer average values ascended to $2.95 \pm 0.09\,g\,l^{-1}$ when using SM, and to $1.84 \pm 0.07\,g\,l^{-1}$ when using VM. An increase of 37% in the cellulose production was obtained when using SM, but after the statistical analysis of change rates through 68 h of fermentation, a difference of means ($p > 0.005$) was not found between SM and VM. As in treatment 1, the highest production values of biopolymer were obtained in this treatment after 120 h of fermentation with SM (Figure 2).

Comparing the global yield of cellulose ($g\,l^{-1}$) after 168 h, it was estimated that a major yield would be observed in both treatments if using VM. The obtained results confirmed that estimation: treatment 1 showed a cellulose increase of 32%, treatment 2 bettered the result by 9%. This result may be due to the forces of centrifugation that

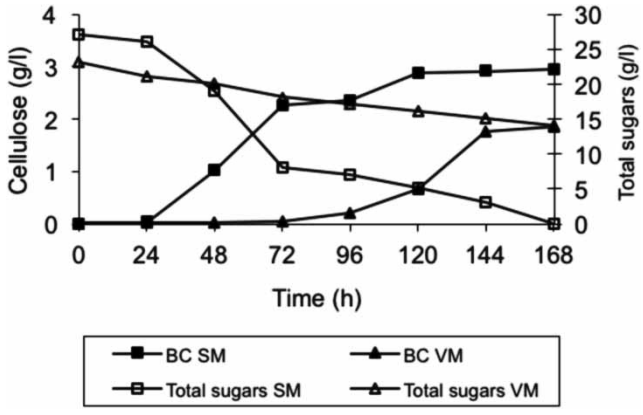

Figure 2 | Bacterial cellulose (BC) production ($g\,l^{-1}$) and total sugars consumption ($g\,l^{-1}$) in a discontinuous fermentation with SM (Standard Medium) and VM (Experimental Medium) in a shaker for 168 h and 250 revolutions per minute (treatment 2).

are directly related to the cellulose-negative (Cel^-) mutant generation, and these mutants do not produce cellulose (Ross *et al*. 1991). The properties of vinasse as a good substrate for fermentation had been observed previously, e.g. when producing biomass to obtain mushroom mycelium, when using it as a protein and fat source (Falanghe 1962), in Upflow Anaerobic Sludge Blanket (UASB) digesters to obtain biogas and compost (Chamy *et al*. 2007; Thanikal *et al*. 2007) and in a static semi-continuous fermentation to produce cellulose by *Gluconacetobacter* sp. GM5 (Velásquez-Riaño & Lombana-Sánchez 2009).

The cellulose production yield observed in the present study confirms the yields obtained by Son *et al*. (2001) for treatment 2 (comparing standard media), who produced 2.2 g l^{-1} of BC using *Acetobacter* sp. A9 after 168 h using a similar methodology (shake-flask fermentations, at 30 °C and 2% of glucose as carbon source). However, with *G. xylinus* BPR2001, Shigematsu *et al*. (2005) obtained a global yield of 3.45 g l^{-1} of BC (1.5-fold higher than that produced with *Gluconacetobacter kakiaceti* GM5) when using a corn step liquor (as experimental medium) that contained a saccharified solution made of sweet potato pulp (SPP) with a glucose concentration of 4% after 57 h of submerged process in a jar fermentor with a continuous air supply, maintaining the pH value constantly at 5.0 and treating the substrate previously with enzymes, thus obtaining a purified product, whereas the vinasse employed in our study was not pre-treated. The use of SPP from the starch production process has confirmed the thesis that industrial organic wastes can be well used as good substrates for BC production.

Bacterial growth kinetics

To study the bacterial kinetics, the cellulose production yield data were employed by sample time. The kinetic parameters analyzed in this study for both treatments (1 and 2) have been examined in Table 1. Even though similar results in the cellulose production yield were obtained when using both SM and VM, a comparison of these parameters clearly demonstrated that the growth velocity constant k decreases almost to a third when using the experimental VM: in treatment 1, the value dropped from 0.15 h^{-1} in SM to 0.05 h^{-1} in VM, whereas in treatment 2 it went down from 0.14 h^{-1} in SM to 0.05 h^{-1} in VM. The mean duplication time g decreases in a similar way. This pattern can be clearly appreciated in Figures 1 and 2, which compare cellulose production vs. fermentation time. In this pattern, it is possible to see how short the lag phase is in SM, that the log phase began at 24 h until 120 h approximately and that k declines at the same time; in VM, however, the lag phase lasts longer than in SM (96 h approximately), and the log phase could continue after 168 h.

