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Full Length Research Paper

Coculture fermentation of banana agro-waste to ethanol by cellulolytic thermophilic *Clostridium thermocellum* CT2

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Banana is a major cash crop of many regions generating good amount of waste after harvest. This agro waste which is left for natural degradation is used as substrate for single step ethanol fermentation by thermophilic, cellulolytic, ethanologenic *Clostridium thermocellum* CT2, a new culture isolated from elephant droppings. Scanning electron microscopic pictures clearly indicate cellulolysis and close interaction of selected isolate CT2 with cellulose. The optimum conditions for cellulose fermentation were 60°C, pH 7.5, inoculums size 5% and incubation time 5 days. Ethanol produced and reducing sugars were estimated by gas chromatography. *Clostridium thermosaccharolyticum* HG8 and *Thermoanaerobacter ethanolicus* ATCC 31937 were used in coculture fermentation with CT2. Coculture fermentation of CT2 with HG8 was more efficient in terms of ethanol production, cellulose degradation and reducing sugars utilization. A maximum ethanol yield of 0.41g/g substrate used was obtained on coculturing CT2 with HG8 on alkali treated banana waste. Coculture was active even at substrate concentrations up to 100 g/l, a maximum ethanol of 22 g/l was obtained at 100 g/l substrate concentration on coculturing CT2 with HG8. This is the first report on anaerobic single step conversion of banana waste to ethanol by *C. thermocellum*.

Key words: *Clostridium thermocellum*, coculture fermentation, banana waste, ethanol, thermophile.

INTRODUCTION

Over the last century, energy consumption has increased progressively as the result of growing world population and industrialization (Sun and Cheng, 2002). Ethanol is a renewable energy source produced through fermentation of sugars unlike the fossil fuels. Owing to the realization of diminishing natural oil and gas resources, interest in the bioconversion of renewable cellulosic biomass into fuel ethanol as an alternate to petroleum is rising around the world (Lin et al., 1998; Stevenson and Weimer, 2002). Biomass is the earth's most attractive alternative among fuel sources and sustainable energy resource. Banana is the major cash crop of many tropical regions around the world. After harvest of crop, the whole plant (leaves and pseudo stem) is left in the field for natural degradation which takes several months. However these agro-wastes can be utilized for production of useful products like ethanol.

Fermentation of sugars by microbes is the most common method for converting sugars inherent within biomass feed stocks into liquid fuels such as ethanol. In the current trend, ethanol produced from biomass is the most widely used biofuel when blended with gasoline. Conventional techniques to achieve bioconversion include acid or enzyme hydrolysis of cellulose followed by fermentation of the resulting soluble sugars into ethanol (Szczodrak and Fiedurek, 1996). According to Lovitt et al. (1984) a single step conversion of cellulosic biomass to ethanol with cellulolytic bacterium such as *Clostridium thermocellum* would be the most economical method of production. *C. thermocellum* is a thermophilic, anaerobic bacterium that performs a mixed acid fermentation of cellulose to produce ethanol and acetic acid as major end products (Ng et al., 1977). This bacterium is of interest as an agent for the conversion of biomass materials to fuel ethanol and other value-added products (Lynd et al.,

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2002). However *C. thermocellum* is unable to utilize pentose sugars such as xylose formed during hemicellulose degradation, hence eventually ethanol yields will be low. Combination of two processes, that is, cellulose utilization and hemicellulose hydrolysis into a single step process could be accomplished by a coculture of two compatible organisms (Ng and Zeikus, 1982). Such coculture types could be expected to be stable in light of each organism, having a substrate which is likely to get utilized. Cocultures between cellulolytic and pentose utilizing microbes are common in nature and offer improved hydrolysis in the laboratory relative to pure cultures of cellulolytic bacteria (Lynd et al., 2002). Coupling cellulose fermentation by *C. thermocellum* with a noncellulolytic, hexose and pentose fermenting secondary bacterium such as *Thermoanerobacter ethanolicus* (Ljungdahl et al., 1981; Wiegel and Ljungdahl 1981) and *C. thermosaccharolyticum* resolves the problem of non-utilization of xylose, pentose sugars as well as better utilization of dissolved sugars (Baskaran et al., 1995). Here we report the fermentation of banana waste to ethanol by co-culture fermentation using *C. thermocellum* in combination with *C. thermosaccharolyticum and T. ethanolicus* bacterial species.

