GENETICALLY ENGINEERED *ESCHERICHIA COLI* FBR5 TO USE CELLULOSIC SUGARS: PRODUCTION OF ETHANOL FROM CORN FIBER HYDROLYZATE EMPLOYING COMMERCIAL NUTRIENT MEDIUM

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Transportation biofuel ethanol was produced from xylose and corn fiber hydrolyzate (CFH) in a batch reactor employing *Escherichia coli* FBR5. This strain was previously developed in our laboratory to use cellulosic sugars. The culture can produce up to 49.32 g L^{-1} ethanol from approximately 125 g L^{-1} xylose. Use of commercial nutrient sources such as corn steep liquor (CSL) and soy peptone (SP) was also studied and SP was found to be superior than CSL. SP at a concentration of 15 g L^{-1} resulted in the production of 42.2 g L^{-1} ethanol with ethanol yield and productivity of 0.49 and 0.74 g L^{-1} h^{-1}, respectively. Corn fiber (CF) was pretreated with dilute H_2SO_4 and hydrolyzed using commercial cellulases. Employing CFH as a sole substrate, the culture produced 35.33 g L^{-1} ethanol with a productivity and yield of 1.01 g L^{-1} h^{-1} and 0.54, respectively, leaving behind no residual sugars in the medium. This productivity is 40 % higher than when using xylose as the carbon source in the control experiment.

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Introduction

In the United States approximately 13.3 billion (13.3 x 10^9) gallons of ethanol was produced in 2013 from corn.[1] In order to meet nation's annual transportation fuel demand (138 billion gallons)[1] it is necessary to produce more biofuel from renewable resources. However, further increase in ethanol production from corn is not possible as it creates food and feed vs. fuel competition thus increasing food and feed prices. For this reason ethanol should be produced from economically available agricultural biomass such as corn stover, corn fiber, or other residues. However, one of the major problems associated with these residues is that they contain pentose sugars which are not fermented by natural yeast such as *Saccharomyces cerevisiae*. Fermentation of pentose sugars, which make up to 35 % of the sugars present in cellulosic residues, is essential for economic reasons. Hence, for the purpose of fermenting hexose (glucose, mannose, and galactose) and pentose (xylose, and arabinose) sugars, *Escherichia coli* FBR5 was developed in our laboratory.[2] Fermentation of both, hexose and pentose, sugars to ethanol would dramatically improve the ethanol yield and economics of this biofuel production. It should be noted that prices of cellulosic biomass are much lower than corn or other feedstocks such as cane or beet molasses.

Another challenge with the economic production of ethanol is the use of costly nutrients such as yeast extract and tryptone. To make ethanol production more economical these ingredients should be replaced with commercial nutrients such as corn steep liquor (CSL) and soy peptone (SP). CSL is a by-product of corn wet milling process and is available for more economical prices than yeast extract and tryptone. Similarly, SP is a product of soy protein which is obtained after enzymatic hydrolysis.

The development in fermentation and bioreactor technologies and/or parameters can reduce the cost of production of this biofuel. These fermentation parameters include ethanol concentration in the final product, ethanol productivity, and yield. A higher ethanol concentration in the broth results in more economical recovery by distillation. Improved productivity results in the decrease of reactor size and hence reduced capital and operational costs.[3] Similarly, a higher product yield also improves the economics of ethanol production. All these three factors should be optimized by using appropriate nutrient levels in the batch reactor. The objectives of these studies were multiple and included: i) use of corn fiber hydrolyzate (CFH) as a substrate for ethanol production; ii) evaluation of commercially available nutrient media for ethanol fermentation; and iii) improvement in ethanol concentration, productivity, and yield.

Materials and Methods

Microbial Culture, Cell Maintenance, Chemicals, and Nutrients

Escherichia coli FBR5 was developed in our laboratory[2] and its details on culture maintenance, propagation, and culture medium have been provided in this reference.[2] Fermentation studies were performed in a 2 L New Brunswick Bioreactor (BIOFLO 3000, New Brunswick Scientific Co., New Brunswick, NJ, USA) with 1000 mL working volume.

Xylose, yeast extract and betaine were obtained from Sigma Chemicals (St. Louis, MO, USA) and tryptone was from Becton Dickinson & Co. (Sparks, MD, USA). CSL (also called Solulys-AST; Roquette Corporation, Gurnee, IL, USA), and SP (Nutricepts, Inc., Burnsville, MN, USA) were used as commercial nutrient media for cell growth and fermentation. CSL or SP (10-30 g) was dissolved in 200 mL distilled water and autoclaved separately at 121 ^{o}C for 15 min. Approximately 900 mL xylose solution (containing 100 g xylose) was autoclaved separately and cooled to 25 ^{o}C before adding the calculated amount of pre-sterilized CSL or SP solution to the desired concentration. To make up the final volume of the medium to the desired level, sterilized and cooled distilled water was added. pH of the xylose solution or CFH which contained CSL or SP was adjusted to 6.5 before and after inoculation. Addition of CSL or SP solution resulted in reduced total sugar concentration in the CFH medium, which was increased to the original hydrolyzate sugar level by supplementing with the concentrated sugar solutions in the same proportion as measured in the original CFH.

Corn Fiber Pretreatment and Hydrolysis

Corn fiber (CF) was obtained from A. E. Staley Manufacturing Company (Decatur, IL, USA; Now Tate & Lyle, Decatur, IL, USA). The moisture content of CF was 16 % (w/w) and it's starch content was 0.40 % (w/w). The CF was ground in a commercial grinder to a uniform size of 1.27 mm. Approximately 95-100 g CF was soaked in 900 mL of 0.5 % (v/v) H_2SO_4 for 15 min in a Pyrex dish and the suspension was covered with a single layer of aluminum foil.

