Biocatalysis and Agricultural Biotechnology 21 (2019) 101294

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Production and optimization of xanthan gum from three-step sequential enzyme treated cassava bagasse hydrolysate

B. Sujithra, S. Deepika, K. Akshaya, V. Ponnusami *

Center for Bioenergy, School of Chemical & Biotechnology, SASTRA Deemed University, Thanjavur, 613401, India

ARTICLE INFO

Keywords:
Cassava bagasse
Sequential enzyme pretreatment
Xanthamonas campestris
Xanthan gum

ABSTRACT

Xanthan gum was produced by microbial fermentation of enzyme hydrolyzed cassava bagasse. Cassava bagasse (CB) contains about 55–60% starch bound in a complex cellulose – hemicelluloses matrix. A mixture of pectinase and cellulase was used to release bound starch which was further hydrolyzed by alpha amylase and glucoamylase to recover maximum reducing sugar from CB. Up to 68% reducing sugars can be recovered from CB by this three step sequential enzyme hydrolysis. This was about 40% higher than that obtained by the acid hydrolysis. Cassava Bagasse hydrolysate (CBH) obtained after enzyme hydrolysis was used for xanthan gum production using *Xanthamonas campestris* NCIM 2956. Inoculum size, initial sugar concentration, nitrogen source and C/N ratio were optimized for xanthan gum production using enzyme CBH. Xanthan gum obtained was characterized using ^{1}H NMR, FTIR, and gel filtration chromatography. Under optimized conditions, xanthan gum yield obtained from enzyme treated CBH was 0.469 g xanthan gum/g CB.

1. Introduction

Xanthan gum is only the second microbial polysaccharide to be approved by FDA for use in food additives as stabilizer and thickener. It is a water soluble exo-polysaccharide (EPS) produced by a plant pathogenic bacterium, *Xanthomonas campestris*. Though *Xanthomonas* spp. like *X. phaseoli, X. malvacearum,* and *X. carotae* also produce xanthan gum, *X. campestris* can utilize wide range of carbohydrates to produce xanthan gum (Schmid et al., 2015). Therefore, *X. campestris* is widely used for xanthan gum production. Xanthan gum has a wide range of application in pharmaceutical, cosmetic, food industries and so forth (Kumar et al., 2018). Xanthan gum based nanocomposites are recently used as scaffolding materials in tissue engineering. Cellulose nanocrystal reinforced xanthan gum – sodium alginate and Xanthan gum/silica glass nanocomposites are recognized as promising scaffolding materials for tissue engineering application recently (Kumar et al., 2017a, 2017b).

On a commercial scale, xanthan gum is usually produced from simple sugars like sucrose and glucose (Palaniraj and Jayaraman, 2011). As cost of raw materials contributes significantly to the overall production cost, various low-cost agrowastes like cheese whey, sugar beet pulp, palm date, sugarcane stalks, peach pulp, molasses etc., have been considered as alternative feedstock for the production of xanthan gum in the recent past (Gilani et al., 2011; Niknezhad et al., 2015; Papi et al., 1999; Salah et al., 2011; Silva et al., 2009; Yoo and Harcum, 1999). Among various agrowastes, cassava bagasse is quite attractive for bio-conversion processes as it contains about 45–65% wt starch and has very low lignin content.

Cassava is a commercial crop and abundantly available in several countries including India, Brazil, Thailand etc. Cassava tubers are used for the production of starch and starch derivatives. Nearly 20% of the tuber is left as a fibrous residue after the recovery of starch. Starch granules present in the root cells are not easily recovered and wasted along with fibrous residues (Srikanta et al., 1987). This fibrous residue, known as cassava bagasse (CB), typically contains about 55–60% starch, 17–19% cellulose and 8–9% hemicellulose (Gunasekar et al., 2014). Bio-conversion of CB to value added products like xanthan gum not only helps to reduce production cost of bio-products, but it also prevents possible land/air pollution by providing safe disposal for CB.

For effective and maximum utilization of CB, suitable pretreatment is essential to recover reducing sugar from CB for subsequent fermentation (Chen et al., 2008; Cuevas et al., 2014). Various chemical, thermal and enzyme pretreatments are possible and in practice (Kapoor et al., 2015; Shen et al., 2011; Kamalini et al., 2018). Among chemical methods, acid treatment is reported to be superior when compared to alkali or hot water treatment (Yu et al., 2018). Acid treatment often requires harsh reaction conditions and result in the formation of intermediate

* Corresponding author. School of Chemical & Biotechnology, SASTRA University, India.
E-mail addresses: ponnusamiv@gmail.com, vponnu@chem.sastra.ac.in (V. Ponnusami).

https://doi.org/10.1016/j.bcab.2019.101294
Received 1 June 2019; Received in revised form 27 July 2019; Accepted 18 August 2019
Available online 19 August 2019

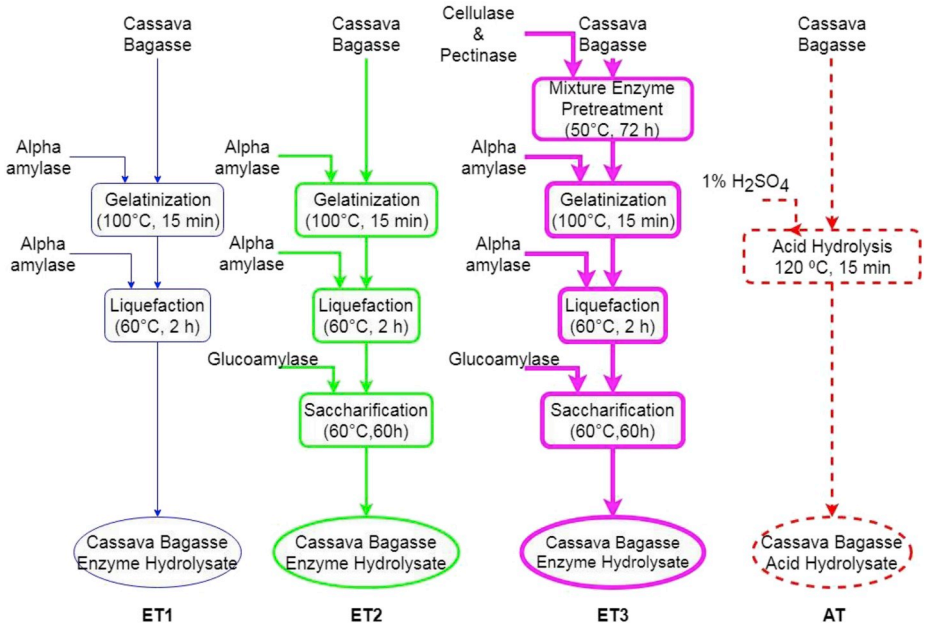

Fig. 1. Schematic Process diagrams illustrating different pretreatments ET1, ET2, ET3 and AT used for reducing sugar recovery from cassava bagasse.