MATERIALS AND METHODS

Microorganisms and culture conditions

The bacterial isolates *C. thermocellum* CT2 was isolated from droppings of elephant (at Nehru Zoological Park, Hyderabad, India) respectively by enrichment culturing using CMS medium (Rani et al., 1996). The isolates were grown in 120 ml serum vials with 20 ml of pre-reduced cellulose mineral salt (CMS) medium pH 7.5 (Ram and Seenayya, 1991) containing (g/l); KH_2PO_4, 1.5; K_2HPO_4, 2.0; Urea, 2.0; $MgSO_4$, 0.8; $CaCl_2$, 0.15; sodium citrate, 3.5; cysteine HCl, 0.15; yeast extract, 4.0; resazurin, 0.002; cellulose, 10.0; in N_2 atmosphere. Cellulose substrate was replaced with same concentration of banana leaves in CMS medium. The medium was sterilized by autoclaving at 121°C for 30 min. *C. thermosaccharolyticum* HG8 and *T. ethanolicus* ATCC 31937 were grown in CMS medium with Whatman No.1 Filter paper as the substrate.

The cultures were stored at 4°C in refrigerator and repeatedly sub-cultured once in a month in CMS medium. In co-culture system the organism used for coculture fermentation was inoculated after 24 h of fermentation. All the fermentation experiments were carried out by taking actively growing 5% (v/v) inoculum at 60 ± 2°C for 5 days of incubation and the ethanol produced, reducing sugars and substrate degraded were estimated.

The cellulolytic and ethanologenic bacterial isolate CT2 was identified by morphology, staining, cultural and biochemical characteristics in comparison to those characteristics of *C. thermocellum* (McBee, 1950; Hippe et al., 1992; Hensyl, 1994).

Treatment of cellulosic banana agro-waste

The banana-agro waste (leaves) was cut approximately into 1 cm pieces and these were subjected to treatment.

Preparation of dried banana agro-waste

About 250 g of banana agro waste was taken in a 500 ml beaker and dried in a hot air oven to constant weight. Dried material was cut into 1 x 1 cm size pieces and used as substrate for fermentation.

Preparation of water treated banana-agro waste

About 250 g of banana –agro waste (dried pieces) was taken in 1 liter conical flasks, containing 500 ml of distilled water and boiled for 30 min. The supernatant was decanted and the residue was thoroughly washed with distilled water until the colouring compounds were removed. The residue was then dried at 60°C to constant weight.

Preparation of alkali or acid treated banana agro-waste

About 250 g of the banana agro-waste (dried pieces) was taken separately in 1 liter conical flasks containing 500 ml of 1% of NaOH or 1% H_2SO_4 and autoclaved at 121°C for 15 min. The supernatant was decanted and the residue was neutralized with 1% H_2SO_4 1% NaOH. The residue was thoroughly washed with distilled water until no colour was imparted to the water and dried at 60°C to constant weight.

Physico chemical characterization of banana waste

Estimation of moisture, total solids and phenol

Total moisture and solids estimation was done according to standard methods described in AOAC (AOAC, 1980). The starch present in the banana waste was estimated by method of Sadashivan and Manickam (1997). Phenol present in the banana agro-waste was checked according to the protocols described by Malick and Singh (1980).

Estimation of cellulose, hemicellulose and lignin

Cellulose was estimated as per method described by Weimer and Zeikus (1977), hemicellulose and lignin contents were estimated by method of Han and Anderson (1975).

Estimation of ethanol and reducing sugars

For the estimation of ethanol 10 ml of fermented broth was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant solution was acidified with 1 ml of 2 N phosphoric acid and 2 µl was injected into a chromosorb 101 column, 80 - 100 mesh in a CIC gas chromatograph equipped with flame ionization detector. The following parameters were chosen for analysis: Oven temperature, 160°C; injector temperature, 170°C; carrier gas, N_2 and flow rate, 20 µl per min (Ramesh et al., 2004). The reducing sugars were estimated by DNS reagent as described by Miller (1959). All the experiments were performed in triplicates.

