Int J Pharm Bio Sci 2016 April; 7(2): (B) 294 - 301

Original Research Article Biotechnology

International Journal of Pharma and Bio Sciences ISSN 0975-6299

CONSOLIDATED BIOPROCESS BY CLOSTRIDIUM THERMOCELLUM DSM 1313 IN IN SITU STERILISABLE STIRRED TANK FERMENTER FOR ENHANCED DIGESTIBILITY AND INCREASED BIOETHANOL PRODUCTION FROM SUGARCANE BAGASSE

NISHA MUKUND[1], MUKUND SHANKAR [2], SARANYAH .K[1] AND LILLY M.SALEENA[*1]

[1]Department of Bioinformatics, School of Bioengineering, SRM University, Kattankulathur - 603203, Tamil Nadu, India
[2]Department of Chemical Engineering, School of Bioengineering, SRM University, Kattankulathur - 603203, Tamil Nadu, India

ABSTRACT

Consolidated bioprocess by *Clostridium thermocellum* DSM1313 is studied here for the hydrolysis of pre-treated sugarcane bagasse and banana pseudostem for ethanol production. Ethanol titre of 26.27 and 9.12mM and release of 4.770±0.107and 3.281±0.109g/L of reducing sugars from sugarcane bagasse and banana pseudostem respectively were observed in flask level studies. Sugarcane bagasse was taken further for scale-up studies in a 3L in situ sterilisable industrial fermenter. An increased growth rate with two fold increase in release of reducing sugars and a final ethanol titre of 54.35mM highlights the efficient hydrolysis of the substrate not only by the organism but also due to the direct steam-substrate contact during sterilisation. Due to the thermophilic conditions of the broth, ethanol recovery was found to be 3.43% efficient than the control. This paper emphasizes the importance of a controlled environment for efficient lignocellulosic hydrolysis, and an energy efficient ethanol recovery.

KEYWORDS: Clostridium *t*hermocellum, fermentation, scale-up, fermenter, ethanol, reducing sugars

LILLY M.SALEENA
Department of Bioinformatics, School of Bioengineering, SRM University,
Kattankulathur - 603203, Tamil Nadu, India

INTRODUCTION

Over, 80% of South Indian agricultural wastes constitute rice straw and husk, wheat straw, sugarcane tops, bagasse and banana pseudostem.[1,2] These lignocellulosic wastes as raw materials are very cheap and have an economical advantage over traditional biofuel feed stocks like corn starch, soybeans, and sugar cane molasses.[3] Majority of the ethanol producing microorganisms depend only on sugars released from carbohydrate moieties of lignocellulosic substrates[4,5] necessitating better pre-treatments of the latter. However pre-treatments studied in the past suffer with effluent issues or higher energy/cost involvements. Utilisation of glycosidases to hydrolyse substrates to fermentable sugars[1,6,7] is a preferred green alternative but commercial production of these enzymes reduce the economic viability of ethanol production. Even though many of the pre-treatments and fermentation for ethanol production have been very well streamlined and documented, research oriented towards industrial scale conversion of complex lignocellulosic biomass to bioethanol is in its infancy.[8] Results of lab scale studies needs to be further supplemented with pilot scale studies to actually translate the results into a commercial viable product in the market. To serve the purpose, the potentials of *Clostridium thermocellum* a cellulolytic, thermophilic anaerobic bacterium can be exploited. This organism consists of multiple hydrolytic enzymes (cellulosomes) enabling it to degrade even the raw substrates and consolidating all the major steps involved, like saccharification of exposed cellulose and hemicelluloses molecules into sugar moieties, its fermentation, and finally to ethanol production in one pot.[9,10] One of the cost involving steps in industrial scale aerobic fermentation like continuous air/oxygen[10] supply can be dilated with anaerobic fermentation and facilitate energy recovery. Many works related to *Clostridium thermocellum* are happening in India [11–14] but very few detailed studies are done in regarding ethanol production using the residual wastes found in agricultural lands of India directly with the organism. Our work deal in scale-up studies utilising the natural potential of *Clostridium thermocellum* that can even hydrolyse lignin to a greater limit.[15] With a concept of consolidated bioprocess, sugarcane bagasse and banana pseudostem were selected to analyse the organisms' adaptation to these commonly found lignocelluloses. Also the use of a lab scale in situ sterilisable fermenter simulates the actual conditions that can be only envisioned in pilot scale fermenters which require a substantial investment before R&D.