substances/byproducts, which may inhibit subsequent fermentation reactions. The poor fermentability of acid Cassava Bagasse Hydrolysate (CBH) and low product yield may be attributed to high concentration of 5- HMF, furfural and sugar degradation compounds (Gunasekar et al., 2014; Mohsin et al., 2018; Taherzadeh et al., 1997; Yu et al., 2018). But as enzymes are very specific, and therefore, such inhibitors are not produced during enzyme pretreatment. Therefore, fermentability of enzyme treated CBH is superior to acid treated CBH. (Wang et al., 2017a). As the major component of CB is starch, amylolytic enzymes like alpha amylase and amyloglucosidase are most commonly used (Valeriano et al., 2018; Ona et al., 2019). However, since starch is bound in cellulose/hemicelluloses fibers prior to amylolytic enzyme treatment often mild hydrothermal/acid (Yu et al., 2018)/alkali/microwave treatments (Pooja et al., 2018; Sudha et al., 2017) are used. But, this would lead to formation of inhibitory substance again. Thus, the objective of the present work is to completely eliminate thermal/acid treatment and to use only enzyme treatment for efficient reducing sugar recovery. Enzymes like pectinase had been shown to possess synergy with cellulase and improve cellulose hydrolysis in various lignocellulosic residues (Li et al., 2014; Zhang et al., 2013). Thus, cellulase and pectinase enzyme mixture is used in this study prior to use of amylolytic enzymes to improve the overall fermentable sugar recovery efficiency and to minimize inhibitory substances. The resulting hydrolysate was then subjected to fermentation to produce a commercially important microbial exopolysaccharide, xanthan gum.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals

Acetonitrile (HPLC grade) was obtained from Sigma-Aldrich Co. St Louis, Missouri, USA. All other chemicals used were obtained from Merck & Co., Inc., Kenilworth, New Jersey (USA). Cellulase, pectinase, xylanase, glucoamylase were purchased from Thai biotech, Thiruchirapalli (India). Standard pullulan kit WAT034207 was purchased from Waters, Massachusetts, US.

2.1.2. Microorganism and culture media

The organism used in this study, *Xanthamonas campestris* NCIM 2956, was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. This culture was maintained on MGYP medium and was sub-cultured once in every two weeks. Composition of MGYP medium is as followed: (g/l) malt extract - 3; glucose - 10; yeast extract - 3; peptone - 5; and agar - 20.

Production media has the following composition: (g/l) carbon source - 20, yeast extract - 10, calcium carbonate - 3, Magnesium sulphate heptahydrate - 0.25, potassium nitrate - 1%. Either dextrose or CBH was used as carbon source in the production medium as required.

2.1.3. Substrate collection

Cassava bagasse was collected from a sago industry, located in Salem District, Tamil Nadu, India. This CB was dried, milled, and sieved and BSS #150/170 mesh size particles were collected. The particles were then dried to a constant weight at 80 °C and stored in moisture free condition.

2.1.4. Acid hydrolysis

Dilute acid hydrolysis was performed by adding 1% H_2SO_4 to the CB slurry containing 200 g CB/l and the mixture was maintained at 120 °C and 15 psi for 15 min. The pretreated slurry was vacuum filtered to obtain a clear hydrolysate. The filtered hydrolysate was treated with activated charcoal at 60 °C for 30 min to remove soluble impurities from the hydrolysate.

2.1.5. Enzyme hydrolysis

A three step enzyme pretreatment with cellulase, pectinase, alpha amylase and glucoamylase was carried out. Optimum pH for cellulase, pectinase, α amylase and glucoamylase are 4.8, 4.8, 6 and 4.7 respectively. Therefore, in each step pH was adjusted to the corresponding optimum level. Cellulase 100 Filter Paper Units (FPU)/g dry CB and pectinase 33 (Polygalactouronase units) PGU/g dry CB each were added to 20 wt % CB slurry in the first step. The slurry pH was adjusted to 4.8 and the mixture was maintained at 50 °C for 72 h. In the second step, 50 U/g dry CB of alpha amylase was added to the mixture. The mixture pH was adjusted to 6and it was maintained at 100 °C for 15 min to reduce the viscosity of the hydrolysate to facilitate subsequent enzyme hydrolysis. The slurry was then cooled to 60 °C and another 200 U/g dry CB alpha amylase was added to the slurry and the mixture was maintained at 60 °C for 2 h. In the third step, glucoamylase (300 U/g dry CB) was

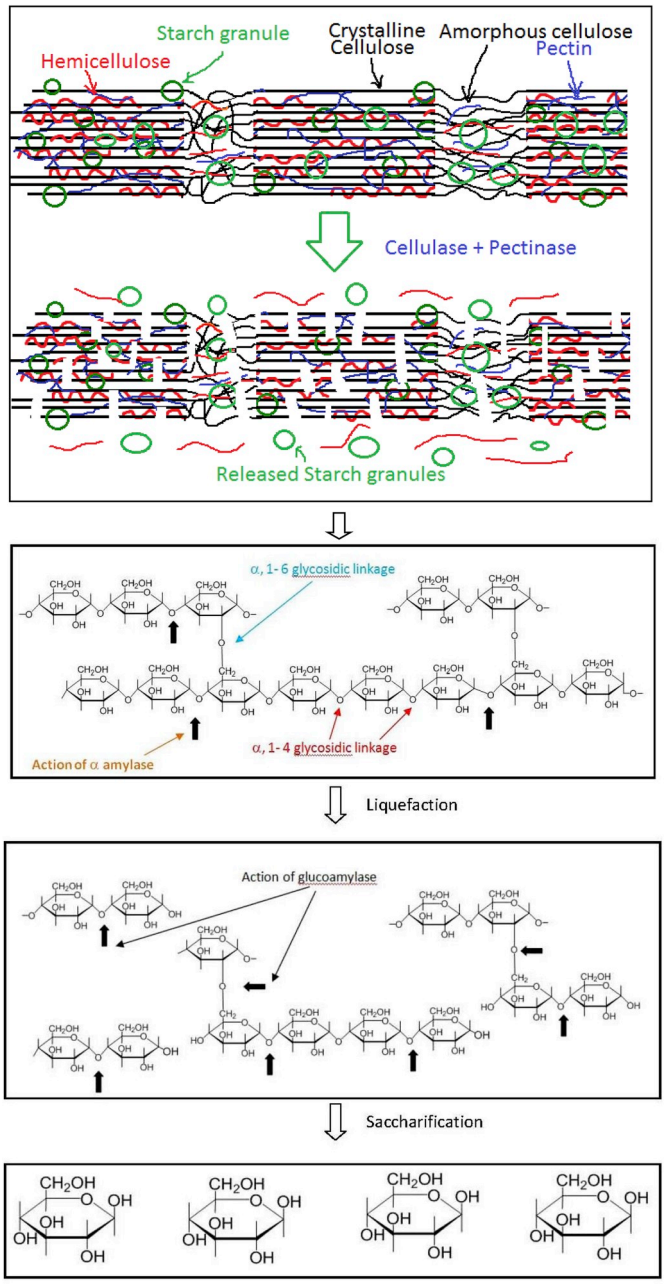

Fig. 2. Schematic diagram showing the mechanism of enzyme actions on the substrate.

added to the liquefied slurry and the mixture was kept at 60 °C and pH 4.7 for 60 h. Both α amylase and glucoamylase are starch degrading enzymes. While α amylase is an endo-amylase, glucoamylase is an exo-amylase. Once starch is released from the substrate (CB), enzyme hydrolysis of starch is carried out in two steps as shown in Fig. 1. In the first step, starch is gelatinized and liquefied by α amylase to produce water soluble dextrin. During this step, α amylase breaks linear amylose chains (α, 1 → 4 linkages) at random positions but does not break branch chains linkages (α, 1 → 6 linkages) to produce dextrin, a mixture of low molecular weight carbohydrates. In the second step, glucoamylase convert dextrin to more fermentable monomeric sugars by breaking α, 1 → 4 and α, 1 → 6 glucosidic linkages. Thus, α amylase followed by glucoamylase is the preferred sequence. There are reports on simultaneous use of these two enzymes but as the optimum pH and temperature differ for these enzymes two step process is recommended. Schematic representation of the mechanism is shown in Fig. 2. The resulting hydrolysate was vacuum filtered and the pH of the filtrate was adjusted to 7. The neutralized hydrolysate was stored at – 20 °C until further use. For the purpose of comparison alpha amylase and amyloglucosidase enzyme treatments were used without cellulase and pectinase treatment independently as shown in Fig. 1.