Cellulase assay

10 ml of fermented broth was taken and centrifuged at 10,000 g for 15 min and the supernatant was used as crude enzyme. The cellulase enzyme activity, endoglucanase (CMCase) exoglucanase (filter paperase activity (FPase) was measured using Carboxy methyl cellulose and Whatman no 1 filter paper (1% w/v) as substrates respectively according to the method of Mandels et al. (1976).

Scanning electron microscopic (SEM) studies

The Scanning Electron Microscopic studies of Banana agro waste leaves before and after inoculation of cellulolytic bacteria *C. thermocellum* CT2 was done.

For SEM microscopic studies the samples were transferred to vials and fixed with 2.5% gluteraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 h at 4^0C and post fixed with 2% aqueous osmium tetroxide in the same buffer for 2 h. Then the samples were dehydrated in series of graded alcohol and dried to critical point drying with Electron Microscopy Science CPD unit. Then dried samples were mounted over the stubs with double sided conductivity tape. Finally a thin layer of gold metal was applied over the sample using an automated sputter coater (JEOL JFC-1600) for about 3 min. Samples were then scanned in scanning electron microscope (Model: JOEL-JSM 5600, JAPAN) at various magnifications at RUSKA Lab, College of Veterinary Sciences, SVUU, R'Nagar, Hyderabad, India.

RESULTS

Morphological and culture characteristics of *Clostridium thermocellum* isolates

The selected *C. thermocellum* isolate CT2 was isolated from dropping of elephant by enrichment technique in CMS medium. The cellulolytic ethanologenic isolates CT2 was identified as *C. thermocellum* by morphology, staining, cultural and biochemical characteristics, in comparision to those characteristics of *C. thermocellum* (McBee, 1950; Hippe et al., 1992; Hensyl, 1994). The isolate was obligately anaerobic, motile, spore forming rod shaped (curved rods) Gram negative bacteria with terminal oval spores and did not grow even under microaerophilic conditions suggesting that they belong to the *Clostridium* (Cato et al., 1986). The isolate produced yellow colour pigment when grown on cellulose. This isolate used a wide variety of carbon sources including glucose, mannose, fructose, sucrose, cellobiose, lactose, arabinose, sorbitol, mannitol and maltose. However they could not ferment pentose sugars. The major metabolic products on cellulose included ethanol, CO_2 and small amounts of acetic acid. The optimized conditions for growth and cellulose degradation for CT2 was 60^oC. Growth was less below 45^oC. The strain showed a pH tolerance between 6-9.5 and maximum cellulose degradation and ethanol yield was observed at pH 7.5.

The microorganism *Thermoanaerobacter ethanolicus* is an extreme thermophilic, non-spore-forming anaerobic bacterium which ferments a variety of carbohydrates to ethanol as the main product (Wiegel and Ljungdahl, 1981). *T. ethanolicus* under anaerobic and thermophilic conditions continuously ferment substrates such as cellobiose, glucose, xylose and other pentose sugars to produce recoverable amounts of ethanol. These are ethanol tolerant up to 10% (v/v) during fermentation. Where as *C. thermosaccharolyticum* HG8 is Gram negative rod shaped, thermophilic, motile and noncellulolytic with ethanol tolerance up to 8%(v/v) (Baskaran et al., 1995).

Characterization of banana waste

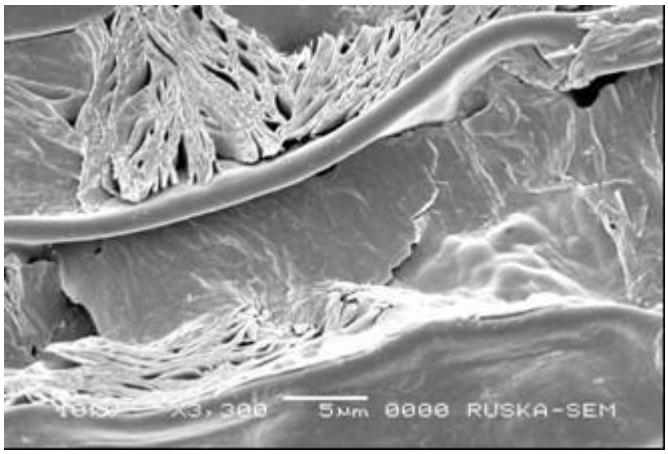
Banana agro-waste (leaves) was collected from local farm following harvest. The waste was washed thoroughly with water and dried. It was cut in to 1 x 1 cm pieces uniformly for further studies. The physical characters of banana agro waste like the moisture content (70.76%), total solids (20.24%) and chemical characteristics like starch (9.3%), phenols (0.0548%), cellulose (28.92%), hemicellulose (25.23%) and lignin (10.56%) were estimated . High concentration of hemicellulose and lignin inhibits the availability of cellulose for fermentation by the isolates. Hemicellulose and lignin are complex polymers and are not easily degraded by bacteria hence pretreatments of agrowaste was aimed at increasing the surface area of cellulose by removing lignin seal, solubilizing hemicellulose and disrupting crystallinity. Pretreated cellulosic biomass like water treated, alkali treated, acid treated and also dried banana agrowaste were used for fermentative production of ethanol.