MATERIALS AND METHODS

Lignocellulosic substrates

Fresh banana pseudostem and sugarcane bagasse were collected from the local farmers, Kattankulathur (Kanchipuram Dist.,India). Sugarcane bagasse and banana pseudostem were dried overnight at 50°C in hot air oven. Samples were chopped into fine pieces with kitchen aid mixer and sifted through BS-410- 36 mesh. Substrates were then subjected to mild alkali treatment with 1% NaOH and autoclaved.[16]

Clostridium thermocellum DSM 1313 and its growth conditions

Clostridium thermocellum DSM 1313 was acquired from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The culture was revived in an equal volume of 1x phosphate buffered saline (PBS) incubated at 55°C, 200 rpm for 4 hours. This was subsequently transferred to screw capped 20 mL bottles containing standard 122 growth medium and the incubation conditions were maintained as recommended by the DSMZ. Sub culturing was performed with 10% inoculum transferred under gassed nitrogen environment to the sterile media pre-heated to 55°C. For maintenance and storage filter sterilised cellobiose solution was used instead of cellulose. For plating, solidifying agent was prepared with gelzan (0.7% w/v).

Lignocellulose substrate (LCS) based media

The LCS medium composition was similar to 122 medium with pure cellulose powder replaced with LCS. Cellulose content in the raw LCSs was determined and the weight equivalent to 10g/L cellulose was calculated and added to respective media. 23.6g/L of bagasse (SB medium) and 18.6g/L of banana pseudostem (BP medium) were considered for the same.

LCS media optimisation experiments

Experiments were performed by altering various incubation and medium conditions to find the optimum level at which the microorganism produced maximum reducing sugar from the added LCS. Media with the aforementioned grams of respective LCS were taken in different screw capped vials and the initial pH was adjusted to 6.0, 6.5, 7.0 and 7.5 and incubated under standard DSM recommended incubation conditions. Temperature optimisation experiments were performed with the pH optimised medium by incubating the same at 50, 55, 60 and 65°C respectively. The pH and temperature optimised broth was fermented for a period of 72 to 168 hours to check for better hydrolysis of substrates.

Upscaling of LCS fermentation seed

Seed culture was prepared in SB medium for early catch-up of culture in the stirred tank fermenter. The medium was prepared in 500mL glass fermenter (Duran) and incubated in temperature controlled water bath at 60°C with intermittent stirring for every 6 hours with a rotational speed of 120 rpm. Subsequently the seed was inoculated into 3L fermenter in its mid-log phase.

Anaerobic fermenter

The scale-up studies were performed in an in situ sterilisable bench scale-stirred tank fermenter (Scigenics India Pvt. Ltd.) The function and design considerations of the vessel satisfied ASTM specifications and standards which is substantiative to an industrial model. The design is with an aspect ratio of 1:2.5, diameter to height respectively and the gross volume 3L with 70-80% as working volume. The material of fabrication used is stainless steel 316L grade for all wet contact parts. The fermenter shell had a dished bottom and a flanged top plate with a variable

speed controlled agitator shaft sealed with a single dry mechanical seal. The agitator designed could meet a maximum tip speed of 300M/min. The shaft was equidistantly screwed with adjustable Rushton turbine impellers, three in numbers and held three baffles (aspect ratio 1 /10th of vessel I.D) for the uniform mixing of the media. Tubular ring sparger had been deliberately positioned below the last impeller for effective gas mixing. The outer shell ensured the maintenance of the temperature conditions. All piping was in accordance to the recommended standards and used actuated valve types suited for the purpose. In situ sterilisable PTFE gas filters (size: 2.5", pore size: 0.2µ) at the inlet and the vent line ensured containment of the system. The sterilisation and fermentation temperatures were controlled through a PID controller with a parallel online pH & pO_2 sensors to monitor pH and oxygen levels in the fermentation medium respectively. A 2vvm of the gas was sparged intermittently until the point where the effective dissolved oxygen in the medium was 0%. 10% seed culture from the glass fermenter was aseptically transferred to the 3L fermenter with incubation conditions maintained at 60^{o}C, pH: 7.0±0.1, pO_2: 0% with an agitation of 120 rpm. Dependent parameters were recorded for every 24 hours like the pH drift, cell O.D., reducing sugar concentration, and the total unused cellulose at the end of the batch.