2.1.6. Fermentation

Fermentation was done with 100 ml of production media in 250 ml Erlenmeyer flasks without pH control. Production media was autoclaved at 120 °C, for 15 min and inoculated with 10% (v/v) of *X. campestris* culture. The culture was kept at 30 °C at 200 rpm in an incubated shaker for five days.

2.1.7. Analytical determinations and characterization

2.1.7.1. Determination of cell dry weight. Samples were collected at regular intervals and centrifuged at 10,000 rpm for 10 min to remove cells. Cell dry weight was obtained by washing and drying the recovered cells to a constant weight at 90 ± 1 °C.

2.1.7.2. Determination of sugars in the fermentation broth. Waters HPLC system, equipped with a Waters 2535 Quaternary gradient pump, and a 2414 Refractive Index detector was used to determine reducing sugar composition. An analytical amino column (4.6 × 250 mm) was used as stationary phase and Acetonitrile – Water (80:20) mixture was used as mobile phase at a flow rate of 1 ml/min to determine reducing sugar composition. Glucose, galactose, arabinose, xylose and mannose HPLC standards were used for the calibration of the sugars.

2.1.7.3. Recovery of xanthan gum. Samples collected from fermentation broth were autoclaved at 120 °C for 15 min and centrifuged at 10,000 rpm for 15 min to obtain a cell free supernatant. The cell free supernatant was added with three volumes of ice cold acetone and left undisturbed for 2 day at 4 °C to allow precipitation to occur. The resulting precipitate was vacuum filtered, dried at 55 °C and stored. The process flow is shown in Fig. 3. The yield was calculated on the basis of raw CB according to the equation given below:

$$\%Y = \frac{Xanthan\ gum\ obtained\ (g/L)}{reducing\ sugar\ used\ for\ fermentation\ (g/L)} \times \frac{reducing\ sugar\ obtained\ (g/L)}{CB\ feed\ (g/L)} \quad (1)$$

2.1.7.4. Characterization of xanthan gum using HPSEC, FTIR and ^1H NMR. Average molecular weight of the product was determined using high pressure size exclusion chromatography (HPSEC). A Waters SEC (Ultrahydrogel 1000, 7.8 × 300 mm) column with 0.5 M sodium nitrate was used for determining the molecular weight of the water soluble xanthan gum. Standard pullulan kit was used for calibration. Fourier Transform Infrared Spectroscopy (FTIR) was used for functional group analysis. KBr pellet method was used for preparing the sample and was operated in a spectral window of 400–4000 waves/cm. To confirm the structure of the obtained polymer ^1H NMR analysis was performed. 20 mg of xanthan gum sample was solubilized in deuterated water and scanned at room temperature in Bruker 500 MHz instrument.

3. Results and discussion

3.1. Enzymatic pretreatment and hydrolysis

It can be seen from Fig. 4 that based on reducing sugar yield the four treatment methods can be arranged in the following order ET3 > AT > ET2 > ET1. Total reducing sugars obtained from ET 3 enzyme pretreatment (135.98 g/l) was 1.4 times higher than that obtained from acid pretreatment (96 g/l). Lower RS yield obtained in ET1

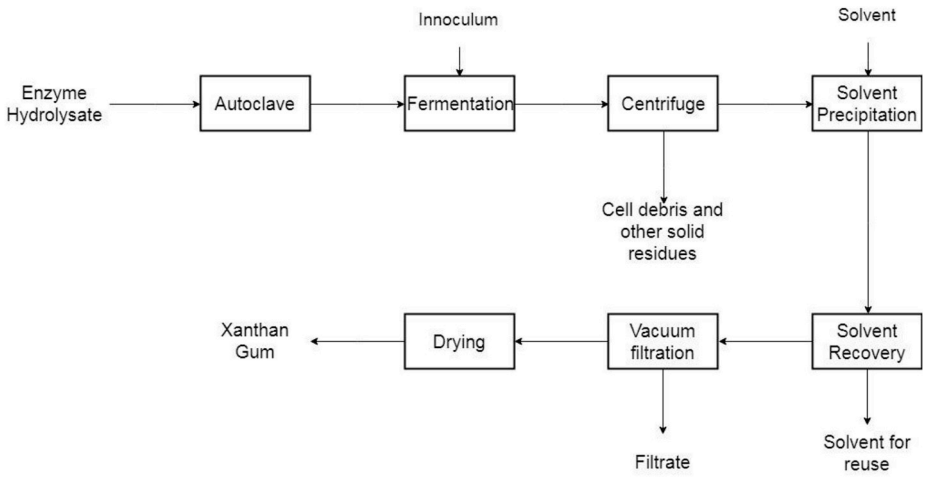

Fig. 3. Block diagram of fermentation and product recovery.

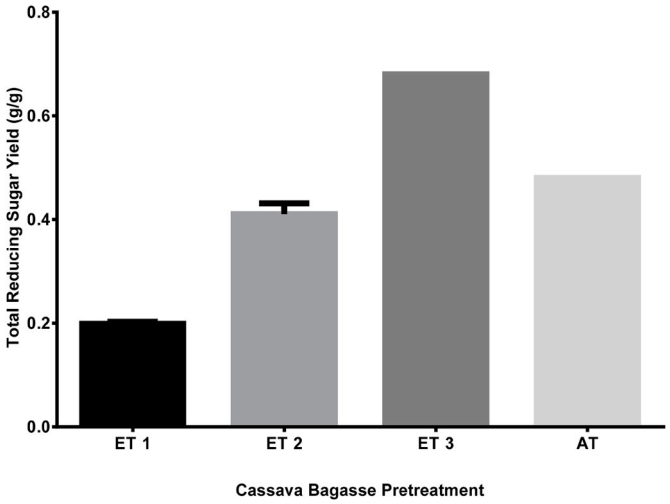

Fig. 4. Total reducing sugar yield from different pretreatment methods. (ET1, ET2, ET3 and AT refer to Enzyme and Acid pretreatment conditions as illustrated in Fig. 1.)