Cellulase activity

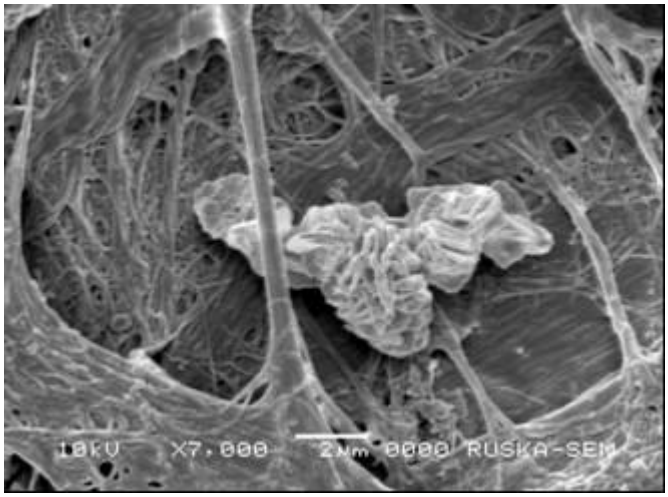
Whatmann No 1 filter paper, dry banana waste, water extracted banana waste, alkali treated banana waste and acid treated banana wastes were used in the range of 10-100 g/l concentration as substrates wherever required in fermentation process. A significant growth was observed when *C. thermocellum* CT2 was grown in the presence of CMS medium containing Whatmann No. 1 filter paper and banana leaves as the substrate. The corresponding endoglucanase and exoglucanase activity were estimated and the results of the same are presented in Figure 1. The endoglunase activity was more than exoglucanase activity and maximum cellulase enzyme activity was observed when CT2 was grown on alkali treated banana waste (Figure 1).

Ethanol fermentation in mono and coculture fermentation

C. thermocellum CT2 in monoculture fermentation produced maximum ethanol of 0.351 g/g cellulose used with whatman no 1 filter paper as the substrate in the medium (Figure 2). Cellulolytic activity of CT2 and its colonization was observed in scanning electron micrographs in Photograph 1b compared to the control in Photograph 1a. CT2 was able to hydrolyze up to 70% of the total substrate, However CT2 could not utilize all the reducing sugars released from cellulose hydrolysis because of feed back inhibition (Figure 3). To overcome the obstacles of inhibition and growth, a coculture fermentation process has been chosen using CT2 in combination with known sachharolytic, ethanologenic bacteria, *C. thermosaccharolyticum* HG8 and *T.*



Photograph 1a. Scanning electron microscopic picture of Banana leaves before inoculation with cellulolytic bacteria



Photograph 1b. Scanning electron microscopic picture of Banana waste after cellulolysis by *Clostridium thermocellum* CT2