Optimisation of the type of gas sparged

The efficacy of different types of gases sparged into the media was studied and recorded for the changes in the growth pattern every 12 hours. Two kinds of gases compared were mixed gas (80% N_2+ 15% CO_2 + 5% H_2) and pure N_2 gas.

Colorimetric assay quantification for reducing sugar and cellulose

Sugar hydrolysates were analysed according to the DNS method of Miller G.L. (1956).[17] Development of deep orange colour due to the formation 3 amino, 5 – nitro salicylic acid was read at 540 nm using multi-plate reader (Multi-Skan Go, Thermo scientific). Cellulose content was assayed in accordance to the method of David M. Updegraff (1969).[18]

Recovery of ethanol1L out of the 2.5L fermenter broth was immediately transferred to a Rotary evaporator (Heidolph, Germany) and the distillate collected was taken for ethanol analysis.[19] For ethanol recovery studies known amount of ethanol (100mM, 500mM) in 1L of distilled water were rotary evaporated to check for (%) ethanol recovery.

HPLC chromatographic validation of ethanol

In this study ethanol was analysed with HPLC (1260 infinity, Agilent technologies) equipped with autosampler and a DAD detector (G1315D). It is a modified methodology of Joseph M. Betz et al (1987)[20] (protocol yet to be published) an indirect U.V. photometric detection technique. Column used was Zorbax Eclipse Plus C18 column (dimension 4.6 x 250mm and 5µm in size). Mobile phase was a combination of acetone and water.

Statistical analysis

Mean reducing sugar yield values for both the substrates were analysed by one-way analysis of variance (anova) and Tukey's test at the 5% level of significance.[21]

RESULTS

Clostridium thermocellum revived from PBS buffer showed a tendency to reach log phase early than direct sub culturing from glycerol stock. Culture harvested from PBS buffer reached 0.2 O.D.(A_{600}) within 30 hours whereas the latter required 48 hours to attain the same. The presence of inorganic phosphate (Pi) can induce germination of endospores.[22]

Substrate preparation

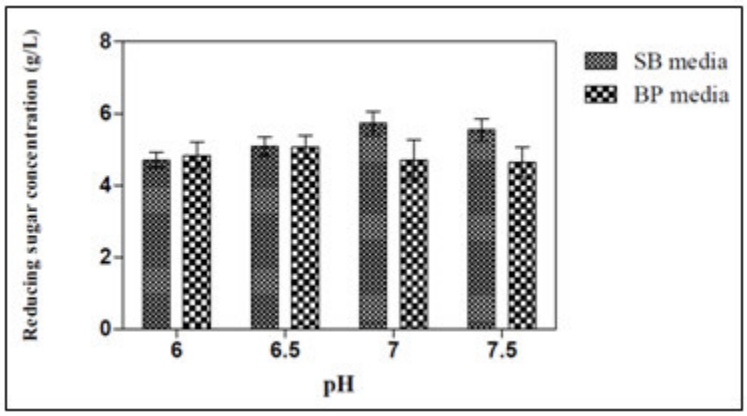
In sugarcane bagasse, the cellulose content was analysed to be 42.38±1.4% and banana pseudostem with 53.77±1.7% which was subsequently added to the respective media to meet the requirement of 10g/L cellulose. It was then subjected to alkali pre-treatment.

Optimisation experiments

pH

The standard 122 media has a pH of 7.2. pH above 7.5 would give gradual inhibitory effects on the organism whereas the lower pH ranges can be bearable due to its inherent capacity to produce acids during fermentation viz. acetic acid and lactic acid.[23] Thus the effect of pH for better hydrolysis of the substrates was considered for a narrow range of 6.0 to 7.5. The reducing sugar concentration of BP media for all the pH ranges under study were more or less similar since the respective mean reducing sugar values for different pH trials was within the inter deviational range with a mean value of 4.808g/L, whereas in SB media . Though there was no significant difference ($p>0.05$) pH 7.0 was found to be optimum with a maximum release of 5.542±0.319g/L of reducing sugar and least observed for pH 6.0(Fig. 1).