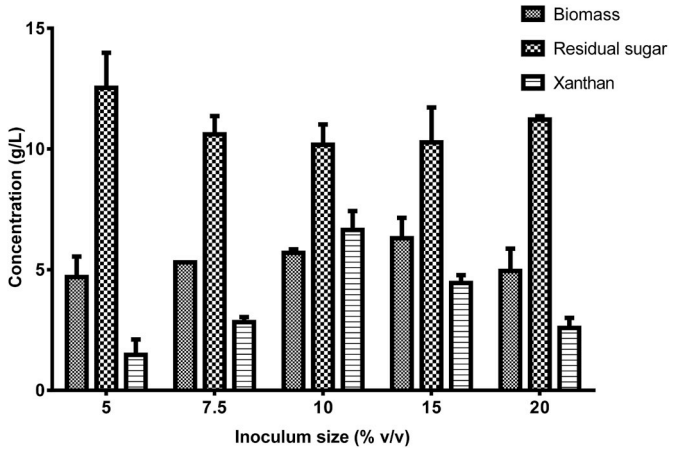

Fig. 5. Effect of inoculum size using enzyme treated Cassava Bagasse Hydrolysate on biomass, xanthan gum, residual sugar (pH 7, 30 °C, 5 days, 200 rpm, inoculum size – 5–20% v/v, initial sugar concentration for- 25 g/l).

Table 1
Cost of enzyme used for pretreatment in ET3 (Basis: 1 kg of cassava bagasse feed).

Enzyme	Enzyme added (Units)	Cost of enzyme (in Indian Rupees)
Cellulase	500	2.51
Pectinase	165	0.61
Alpha amylase	250	0.96
GAM	1500	7.94
Total cost		**12.05**

and ET2 is due to the fact that the starch content in CB is not accessible to starch degrading enzymes owing to the complex structure of CB. Enzyme hydrolysis after pretreating with pectinase and cellulase, ET3, showed threefold increase in the total reducing sugar yield than ET1 & ET2 (Fig. 4). This can be attributed to the action of pectinase and cellulase on the complex cellulose – hemicellulose – starch three dimensional network structure of CB leading to efficient release of bound starch. Cellulose degrading enzymes like cellulase and pectinase enable the release of the trapped starch granules from the fibers leading to enhanced starch recovery (George et al., 1991). Once starch is released, alpha amylase and glucoamylase have improved access to the starch and this leads to better enzyme-substrate interaction and enhanced sugar yield. The composition of hydrolysate obtained from ET3 was 95.4% glucose and 4.6% galactose.

Woiciechowski et al. (2004) obtained 62.4% conversion of CB into reducing sugar by acid hydrolysis and 64.75% by enzyme hydrolysis using alpha amylase and glucoamylase. Gaewchingduang et al. (2010) converted 57% CB to sugar by acid hydrolysis along with hydrothermal treatment. Kosugi et al. (2009) reported 61.1% conversion of CB to sugars using cellulase, alpha amylase and glucoamylase. Shamala and Sreekantiah (1986), showed that by using *Aspergillus ustus* enzyme preparation having cellulase, xylanase, glucosidase, alpha amylase, amyloglucosidase and pectinase activities, 45% CB could be converted to reducing sugar. Single and two step pretreatment process used with commercial enzymes namely Liquozyme® SC DS, Spirizyme® Fuel, Novozyme® NS 50,012 and Novozyme® NS 50,013 were found to release 0.37–0.51 g/g RS (Virunanon et al., 2013). Cassava peel waste upon hydrolysis by Cellulase, alpha amylase and amyloglucosidase enzymes produced 0.58 g of RS/g (Aruwajoye et al., 2019). A two step enzyme treatment of cassava peels by amylolytic enzymes (amylase and glucoamylase) followed by cellulase enzymes resulted in 62% reducing sugar yield (Ona et al., 2019). In our work, 48% CB has been converted to sugar by acid hydrolysis and 68% by enzymatic hydrolysis. This sugar yield obtained from the 3 step enzyme hydrolysis is higher than earlier reports in literature thus proving that the multi enzymatic hydrolysis enhanced the conversion of CB to sugar. Reducing sugars produced from

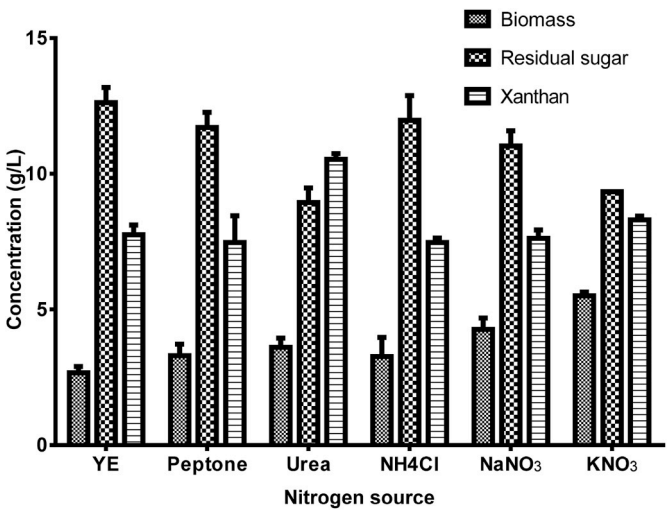

Fig. 6. Effect of nitrogen source using enzyme treated cassava bagasse hydrolysate on biomass, xanthan gum, residual sugar (pH 7, 30 °C, 5 days, 200 rpm, initial sugar concentration- 25 g/l, C/N ratio- 25, inoculum size- 10% v/v), Y. E. - Yeast extract.

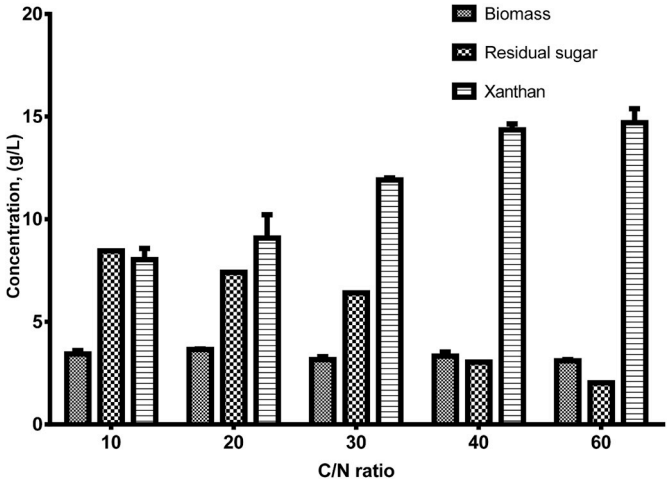

Fig. 7. Effect of C/N ratio on (a) biomass, xanthan gum, residual sugar (b) product yield (pH 7, 30 °C, 5 days, 200 rpm, initial sugar concentration- 30 g/l, inoculum size – 10% v/v, nitrogen source – urea, substrate: Enzyme treated cassava bagasse hydrolysate).

1 kg CB was 0.68 kg. At a conservative price of Rs. 60 per kg of glucose, the cost of sugar produced from 1 kg of CB is estimated to be around Rs. 40.8. The actual cost of enzyme used in the process was Rs. 12.05 per kg of CB used (Table 1). Thus, enzyme cost is only about 30% of the product value. Considering the fact that CB is a waste to be disposed off, the process has very good economic potential.

3.2. Fermentation

Enzyme hydrolysate of CB was subjected to fermentation as described above to investigate the effect of fermentation conditions on the exo-polysaccharide production. Effect of process variables like inoculum size, sugar concentration, nitrogen source and C/N ratio were studied.