Table 1 | Kinetic parameters derived from the model of maximum similarity between treatments 1 and 2 using SM (Standard Medium) and VM (Experimental Medium)

	Kinetic parameters	
	k (h^{-1})	g (h)
Treatment 1		
SM	0.15	4.53
VM	0.05	13.98
Treatment 2		
SM	0.14	4.81
VM	0.05	15.50

Kongruang (2008) reported growth kinetics for three different strains of *A. xylinum* when facilitating static fermentation in coconut and pineapple juice (containing approximately 20% of total residual sugars) at 30 °C for 2 weeks: *A. xylinum* TISTR 998, *A. xylinum* TISTR 975, and *A. xylinum* TISTR 893; the specific growth rate (μ, h^{-1}) was nine-fold higher when using coconut juice than with VM. When using pineapple juice, however, the results were two-fold lower than with VM. Although these three organic residues (vinasse, coconut and pineapple juice) appear to be good for producing cellulose, we were unable to compare the efficiency of these substrates based on their growth kinetics, because in the latter study the BC production was reported as wet weight.

Gluconacetobacter kakiaceti GM5 growth unfolded faster in SM. It was as fast as expected because this microorganism did not need additional steps to start specific cellulose formation: the carbon source (glucose) was as a single source. VM, on the contrary, contains many carbon sources such as sugars, acids and alcohols (Decloux & Bories 2002).

pH and COD changes during fermentation

Other variables studied in treatment 1 were total sugars (g l^{-1}), pH and COD (g l^{-1}). In SM medium, the pH quickly decreases until reaching 3.2 after the first 24 h, and this value was maintained at a constant level throughout the process. On the other hand, an increase in the pH level (obtained values close to 5.2 after 168 h) was observed in VM (Figure 3). After 168 h, residual total sugars were consumed almost totally in SM, but VM had less than half the

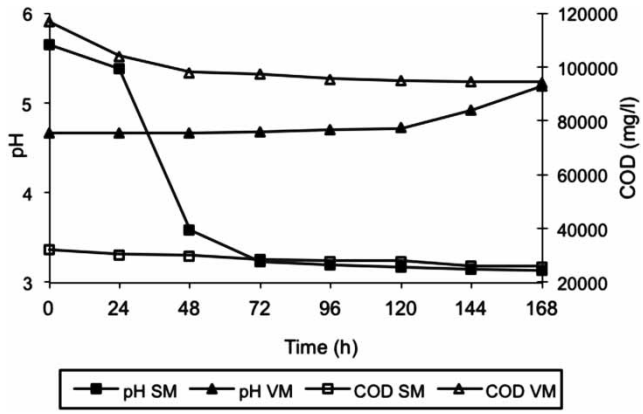

Figure 3 | pH and COD (mg/l) evolution with SM (Standard Medium) and VM (Experimental Medium) after 168 h of fermentation in treatment 1.

amount of sugars to be consumed, almost 9 g l^{-1} (Figure 1). COD was slowly reduced, reaching a 20%-reduction at the end of the process (Figure 3). In treatment 2, the change observed in all the variables was similar to that of treatment 1 (Figure 2), but it was the pH of VM which presented the highest variation: the increase was close to 5.8 after 72 h and it was maintained at a constant level until after 168 h (data not shown).

The trend towards alkalinity observed when using VM suggests that parts of the acids present in this medium were simultaneously consumed with total sugars. This trend clearly increased when using a rotary shaker in the discontinuous fermentation process (treatment 2). Hwang *et al.* (1999) reported a preferred pH of 5.5 for cell growth and cellulose production by *A. xylinum* BRC5 on the basis of accumulated gluconic acid in a medium containing 2% of glucose as carbon source in batch agitated cultures. They also showed that in comparison with batch cultures kept at constant pH-levels, shifting the pH from 4.0 to 5.5 during the cellulose production phase in batch cultures improved cellulose production and reduced the total fermentation time.

The decrease in COD values is another important advantage of working with *Gluconacetobacter kakiaceti* GM5. In the present study, we found a 20%-reduction of COD after 7 days of process. This is an excellent value for this organic waste product if compared with the 47.48%-reduction after 32 days at 25 °C when using *Phanerochaete chrysosporium* (Potentini & Rodriguez-Malaver 2006). Some processes aimed at obtaining biogas from vinasse using methanogens and UASB technologies have been previously studied (Lalov *et al.* 2001; Chamy *et al.* 2007; Thanikal *et al.* 2007), showing a COD removal of 92%, but all these processes are very complex and slow, because the bacteria consortia need at least 90 days to adapt to vinasse under strict anaerobic conditions.

CONCLUSIONS

Ethanol production poses an excellent economic alternative to the traditional fuel industry in many emerging countries. An important aspect to consider is how to employ its byproducts efficiently, especially if we keep in mind that the production of every single litre of ethanol goes together with the production of 14 l of vinasse. This study presents a biological, low cost alternative for the use of vinasse (the principal organic contaminant of bio-alcoholic distilling processes) within the bounds of cellulose production (the most commonly used biopolymer on the planet) by employing a newly isolated strain from Kargasok's tea that could be used to produce good quantities of biopolymer at huge scale by fermentation under designed conditions.

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