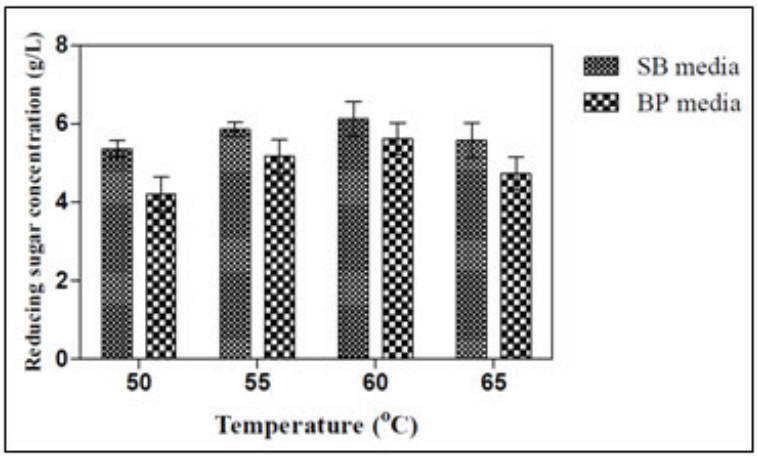
Figure-1
Effect of pH during fermentation of SB media and BP media



Temperature

Being thermophilic, the organism has the capability to survive temperature to a maximum of 68°C.[24] Higher temperatures may facilitate easier bond breakages between the substrates and thus efficient release of sugars.[25] However prolonged exposure to higher temperature will result in charring of the substrates which is undesirable.[26] Thus to find an optimum limit for maximum substrate utilisation at higher temperatures, experiments were carried out by incubating the vials in temperatures ranging from 50 to 65°C. The SB and BP media showed a maximum reducing sugar release of (5.569±0.448g/L and 4.722±0.421g/L respectively) at 60°C even though there no significant difference ($p>0.05$) between the narrow range of temperature difference (Fig.2). An early charring (darkening of the media) was observed indicating the degradation of sugars in the media at 65°C. Lesser growth and reducing sugar concentration were observed below 55°C with the least at 50°C.

Figure-2
Effect of different temperature on SB media and BP media fermentation



Analysis of Batch duration

The organisms' growth in the standard 122 medium with cellulose as substrate entered into the late log phase after 96 hours. To ascertain the growth characteristics and subsequent sugar release in the respective LCS media, the fermentation batch studies were extended to a maximum of 168 hours. In the case of SB media an increasing trend of reducing sugar release into the media was observed till 120th hour with corresponding growth entering late log phase by the 96th hour itself. BP media however entered late log phase by the 84th hour and the reducing sugar concentrations only peaked to its maximum till 72nd hour. The sugar release dropped along with the organisms' entry into the stationary phase.

Fermentation experiments

The LCS media with optimised parameters was fermented in flask level studies (100mL). Sugarcane bagasse and banana pseudostem entered into the exponential phase after 30 and 40 hours respectively. Subsequently the growth increased until the 96 and 84 hours respectively. The cellulose utilisation and the ethanol content were calculated and are mentioned in Table1.

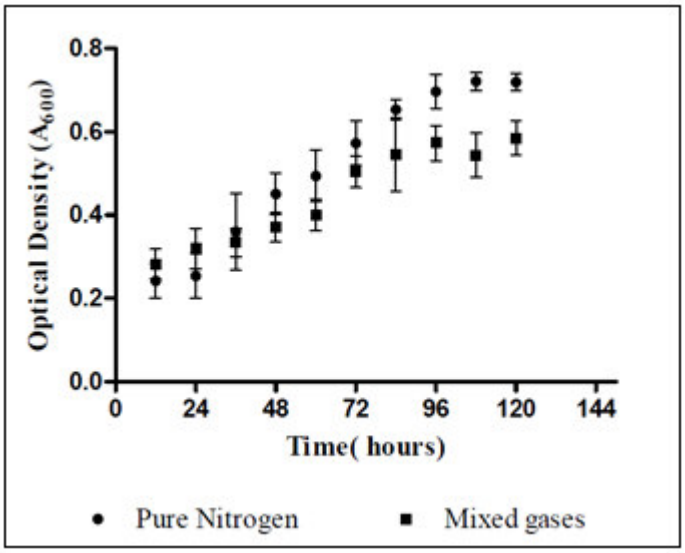
Table 1
Flask level fermentation batch results.