3.2.1. Effect of inoculum size

The effect of inoculum size on xanthan gum production using enzyme hydrolysate as carbon source is shown in Fig. 5. Inoculum size had a significant effect on xanthan gum yield ($p < 0.005$). A maximum yield of 0.181 g/g CB was achieved at an inoculum concentration of 10% v/v. With further increase in inoculum size, xanthan gum yield decreased and biomass concentration increased. Similar trend was reported by Salah et al. (2011) [6] while producing xanthan gum from date juice palm by-products using *Xanthomonas campestris*.

They have reported that optimum inoculum size was 5% for xanthan gum production and beyond this only biomass accumulation was favored. However, inoculum size required for maximum production of a metabolite depends on the strain and the desired product.

3.2.2. Effect of nitrogen source

Nitrogen source had a significant role in the production of xanthan gum. Organic and inorganic nitrogen sources namely, yeast extract, peptone, urea, NH_4Cl, $NaNO_3$, KNO_3 were studied with a C/N ratio of 25. The results are shown in Fig. 6. Urea resulted in most efficient conversion of reducing sugar into xanthan gum. A maximum product yield (Y_{PS}) of 0.287 g xanthan gum/g CB was achieved when urea was used as a nitrogen source. Urea also resulted in the maximum reducing sugar consumption among all the nitrogen sources examined. KNO_3 showed similar sugar utilization but, in this case the sugars were directed mostly towards biomass growth rather than xanthan gum production. Woiciechowski et al. (2004) studied the effect of different nitrogen sources on xanthan gum production from HCl treated CB hydrolysate.

It was reported that among the nitrogen sources yeast extract, peptone, urea, potassium nitrate and ammonium sulphate, urea was the least efficient for biomass production. But for xanthan gum production both urea, potassium nitrate had a positive effect.

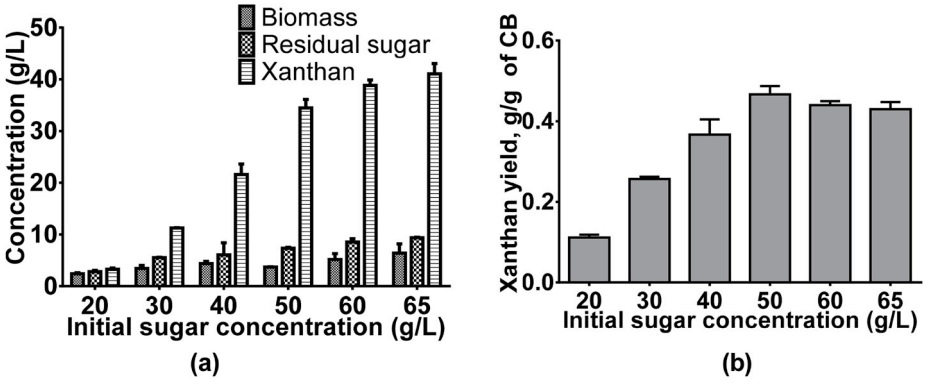

Fig. 8. Fermentation of enzyme treated hydrolysate. Effect of initial sugar concentration using enzyme treated cassava bagasse hydrolysate on (a) biomass, xanthan gum, residual sugar and (b) product yield (pH 7, 30 °C, 5 days, 200 rpm, inoculum concentration – 10% v/v, initial sugar concentration- 20–65 g/l, nitrogen source - urea).

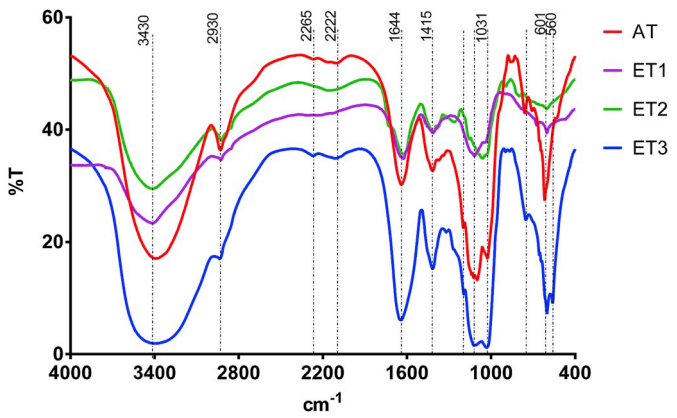

Fig. 9. FTIR spectra of xanthan gum from enzyme and acid treated hydrolysates. (AH –Acid pretreated cassava bagasse hydrolysate; ET1 - Enzyme pretreatment 1; ET2 Enzyme pretreatment 2; ET3 - Enzyme pretreatment 3).

3.2.3. Effect of carbon to nitrogen ratio

Carbon to nitrogen (C/N) ratio was varied as 10, 20, 30, 40 and 60 using urea as carbon source. The results are shown in Fig. 7. Upon increasing the C/N ratio, xanthan gum production increased constantly. Sugar utilization also increased resulting in 2.05 g/l residual reducing sugar for the C/N ratio 60. Biomass production did not change significantly and the utilized sugars were directed only towards xanthan gum production. Nitrogen is essential for cell growth but is not needed for xanthan gum formation. Therefore, lower C/N ratio favors cell growth while higher C/N ratio favors xanthan gum formation. By limiting nitrogen availability, metabolic flux is shifted towards xanthan gum formation rather than cell growth. Mostly, a C/N ratio between 35 and 40 has been widely reported as optimum C/N ratio in previous literature for different nitrogen sources like, NH_4NO_3, $(NH_4)_2PO_4$, peptone (Casas et al., 2000; Garcia-Ochoa et al., 1995; Garcıa-Ochoa et al., 2000). A maximum of 77 C/N ratio was used by Yoo and Harcum (1999) to for waste sugar beet pulp to obtain a xanthan gum yield of nearly 80%. Since the xanthan gum yield did not improve significantly when C/N ratio was changed from 40 to 60 for further studies C/N ratio of 40 was used. With a C/N ratio of 40, xanthan gum yield obtained was 0.390 g/g of CB.

3.2.4. Effect of sugar concentration

To study the effect of sugar concentration on biomass and polysaccharide yield, the purified and concentrated hydrolysates were diluted to 20, 30, 40, 50, 60 and 65 g/l initial reducing sugar concentrations using distilled water. For effective fermentation, initial sugar concentration is critical. Sugars will be used for the formation of cell components and product synthesis and for cell maintenance. Product yield typically increases with increasing sugar concentration (Li et al., 2016). However, if sugar concentration is too high, substrate inhibition may occur, resulting in low product yield (Leela and Sharma, 2000; Niknezhad et al., 2015).

From Fig. 8 (a) it can be seen that the initial sugar concentration had a significant effect on xanthan gum yield ($p < 0.05$) from enzyme treated CBH. Xanthan gum production had shown increasing trend with increase in initial sugar concentration. However, as evident from Fig. 8 (b) the specific yield of xanthan gum decreased beyond an initial sugar concentration of 50 g/l. Maximum yield 0.469 g/g was obtained at this initial sugar concentration (50 g/L), and then it dropped slowly with further increase in initial sugar concentration. The results were consistent with the previous literature on effect of initial sugar concentration. Carbon source in the range of 1–4% were shown to be conducive for xanthan gum production by several researchers (Palaniraj and Jayaraman, 2011; Souw and Demain, 1980; El Enshasy et al., 2011). The increase in xanthan gum yield obtained may be attributed to two reasons. First, enzyme pretreatment results in higher sugar yield from cassava bagasse without formation of inhibitory compounds. Absence of inhibitory compounds and favorable fermentable sugar composition had a positive effect on xanthan gum yield per gram of cassava bagasse. Second, optimization of fermentation medium and composition, in particular, optimum C/N ratio had a favorable effect on carbon flux towards product formation.