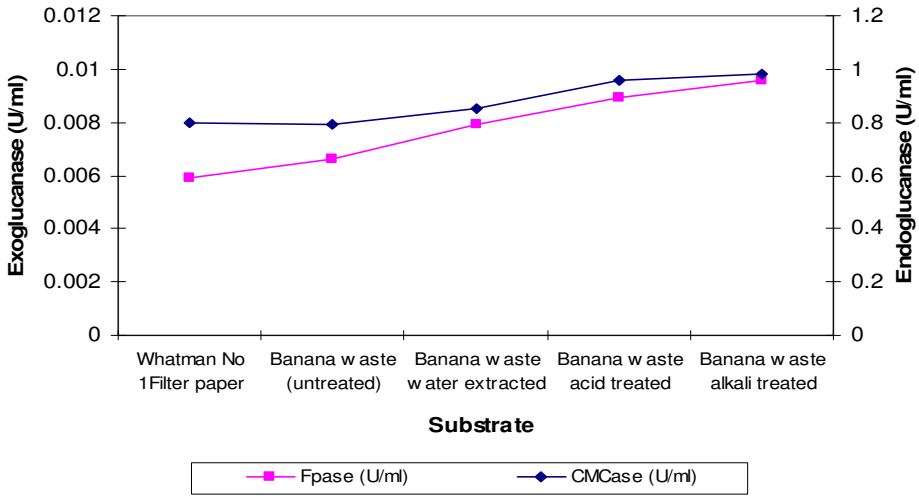


Figure 1. Exo- and endoglucanase activity of *C. thermocellum* CT2 on various substrates.

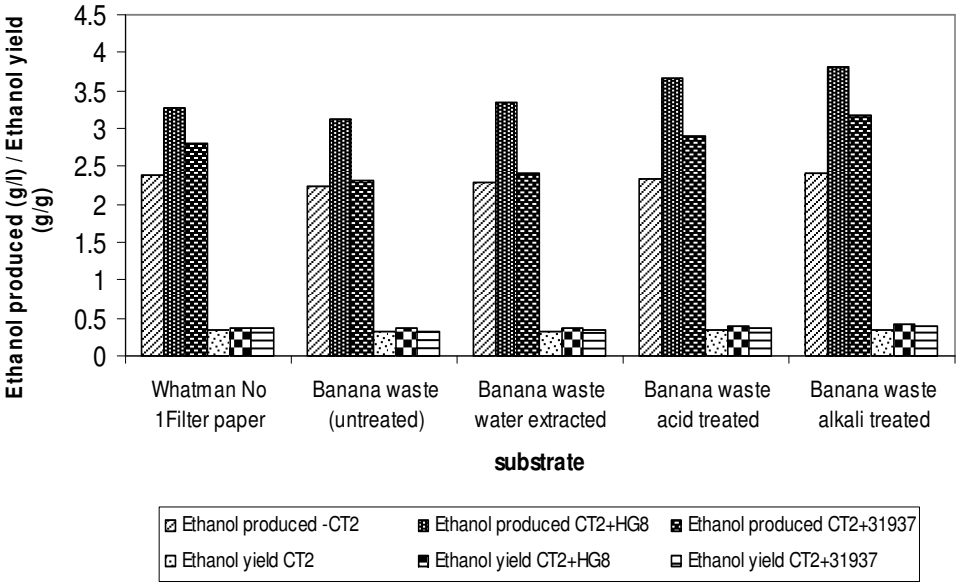


Figure 2. Ethanol produced and ethanol yields in mono and coculture fermentations of banana leaves by *C. thermocellum* CT2 with HG8 and ATCC 31937.