Substrates	Final reducing sugar (g/L) [a]	Specific growth rate (hr^{-1})	Cellulose (%) hydrolysed by the end of the batch	Ethanol (mM)
Cellulose (control)	6.770±0.161	0.1	57.83	24.75
SB	4.770±0.107	0.03	47.68	26.27
BP	3.281±0.109	0.03	29.57	9.12

[a] ***Values are mean ± SE (n=3)***

Scale-up studies
All the standardisation in anaerobic fermenter was carried out in the 122 medium with cellulose as substrate. Mixed gas under study caused a nosedive effect in pH by 2.0 units for every sparge whereas this was not evident when N_2 gas was sparged. Media acidification was observed due to CO_2 which necessitated higher volume of alkali to maintain the pH. Moreover, mixed gas reduced the growth of the organism with late entry into log phase after 40 hours (Fig.3). To maintain the pH under N_2, 0.5N NaOH was added at a rate of 10mL/day only whereas it was 25mL/day for mixed gas.

Figure-3
Influence of mixed gas and N_2 gas upon the growth of Clostridium thermocellumduring fermentation of pure cellulose powder.



LCS medium fermenter batch results
SB media was chosen for further scale-up to a volume of 2.5L in the fermenter. The 60 hours seed inoculum (SB media broth) was aseptically transferred to the fermenter with the sterile SB medium preheated to the incubation temperature of 60°C. An early growth initiation with reducing sugar concentration of 6.042±0.57 g/L at 24th hour was observed and gradually increased to 12.54±1.2g/L by 120 hour. This increase was in proportion to the growth rate. The value was found to be almost consistent (11.952±1.1g/L) till 144th hour indicating the constant release of reducing sugar into the media. The general pH trend was a gradual shift towards the acidic range with a maximum dip of 6.5±0.2 at the end of the batch. The pH of the broth was maintained at 7.0±0.1 by alkali addition till the end of the standardised batch. At the end of the 120 hour batch the unused cellulose content of the sugarcane bagasse was 39.35% and direct ethanol analysis of the batch was 54.35mM.

Crude ethanol recovery
1L of fermenter broth was subjected to rotary evaporation from which 50mL of crude ethanol was obtained. Total ethanol recovery from the fermenter broth was 87.3%.

DISCUSSION

The statistical reports of India have mentioned that out of 101.3 million metric tons of sugarcane bagasse, 6.4 million metric tons can be used for biofuel production.[1] From every hectare of banana plantation nearly 220 tons of plant residues that consist mainly of lignocelluloses material are produced.[27] For sugarcane tops pre-treatment with 3% alkali have been reported to improve reducing sugar yield during enzymatic hydrolysis.[28] We were able to achieve good results with 1% NaOH for sugarcane bagasse though banana pseudostem couldn't provide an observable result for reducing sugar concentration nor ethanol production. Mildly treated residues were further hydrolysed by the