3.3. Production in laboratory scale fermentor

Fermentation was carried out with optimized medium as discussed above under optimized conditions in a 3 L laboratory scale fermentor (Hygiene Bioreactor supplied by Lark Innovative Fine Teknowledge) with 2.5 L working volume. Glucose was autoclaved separately and added to the broth to prevent Millard reaction. Dissolved oxygen and pH were monitored using Broadley James Oxyprobe and Fermprobe, respectively. pH was maintained between 6.4 and 6.8 during fermentation. Five days after inoculation, fermentor was stopped and xanthan

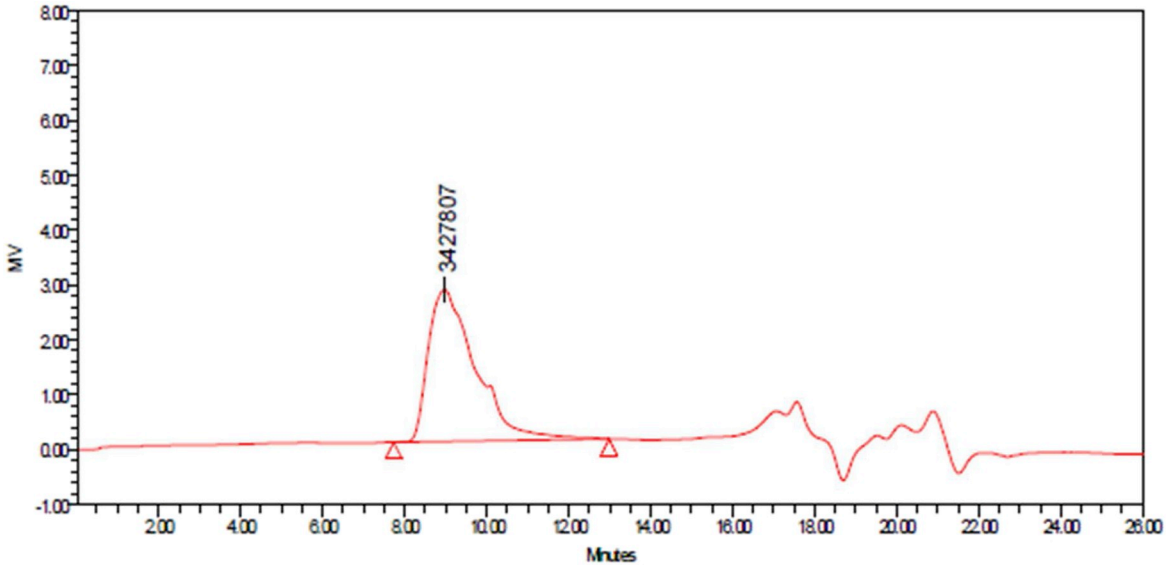

Fig. 10. GPC chromatogram of purified xanthan gum. Peak at retention time (R_T) 9 min corresponds to polymer molecular weight of 3×10^6 Da.

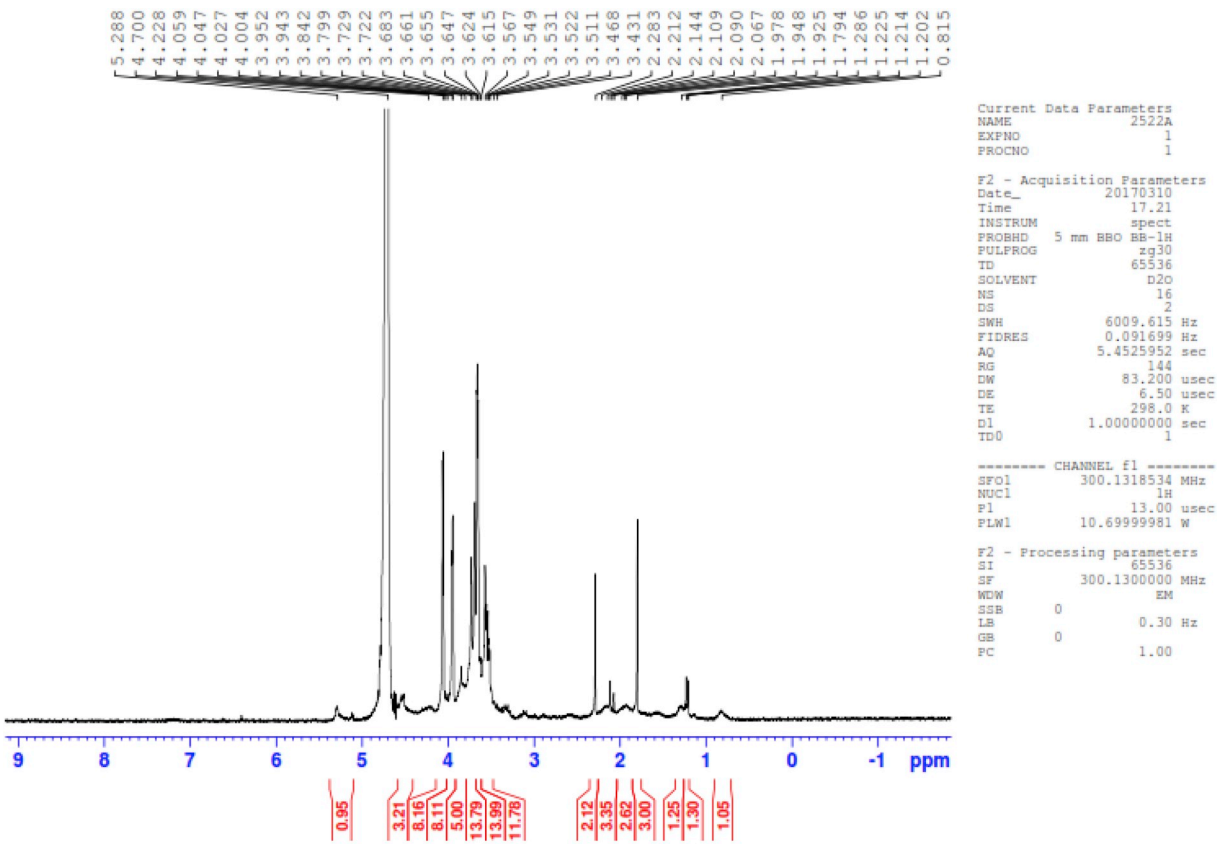

Fig. 11. Proton nuclear magnetic resonance (^{1}H NMR).

gum was recovered from the broth as described in section 2.1.6. Xanthan gum yield was found to be 0.491 g/g CB. At the end of fermentation, residual sugar and biomass concentrations in the medium were found to be 6 g/L and 3.28 g/L., respectively. Viscosity of fermentation broth was measured using Brookfield LV DV –II + Pro extra with spindle S 64 at 200 rpm (shear rate = 264 s^{-1}) at the beginning and end of fermentation. Viscosity was 1.34 cP and 158 cP, respectively at the beginning (0^{th} day) and end (5th day) of fermentation.