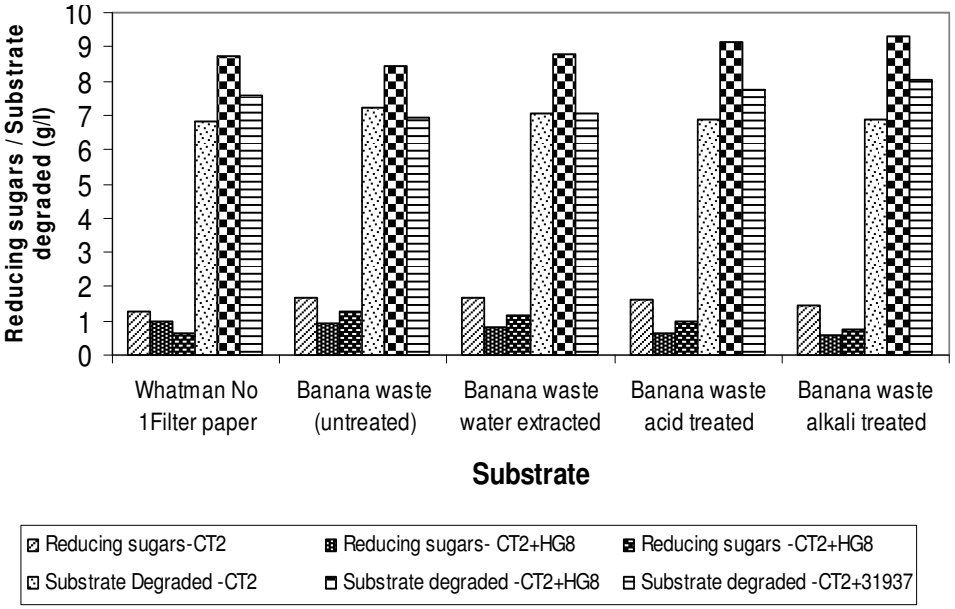


Figure 3. Substrate degraded and reducing sugars formed in mono and coculture fermentations of banana leaves by *C. thermocellum* CT2 with HG8 and ATCC 31937.

ethanolicus ATCC 31937 (Ng et al., 1981). During coculture fermentation there is increased ethanol production, substrate degradation and ethanol yield compared to monoculture fermentation (Figures 2 and 3). The leftover reducing sugars were less in coculture than in monoculture fermentation indicating the efficiency of coculturing organism to utilize them and convert them to ethanol (Figure 3).

Of the two selected coculturing organisms *C. thermosachharolyticum* HG8 and *T. ethanolicus,* HG8 was more efficient in conversion of reducing sugars to ethanol. A maximum ethanol of 3.82 g/l was obtained with alkali treated banana leaves with a ethanol yield of 0.41 gram per gram of cellulose degraded. The ethanol production was 36 - 59% more in coculture with HG8 than with monoculture fermentation in all the substrates used.

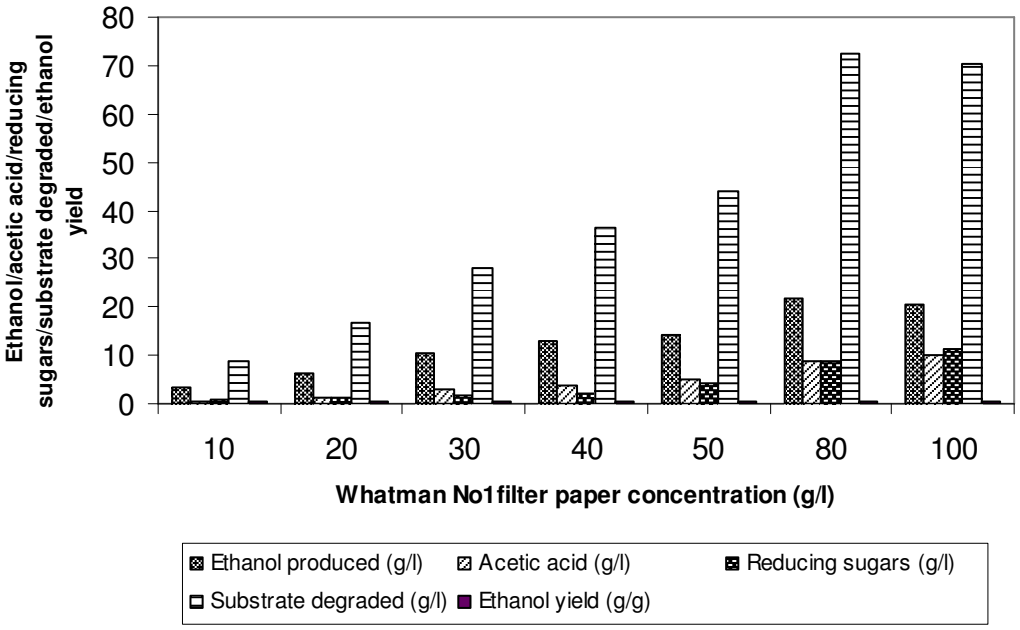


Figure 4. Coculture fermentation of high concentration of Whatman No 1 filter paper to ethanol by *C. thermocellum* CT2 with *C. thermosaccharolyticum* HG8.