whole live organism since it has a consortium of hydrolytic enzymes.[10] During optimisation both substrates showed different properties with respect to fermentation. pH is one of the factor affecting the growth of microorganism, fermentation rate and by-product formation.[29] *Clostridium thermocellum* ATCC 27405 is reported to give 94 – 98% of cellulose degradation in between pH of 7 and 9.[30] This study evaluated the efficiency of banana pseudostem and sugarcane bagasse in the pH range of 6.0 to 7.5 for *C.thermocellum* DSM1313. Organism efficiently adapted the bagasse at a range between 7 and 7.5 in releasing fermentable sugars whereas in pseudostem variable pH showed no difference in hydrolysis pattern. The presence of inhibitors could have prevented the enzymatic action on the pseudostem. Presence of phenolic compounds which are toxic to the majority of alcoholic fermentation micro-organisms from banana pseudostem have been reported earlier too.[31] This infers that the 1% mild alkali treatment did not in any way facilitate the removal of these phenolic compounds and hence was not found feasible for an up scaled study. Temperature is also known to affect the metabolism of microorganism and results in the formation of secondary metabolites.[32] The inherent thermophilic nature of *C.thermocellum* shows better growth even at higher temperatures.[33] In both the substrates the highest reducing sugar concentration was achieved at 60^{o}C and the same temperature was reported optimal by Eric A. Johnson (1982).[34] As per the experiment results the SB medium batch showed a similar growth trend to that of the control. BP medium entered stationary phase 24 hours earlier for the reasons mentioned in the previous section. Optimisations done by researchers using different substrates highlights that the growth conditions for *Clostridium thermocellum* vary, depending on the type and the quality of substrate obtained after initial pre-treatment.[27,35] The optimum conditions achieved to run a fermentation batch with the concerned agricultural residues by *C. thermocellum* DSM1313 was pH 7.0, temperature 60^{o}C for 120 hours. Ethanol production in lab-scale experiments gave observable results and the control gave a titre in accordance to the already published literatures.[24] An attempt has been made to simulate the pilot scale studies using an in situ sterilisable fermenter. The direct supply of steam into the vessel after 100^{o}C during sterilisation not only ensured the sterility of the media but also enabled the direct contact of steam onto the substrate surface. The type of gas sparged for maintaining anaerobiosis has a key role to play in ethanol production. The dip in the pH in case of N_2 fermentation was gradual due to the release of other by-products.[36] Provision of N_2 favours the cultivation and ensures pH stability and is cost effective when compared to mixed gases. Intermittent sparging to ensure anaerobiosis is sufficient which further reduces the expenses involved in continuous air/oxygen sparging as in the case of aerobic fermentation. When SB media was scaled up in the fermenter an early catch up of growth was observed. An increase in the specific growth rate to 0.05 hr^{-1} (fermenter) from 0.03hr^{-1} (flask studies) points out the advantages of scaling up the process in in situ fermenters. This indicates that controlled environment can stimulate the cell growth which is not attainable in flask level hence provides a scope of study to initiate lignocellulosic conversion directly to industrial scale. 2.62 fold increase in the reducing sugar concentration was observed when 100 mL media was scaled up to two litres which explains the adaptation of the organism that was not evident in flask level. Similarly increase in ethanol titre was also evident. In flask level only 26.27mM was accounted whereas after 120 hour in fermenter 54.35mM was recorded. For ethanol recovery studies, initially a known concentration of ethanol (100mM, 1L) was vacuum evaporated and a loss of 15.6% was observed. However the loss was reduced to 8.7% when the concentration of ethanol was increased 5 times. This suggests that the inherent loss can be minimised by increasing the ethanol concentration or the distilling volume. Simultaneous harvest and vacuum evaporation of the 1L fermenter broth in collecting 50mL distillate took 120min whereas the known ethanol samples took 180 min. The reduction in time of vacuum evaporation can be attributed to the thermophilic conditions (60^{o}C) of the broth hence making it energy efficient. Also the reduction in time of vacuum evaporation has increased the ethanol recovery, reducing the loss to 12.7% in comparison to the 100mM ethanol sample.

CONCLUSION

Efficient degradation of cellulose was achieved through batch fermentation using in situ sterilisable fermenter for the production of ethanol with the aid of *Clostridium thermocellum* DSM1313. This experiment highlights the prime requirement of a fermenter to enhance the conversion of cellulose or any cellulosic material like agricultural, domestic or municipal wastes. Thermophilic fermentation has also facilitated faster and higher ethanol recovery with less energy consumption in one of its downstream processing steps. Mass cultivation with controlled parameters to an extended level can compensate the ethanol production despite its slow growth rate. The possibilities of co-cultivation, sequential cultivations can be developed and optimised with the aim of enhanced bioethanol production as well as other products of interest. Furthermore the residual spent medium in such cases can also be further digested with natural microbial consortium in Up flow Anaerobic Sludge Blanket (UASB) reactor[37] for the production of biogas with minimum sludge generation and pollution.