3.4. Characterization of xanthan gum

Fourier transform IR spectra of xanthan gum obtained from all four hydrolysate obtained from different enzyme treatments and acid treatment are shown in Fig. 9. The spectra were nearly identical. While pretreatment was carried out in multiple stages, fermentation was carried out in single stage as shown in the flow sheets (Figs. 1 and 2). We believe that while the composition of fermentation feed (cassava bagasse hydrolysate) influence the product yield, it does not affect the structure of the product. Comparison of FTIR spectra of xanthan gum samples produced from cassava bagasse hydrolysate obtained by all the four different pretreatment methods were nearly identical confirming our hypothesis. Strong and wide absorption bands at 3400 cm^{-1} and 3430 cm^{-1} were due to hydroxyl stretching. Absorption bands between 2900 and 3050 cm^{-1} correspond to the C–H stretching from aldehyde, CH_2 or CH_3 groups. Strong carbonyl group absorption was detected in the wave number range of 1640–1700 cm^{-1}. C–H deflection angle and C–O stretching vibration were observed in the range of 1410–1420 cm^{-1} and 1020- 1125 cm^{-1}. The FTIR spectra of xanthan gum from acid and enzyme hydrolysates were similar to the spectra obtained for xanthan gum in the literature (Gunasekar et al., 2014).

Molecular weight for xanthan gum obtained through HPSEC was 3×10^6 Da (Fig. 10). Typically, average molecular weight of xanthan gum range from 3×10^5 to 7.5×10^6 Da (Cano-Barrita and Leon-Martínez, 2016). At lower C/N ratio, xanthan gum with different molecular weights 4×10^6, 6×10^5 and 1×10^5 were produced. Kalogiannis et al. (2003) observed a molecular weight of 0.8–1.4 $\times 10^6$ Da upon using molasses as substrate. They stated that increase in sugar concentration lead to production of xanthan gum with lower molecular weight (Kalogiannis et al., 2003). Moreover, differences in fermentation conditions impart differences in molecular weight (Z. Wang et al., 2017). Therefore, by adjusting the sugar concentration and other fermentation conditions with yield, desired molecular weight range can be achieved.

^{1}H NMR spectra of xanthan gum (Fig. 11) indicates the presence of H-1α anomeric proton with a peak at 5.28 ppm. The hydrogen located near the hydroxyl group can be identified at 4 ppm. Peak at 1.286 and 2.09 ppm corresponds to pyruvate and acetate groups. The results are consistent with previous literature and confirm the presence of xanthan gum.

4. Conclusion

A three stage enzyme treatment with pectinase, cellulase, α-amylase and glucoamylase was proven to be a more effective way of recovering fermentable sugars from the agro industry waste, cassava bagasse. Reducing sugar obtained by three step enzyme treatment was subjected to microbial fermentation for the production of xanthan gum. Effect of fermentation conditions like initial sugar concentration, nitrogen source, inoculum size and C/N ratio were studied and optimized. Under optimum fermentation conditions enhanced xanthan gum yield of 0.469 g/g CB was obtained. The multi stage enzyme hydrolysate yielded better xanthan gum through maximum utilization of substrate. FTIR and ^{1}H NMR results confirm the structure of xanthan gum. The molecular weight of xanthan gum obtained was 3×10^3 kDa.

Acknowledgements

The authors thank SASTRA Deemed University for providing infrastructure and research facility for the successful completion of this research work. The authors gratefully thank DST for support through FIST programme (SR/FST/ETI-331/2013) and DBT for support through the EMR grant (BT/PR7511/BCE/8/973/2013).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101294.

References

Aruwajoye, Gabriel S., Faloye, Funmilayo D., Gueguim, Evariste, 2019. Process optimisation of enzymatic saccharification of soaking assisted and thermal pretreated cassava peels waste for bioethanol production. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-018-00562-0.

Cano-Barrita, P.d.J., Leon-Martínez, F., 2016. Biopolymers with Viscosity-Enhancing Properties for Concrete Biopolymers and Biotech Admixtures for Eco-Efficient Construction Materials. Elsevier, pp. 221–252.

Casas, J., Santos, V., Garcıa-Ochoa, F., 2000. Xanthan gum production under several operational conditions: molecular structure and rheological properties. Enzym. Microb. Technol. 26 (2–4), 282–291.

Chen, M., Zhao, J., Xia, L., 2008. Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars. Carbohydr. Polym. 71 (3), 411–415.

Cuevas, M., García, J.F., Sánchez, S., 2014. Enhanced enzymatic hydrolysis of pretreated almond-tree prunings for sugar production. Carbohydr. Polym. 99, 791–799.

El Enshasy, H., Then, C., Othman, N., Al Homosany, H., Sabry, M., Sarmidi, M., Aziz, R., 2011. Enhanced xanthan production process in shake flasks and pilot scale bioreactors using industrial semidefined medium. Afr. J. Biotechnol. 10 (6), 1029–1038.

Gaewchingduang, S., Pengthemkeerati, P., 2010. Enhancing efficiency for reducing sugar from cassava bagasse by pretreatment. World Acad. Sci. Eng. Technol. 70, 728–731.

Garcia-Ochoa, F., Santos, V., Alcon, A., 1995. Xanthan gum production: an unstructured kinetic model. Enzym. Microb. Technol. 17 (3), 206–217.

Garcıa-Ochoa, F., Santos, V., Casas, J., Gómez, E., 2000. Xanthan gum: production, recovery, and properties. Biotechnol. Adv. 18 (7), 549–579.

George, M., Padmaja, G., Moorthy, S.N., 1991. Enhancement in starch extractability from cassava (Manihot esculenta crantz) tuber through fermentation with a mixed culture inoculum. J. Root Crops 17 (1), 1–9.

Gilani, S., Najafpour, G., Heydarzadeh, H., Zare, H., 2011. Kinetic models for xanthan gum production using Xanthomonas campestris from molasses. Chem. Ind. Chem. Eng. Q./CICEQ 17 (2), 179–187.

Gunasekar, V., Reshma, K.R., Treesa, G., Gowdhaman, D., Ponnusami, V., 2014. Xanthan from sulphuric acid treated tapioca pulp: influence of acid concentration on xanthan fermentation. Carbohydr. Polym. 102, 669–673.

Kalogiannis, S., Iakovidou, G., Liakopoulou-Kyriakides, M., Kyriakidis, D.A., Skaracis, G. N., 2003. Optimization of xanthan gum production by Xanthomonas campestris grown in molasses. Process Biochem. 39 (2), 249–256.

Kamalini, A., et al., 2018. Optimization of sugar recovery efficiency using microwave assisted alkaline pretreatment of cassava stem using response surface methodology and its structural characterization. J. Mol. Liq. 254, 55–63.

Kapoor, M., Raj, T., Vijayaraj, M., Chopra, A., Gupta, R.P., Tuli, D.K., Kumar, R., 2015. Structural features of dilute acid, steam exploded, and alkali pretreated mustard stalk and their impact on enzymatic hydrolysis. Carbohydr. Polym. 124, 265–273.

Kosugi, A., Kondo, A., Ueda, M., Murata, Y., Vaithanomsat, P., Thanapase, W., Arai, T., Mori, Y., 2009. Production of ethanol from cassava pulp via fermentation with a surface-engineered yeast strain displaying glucoamylase. Renew. Energy 34 (5), 1354–1358.