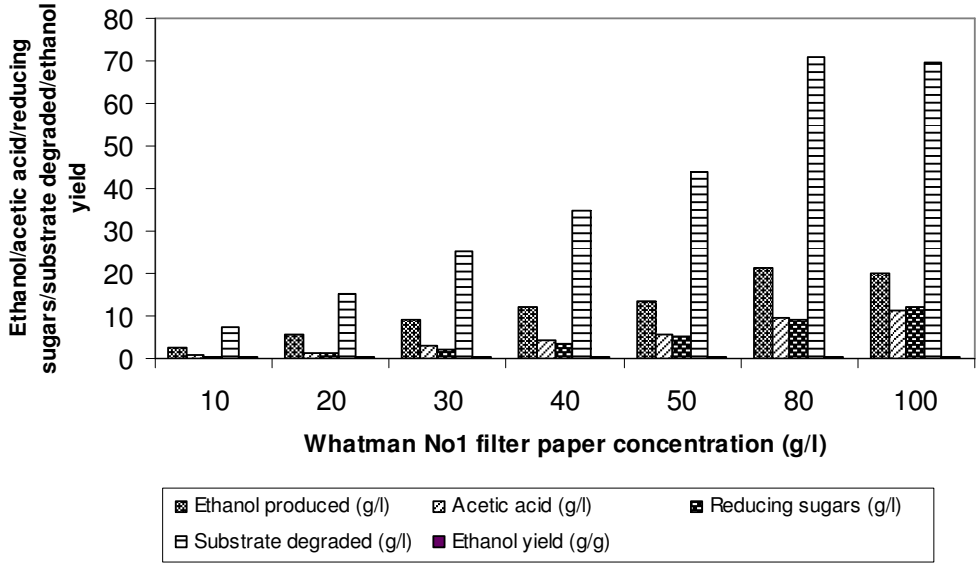


Figure 5. Coculture fermentation of high concentration of Whatman No1 filter paper materials to ethanol by *C. thermocellum* CT2 with *C. ethanolicus* ATCC 31937.

Highest amount of ethanol was produced with alkali treated banana agro waste.

Ethanol fermentation at high substrate (Whatman No 1 filter paper and banana agro waste) concentrations was studied in the range of 10 to 100 g/l. With the increase in substrate concentration the time of incubation has to be increased up to 11 days in some cases for better cellulolysis. Eventually with the increase in substrate degradation the ethanol produced increased along with production of acetic acid (Figures 4 and 5). Similar observation was recorded when banana agrowaste was used (Tables 1 and 2). Ethanol produced was in the range of 2.82 to 22 g/l in coculture of CT2 with HG8 and 2.48 to 22.7 g/l in coculture of CT2 with ATCC 31937. Previously researchers have reported the fermentation of pure and crude cellulosic substrates to ethanol by *C. thermocellum* (Lovitt et al., 1988; Rani et al., 1996). However to the best of our knowledge the present finding

Table 1. Coculture fermentation of high concentration of pre-treated banana cellulosic waste materials to ethanol by *C. thermocellum* CT2 with *C. thermosaccharolyticum* HG8.

Substrate concentration (g/l)	Time (days)	Substrate	Ethanol produced (g/l)	Acetic acid (g/l)	Reducing sugars (g/l)	Substrate degraded (g/l)	Ethanol yield (g/g)
0	5	DBW	2.82	0.38	1.00	7.81	0.362
		WTBW	2.93	0.43	0.786	7.72	0.380
		ACBW	2.951	0.51	0.68	7.36	0.401
		AKBW	3.142	0.46	0.531	7.43	0.423
50	8	DBW	12.13	3.16	6.12	38.94	0.314
		WTBW	12.86	4.24	6.24	39.44	0.326
		ACBW	13.01	4.57	5.12	39.30	0.331
		AKBW	14.64		5.06	43.05	0.340
100		DBW	20.08	12.00	12.39	80.0	0.251
		WTBW	20.29	11.96	11.63	71.696	0.283
		ACBW	20.33	12.29	11.58	76.42	0.266
		AKBW	22.00	12.19	11.12	75.01	0.290

DBW: dry banana waste; WTBW: water extracted banana waste; ACBW: Acid treated banana waste; AKBW: alkali treated banana waste.

Table 2. Coculture fermentation of high concentration of pre-treated banana cellulosic waste materials to ethanol by *C. thermocellum* CT2 with *C. ethanolicus* ATCC 31937.