ACKNOWLEDGEMENT

This work is supported by the Department of Science and Technology (DST) under Women Scientist Scheme A (SR/WOS-A/LS-196/2011(G)), New Delhi for author N.M.

REFERENCES

1. Sukumaran RK, Surender VJ, Sindhu R, Binod P, Janu KU, Sajna KV, et al. Lignocellulosic ethanol in India: Prospects, challenges and feedstock availability. Bioresour Technol [Internet]. Elsevier Ltd; 2010;101(13):4826–33. Available from: http://dx.doi.org/10.1016/j.biortech.2009.11.049
2. Patil R, Kolambe B, Pawar S, Patel J, Prajapati D, Savani N, et al. Development of value added Products from Banana Pseudostem (An Overview of Progress). 2011.
3. Zafar S. Biofuels from lignocellulosic biomass [Internet]. Bioenergy Consult. 2014 [cited 2015 Nov 30]. Available from: http://www.bioenergyconsult.com/what-is-lignocellulosic-biomass/
4. Cardona C a, Sánchez OJ. Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol [Internet]. 2007 Sep [cited 2013 May 23];98(12):2415–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17336061
5. Senthilkumar V, Gunasekaran P. Bioethanol production from cellulosic substrates : Engineered bacteria and process integration challenges. 2005;64(November):845–53.
6. Mbaneme V, Chinn SSM. Consolidated bioprocessing for biofuel production: recent advances. Energy Emiss Control Technol [Internet]. Dove Press; 2015 May 6 [cited 2015 Jun 15];Volume 3:23. Available from: http://www.dovepress.com/consolidated-bioprocessing-for-biofuel-production-recent-advances-peer-reviewed-fulltext-article-EECT
7. Wilson DB. Microbial diversity of cellulose hydrolysis. Curr Opin Microbiol [Internet]. Elsevier Ltd; 2011;14(3):259–63. Available from: http://linkinghub.elsevier.com/retrieve/pii/S13695 2741100052X
8. Gnansounou E, Dauriat A. Ethanol fuel from biomass: A review. J Sci Ind Res (India). 2005;64(11):809–21.
9. Lynd LR. Production of ethanol from lignocellulosic materials using thermophilic bacteria: Critical evaluation of potential and review. Adv Biochem Eng Biotechnol. 1989;38:1–52.
10. Demain AL, Newcomb M, David JH, Wu JHD. Cellulase, Clostridia, and Ethanol. Microbiol Mol Biol Rev. 2005;
11. Das SP, Ghosh A, Gupta A, Goyal A, Das D. Lignocellulosic fermentation of wild grass employing recombinant hydrolytic enzymes and fermentative microbes with effective bioethanol recovery. Biomed Res Int. 2013;2013.
12. Mutreja R, Das D, Goyal D, Goyal A. Bioconversion of agricultural waste to ethanol by SSF using recombinant cellulase from Clostridium thermocellum. Enzyme Res. 2011;2011:340279.
13. Balusu R, Paduru RR, Kuravi SK, Seenayya G, Reddy G. Optimization of critical medium components using response surface methodology for ethanol production from cellulosic biomass by Clostridium thermocellum SS19. Process Biochem [Internet]. 2005 Sep [cited 2013 Jul 26];40(9):3025–30. Available from: http://linkinghub.elsevier.com/retrieve/pii/S13595 11305001121
14. Rani KS, Seenayya G. High ethanol tolerance of new isolates of Clostridium thermocellum strains SS21 and SS22. World J Microbiol Biotechnol. 1999;15:173–8.
15. Blum DL, Kataeva IA, Li X, Ljungdahl LG. Feruloyl esterase activity of the Clostridium thermocellum cellulosome can be attributed to previously unknown domains of XynY and XynZ. J Bacteriol. 2000;182(5):1346–51.
16. Kiyoshi K, Furukawa M, Seyama T, Kadokura T, Nakazato A, Nakayama S. Butanol production from alkali-pretreated rice straw by co-culture of Clostridium thermocellum and Clostridium saccharoperbutylacetonicum. Bioresour Technol [Internet]. Elsevier Ltd; 2015;186:325–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25818258
17. Miller GL. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–8.
18. Updegraff DM. Semimicro determination of cellulose inbiological materials. Anal Biochem [Internet]. 1969 Dec [cited 2015 Apr 14];32(3):420–4. Available from: http://www.sciencedirect.com/science/article/pii/S 0003269769800096
19. Das SP, Deka D, Ghosh A, Das D, Jawed M, Goyal A. Scale up and efficient bioethanol production involving recombinant cellulase (Glycoside hydrolase family 5) from Clostridium thermocellum. 2013;1(1):1.
20. Betz JM, Nikelly JG. Determination of ethanol in alcoholic beverages by liquid chromatography using the UV detector. J Chromatogr Sci. 1987;25(9):391–4.
21. Ren Z, Ward TE, Logan BE, Regan JM. Characterization of the cellulolytic and hydrogen-producing activities of six mesophilic Clostridium species. J Appl Microbiol [Internet]. 2007 Dec [cited 2013 Jul 26];103(6):2258–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18045409
22. Paredes-Sabja D, Bond C, Carman RJ, Setlow P, Sarker MR. Germination of spores of Clostridium difficile strains, including isolates from a hospital outbreak of Clostridium difficile-associated disease (CDAD). Microbiology. 2008;154(8):2241–50.
23. Ruyet P Le, Dubourguier HC, Albagnac G. Homoacetogenic Fermentation of Cellulose by a Coculture of *Clostridium thermocellum* and *Acetogenium kivui*. Appl Environ Microbiol. 1984;48(4):893–5.
24. Akinosho H, Yee K, Close D, Ragauskas A. The emergence of Clostridium thermocellum as a high utility candidate for consolidated bioprocessing applications. Front Chem