Kumar, A., Rao, K.M., Han, S.S., 2018. Application of xanthan gum as polysaccharide in tissue Engineering : a review. Carbohydr. Polym. 180, 128–144.

Kumar, A., Rao, K.M., Han, S.S., 2017. Development of sodium alginate-xanthan gum based nanocomposite scaffolds reinforced with cellulose nanocrystals and halloysite nanotubes. Polym. Test. 63, 214–225.

Kumar, A., Rao, K.M., Kwon, S.E., Lee, Y.N., Han, S.S., 2017. Xanthan gum/bioactive silica glass hybrid scaffolds reinforced with cellulose nanocrystals: morphological, mechanical and in vitro cytocompatibility study. Mater. Lett. 193, 274–278.

Leela, J.K., Sharma, G., 2000. Studies on xanthan production from Xanthomonas campestris. Bioprocess Eng. 23 (6), 687–689.

Li, J., Zhou, P., Liu, H., Xiong, C., Lin, J., Xiao, W., Gong, Y., 2014. Synergism of cellulase , xylanase , and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. Bioresour. Technol. 155, 258–265.

Li, P., Li, T., Zeng, Y., Li, X., Jiang, X., Wang, Y., Xie, Y., Zhang, Y., 2016. Biosynthesis of xanthan gum by Xanthomonas campestris LRELP-1 using kitchen waste as the sole substrate. Carbohydr. Polym. 151, 684–691.

Mohsin, A., Zhang, K., Hu, J., Salim-Ur-Rehman, Tariq, M., Zaman, W.Q., Khan, I.M., Zhuang, Y., Guo, M., 2018. Optimized biosynthesis of xanthan via effective valorization of orange peels using response surface methodology: a kinetic model approach. Carbohydr. Polym. 181, 793–800.

Niknezhad, S.V., Asadollahi, M.A., Zamani, A., Biria, D., Doostmohammadi, M., 2015. Optimization of xanthan gum production using cheese whey and response surface methodology. Food Sci. Biotechnol. 24 (2), 453–460.

Ona, John Ikuba, Halling, Peter J., Ballesteros, Mercedes, 2019. Enzyme hydrolysis of cassava Peels : treatment by amylolytic and cellulolytic enzymes. Biocatal. Biotransform. 37 (2), 1–9.

Palaniraj, A., Jayaraman, V., 2011. Production, recovery and applications of xanthan gum by Xanthomonas campestris. J. Food Eng. 106 (1), 1–12.

Papi, R., Ekateriniadou, L., Beletsiotis, E., Typas, M., Kyriakidis, D., 1999. Xanthan gum and ethanol production by Xanthomonas campestris and Zymomonas mobilis from peach pulp. Biotechnol. Lett. 21 (1), 39–43.

Pooja, N.S., Sajeev, M.S., Padmaja, M.L., G, J., 2018. Bioethanol production from microwave - assisted acid or alkali - pretreated agricultural residues of cassava using separate hydrolysis and fermentation (SHF). 3 Biotech 8, 69.

Salah, R.B., Chaari, K., Besbes, S., Blecker, C., Attia, H., 2011. Production of xanthan gum from Xanthomonas campestris NRRL B-1459 by fermentation of date juice palm by-products (Phoenix dactylifera L.). J. Food Process. Eng. 34 (2), 457–474.

Schimd, J., Sieber, V., Rehm, B., 2015. Bacterial exopolysaccharides : biosynthesis pathways and engineering strategies. Front. Microbiol. 6. Article 496.

Shamala, T., Sreekantiah, K., 1986. Saccharification of tapioca starch residue with a multienzyme preparation of Aspergillus ustus. Starch Staerke 38 (12), 428–432.

Shen, F., Saddler, J.N., Liu, R., Lin, L., Deng, S., Zhang, Y., Yang, Gang, Xiao, Hong, Li, Y., 2011. Evaluation of steam pretreatment on sweet sorghum bagasse for enzymatic hydrolysis and bioethanol production. Carbohydr. Polym. 86 (4), 1542–1548.

Silva, M.F., Fornari, R.C., Mazutti, M.A., de Oliveira, D., Padilha, F.F., Cichoski, A.J., Cansian, Rogério Luis, Luccio, Marco Di, Treichel, H., 2009. Production and characterization of xantham gum by Xanthomonas campestris using cheese whey as sole carbon source. J. Food Eng. 90 (1), 119–123.

Souw, P., Demain, A.L., 1980. Role of citrate in xanthan production by Xanthomonas campestris. J. Ferment. Technol. 58 (5), 411–416.

Srikanta, S., Jaleel, S., Ghildyal, N., Lonsane, B., Karanth, N., 1987. Novel Technique for saccharification of cassava fibrous waste for alcohol production. Starch Staerke 39 (7), 234–237.

Sudha, A., Sivakumar, V., Sangeetha, V., Priyenka Devi, K., 2017. Physicochemical treatment for improving bioconversion of cassava industrial residues. Environ. Prog. Sustain. Energy 37 (1), 577–583.

Taherzadeh, M.J., Eklund, R., Gustafsson, L., Niklasson, C., Lidén, G., 1997. Characterization and fermentation of dilute-acid hydrolyzates from wood. Ind. Eng. Chem. Res. 36 (11), 4659–4665.

Valeriano, I.H., Marques, G.L.L., Freitas, S.P., Couri, S., Penha, E.M., Gonçalves, M.M.M., 2018. Cassava pulp enzymatic hydrolysate as a promising feedstock for ethanol production. Braz. Arch. Biol. Technol. 61, 1–10.

Virunanon, Chompunuch, Ouephanit, Chanika, Burapatana, Vorakan, 2013. Cassava pulp enzymatic hydrolysis process as a preliminary step in bio-alcohols production from waste starchy resources. J. Clean. Prod. 39, 273–279.

Wang, D., Ma, X., Yan, L., Chantapakul, T., Wang, W., Ding, T., Liu, D., 2017. Ultrasound assisted enzymatic hydrolysis of starch catalyzed by glucoamylase: investigation on starch properties and degradation kinetics. Carbohydr. Polym. 175, 47–54.

Wang, Z., Wu, J., Zhu, L., Zhan, X., 2017. Characterization of xanthan gum produced from glycerol by a mutant strain Xanthomonas campestris CCTCC M2015714. Carbohydr. Polym. 157, 521–526.

Woiciechowski, A.L., Soccol, C.R., Rocha, S.N., Pandey, A., 2004. Xanthan gum production from cassava bagasse hydrolysate with Xanthomonas campestris using alternative sources of nitrogen. Appl. Biochem. Biotechnol. 118 (1), 305–312.

Yoo, S.D., Harcum, S.W., 1999. Xanthan gum production from waste sugar beet pulp. Bioresour. Technol. 70 (1), 105–109.

Yu, Zhuping, Du, Yangliu, et al., 2018. Enhancing fermentable sugar yield from cassava residue using a two-step dilute ultra-low acid pretreatment process. Ind. Crops Prod. 124, 555–562.

Zhang, Junhua, Pakarinen, Annukka, Viikari, Liisa, 2013. Synergy between cellulases and pectinases in the hydrolysis of hemp. Bioresour. Technol. 129, 302–307.