Substrate concentration (g/l)	Time (days)	Substrate	Ethanol produced (g/l)	Acetic acid (g/l)	Reducing sugars (g/l)	Substrate degraded (g/l)	Ethanol yield (g/g)
10	5	DBW	2.48	0.89	1.16	7.49	0.331
		WTBW	2.60	0.92	1.11	7.51	0.346
		ACBW	2.59	1.00	0.96	7.11	0.364
		AKBW	2.73	0.89	0.81	7.00	0.39
50	8	DBW	11.83	3.83	7.16	38.16	0.31
		WTBW	12.62	4.89	7.23	39.31	0.321
		ACBW	12.50	5.03	6.14	36.98	0.338
		AKBW	13.53	4.60	6.03	39.44	0.343
100	11	DBW	21.81	11.86	12.98	85.19	0.256
		WTBW	20.36	12.09	12.13	73.23	0.278
		ACBW	21.03	12.11	11.66	78.17	0.269
		AKBW	22.71	12.19	11.91	76.72	0.296

DBW: dry banana waste; WTBW: water extracted banana waste; ACBW: Acid treated banana waste; AKBW: alkali treated banana waste.

is probably the first report on usage of banana agro waste to ethanol in single step coculture anaerobic fermentation.

DISCUSSION

C. thermocellum have the potential of converting cellulose to ethanol in a single step fermentation process (Ramesh et al., 2004). Several strains of *C. thermocellum* and *Clostridium* species are isolated from diverse habitats such as manure, soil, compost, leachate slurry, hot springs, deseeded cotton balls, volcanic soils, sewage digester sludge, biogas digester, insects, fecal droppings of various herbivorous animals and birds are found to produce ethanol from cellulosic substrates (McBee, 1950; Ng et al., 1977; Johnson et al., 1982; Mori, 1990; Sai Ram et al., 1991; Sudharani et al., 1996; Ramesh et al., 2004). Different strains of *C. thermocellum* received increasing attention for their ability to ferment ligno-cellulosic biomass to ethanol and organic acids in a one-step process (Tailliez et al., 1989; Lynd et al., 2002).

Koonin (2006) explained about energy crops and modern fermentation technology including anaerobic fermentation with various cellulolytic organisms to produce useful organic products like ethanol, acetic acid,

etc. Wild strains of *C. thermocellum* so far reported produced 0.08 - 0.37 g ethanol per gram glucose or equivalent fermented (Bender et al., 1985; Freier et al., 1988; Mori, 1990; Sairam et al., 1991; Sato et al., 1992; Sudharani et al., 1996; Ramesh et al., 2004). Avgerinos et al. (1981) reported ethanol production on solka floc and corn stover. Wang et al. (1983) discussed about ethanol formation on pretreated cellulosic substrates. Baig et al. (2004) reported saccharification of banana agrowaste by cellulolytic enzymes for production of useful products like ethanol. However there are no reports on direct fermentation of banana agro-waste to ethanol. Wang and co-workers at Massachusetts Institute of Technology, USA employed coculture fermentations by coupling ethanol tolerant strains of *C. thermocellum* (S-7) with *C. themosacharolyticum* (HG4). In this coculture fermentation ethanol yield was 0.40 (g/g) with solka flock as the substrate. Zeikus and co workers at University of Wisconsin, USA studied coculture fermentation coupling *C. thermocellum* and *C. thermohydrosulfuricum*. Coculture fermentation significantly increased the amount of cellulose degradation, ethanol yield and productivity when compared to monoculture (Zeikus et al., 1983; Mori, 1990). Zyabreva et al. (2001) reported mixed cultures which could utilize cellulose, cellobiose, glucose, maize residue, cotton and flax boon producing ethanol (up to 0.9 g/l) and acetic acid (up to 0.8 g/l). Whereas association of thermophilic anaerobic bacteria produced 0.64 g of ethanol per g substrate utilized. Spinnler has reported 8.5 g/l of ethanol production by *C. thermocellum* at high cellulose concentration and here in the present study we report ethanol production of 19.69 and 21.32 g/l on on Whatmann No 1 filter paper and alkali treated banana waste by *Clostridium thermocellum* CT2 at high substrate concentration.

Banana is one of the major crops in tropical countries which results in accumulation of cellulosic biomass waste in agricultural practices. Coculture fermentation of banana waste to ethanol is very encouraging and an attractive alternative technology for the production of biofuels using cellulolytic, thermophilic and ethanologenic bacteria.

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