[Internet]. 2014;2(August):1–18. Available from: http://journal.frontiersin.org/article/10.3389/fchem.2014.00066/abstract
25. Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol [Internet]. 2009;100(1):10–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0960852408004574
26. Palmer JJ. How to Brew: Ingredients, Methods, Recipes, and Equipment for Brewing Beer at Home. Defenestrative Publishing Company; 2001. 143 p.
27. Ingale S, Joshi S, Gupte A. Production of bioethanol using agricultural waste: Banana pseudo stem Journal: Brazilian J Microbiol. 2012;892:1–23.
28. Chaturvedi V, Verma P. An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. 3 Biotech [Internet]. 2013;3(5):415–31. Available from: http://link.springer.com/10.1007/s13205-013-0167-8
29. Pramanik K. Parametric studies on batch alcohol fermentation using Saccharomyces Yeast extrated from Toddy. J Chin Inst Chem Engrs. 2003;34(4):487–472.
30. Özpinar Ö, Özkan M. Cellulose degradation and glucose accumulation by Clostridium thermocellum ATCC 27405 under different cultural conditions. Ann Microbiol. 2007;57(3):395–400.
31. Souza EL, Liebl GF, Marangoni C, Sellin N, Millena S. Bioethanol from fresh and dried banana plant pseudostem. Chem Eng Trans. 2014;38:271–6.
32. S L-L. Wine and brandy. Biotechnol. 1983;5:81–163.
33. Feeds RSS. Development of ethanol tolerance in Clostridium thermocellum : effect of growth temperature . Development of Ethanol Tolerance in Clostridium thermocellum : Effect of Growth Temperaturet. 1980;
34. Johnson E a., Sakajoh M, Halliwell G. Saccharification of complex cellulosic substrates by the cellulase system from Clostridium thermocellum. Appl Environ Microbiol. 1982;43(5):1125–32.
35. Sindhu R, Binod P, Satyanagalakshmi K, Janu KU, Sajna KV, Kurien N, et al. Formic acid as a potential pretreatment agent for the conversion of sugarcane bagasse to bioethanol. Appl Biochem Biotechnol. 2010;162(8):2313–23.
36. Ulbrik TYL. Cellulolytic Fermentation By Clostridium thermocellum. Georgia Institute of Technology; 1991.
37. Lettinga G, Velsen A van, Hobma S, Zeeuw W de, Klapwjik A. Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnol Bioeng. 1980;22(4):699–734.