The dish containing suspension was placed in an autoclave at 121 ^{o}C for 60 min followed by cooling it to room temperature (25 ^{o}C) and adjusting its pH to 5.0 with 10 M NaOH. Then hydrolytic enzymes [cellulase (Celluclast), and cellobiase (Novo 188); 12 mL each per L mixture; both supplied by Novozymes] were added and the mixture was transferred to a 2 L bioreactor (New Brunswick). The temperature inside the bioreactor was raised to 45 ^{o}C and the suspension was agitated at 200 rpm for 72 h. After hydrolysis, the suspension was filtered through a cheese cloth to remove suspended solids followed by centrifuging the liquid portion at 4000 rpm (Sorvall^{R} RC5C Plus) for 15 min. The liquid was detoxified by overliming method reported elsewhere.[2,4] This was followed by filtering the liquid through a series of filters (11-4 µm) to remove coarse particles in preparation to filter sterilize it. Then the liquid was filter sterilized by filtering through a 0.22 µm filter. The resultant clear liquid was called CFH and was used for fermentation studies. 1.5 mL samples were taken to measure ethanol, sugars, and cell concentration. The samples were centrifuged at 12,000 rpm in a micro-centrifuge (Eppendorf Centrifuge 5417; Hamburg, Germany) to separate cells and supernatant. After centrifugation, the microbial cells were washed with equal volume of pre-sterilized 9 g L^{-1} NaCl solution to remove medium components. Following this, the cells were suspended in equal volume of the above saline solution and mixed (Maxi MixII; Barnstead, Dubuque, IA, USA) before measuring optical density at 540 nm. The supernatant sample was stored at -18 ^{o}C until measurement of ethanol and sugars.

Analyses

Ethanol concentration in the fermentation broth was measured by GC (6890N; Agilent Technologies, Wilmington, DE, USA) using a packed glass column as described elsewhere.[5] Sugars and fermentation acids (by-products) were measured by HPLC.[5] Cell concentration was measured using a predetermined correlation between dry weight cell concentration and optical density (λ 540). Ethanol productivity was defined as ethanol concentration in g L^{-1} divided by the fermentation time h and is expressed as g L^{-1} h^{-1}. Specific productivity (h^{-1}) is expressed as productivity in g L^{-1} h^{-1} divided by the cell concentration in g L^{-1}. Ethanol yield is defined as ethanol produced in g L^{-1} divided by the total sugar utilized in g L^{-1}. Fermentation time is defined as the time period between inoculation, and time when fermentation ceased; i.e. ethanol concentration ceased to increase. The results presented here are an average of two replications and have an error margin of ±3.5-7.5 %.

Results and Discussion

In order to compare the results obtained in these studies, a control or baseline fermentation was run that produced 39.40 g L^{-1} ethanol with a productivity and ethanol yield of 0.72 g L^{-1} h^{-1}, and 0.43, respectively (**Table 1**). For this fermentation, 5 g L^{-1} yeast extract, and 10 g L^{-1} tryptone were used as nutrients. The maximum cell concentration that was achieved in the fermentation was 1.83 g L^{-1}. This cell concentration resulted in a specific ethanol productivity of 0.39 h^{-1}. Since, the high concentrations of these nutrients is impractical for a commercial ethanol production facility, further experiments were run with reduced levels of nutrients with aims to achieve high ethanol concentration and kinetic parameters such as productivity and yield. So the next experiment was performed with only 10 g L^{-1} tryptone which resulted in the production of 44.14 g L^{-1} ethanol with a yield of 0.46. Although, ethanol concentration was higher than the control, it's productivity was decreased to 0.37 g L^{-1} h^{-1} suggesting that the fermentation was considerably slower than the control fermentation. Further, an experiment was performed with 5 g L^{-1} yeast extract concentration in the medium. During the fermentation 42.5 g L^{-1} ethanol was produced with a productivity and yield of 0.50 g L^{-1} h^{-1} and 0.45, respectively. In the next experiment 5 g L^{-1} yeast extract and 5 g L^{-1} tryptone were used. This experiment resulted in the production of 44.10 g L^{-1} ethanol with ethanol yield and productivity of 0.50 and 0.88 g L^{-1} h^{-1}, respectively (**Table 1**). These productivity and yield values are higher than achieved in the control fermentation. In another experiment yeast extract concentration was increased to 7 g L^{-1} with no tryptone supplementation. Although, fermentation was faster with a productivity of 0.78 g L^{-1} h^{-1}, it was at the expense of low final ethanol concentration which was 34.57 g L^{-1}.

Furthermore, two experiments were performed where 2 g L^{-1} yeast extract was supplemented with vitamin, mineral, and buffer stock solutions.[6] These stock solutions have proved to be effective in butanol fermentation and are regularly used[7] and hence were considered for this fermentation as well. As reported in **Table 1**, the results from the supplementation of stock solutions were not beneficial for this fermentation.

Table 1. Ethanol concentration, yield, and productivity obtained using various semisynthetic and commercial nutrient media for ethanol production employing *E. coli* FBR5.

Media	Ethanol [g L^{-1}]	Ethanol yield [-]	Ethanol productivity [g L^{-1} h^{-1}]	Cell conc. [g L^{-1}]
Semi-synthetic media				
Control (YE 5 g L^{-1}, Tryp 10 g L^{-1})	39.40	0.43	0.72	1.83
Tryptone (10 g L^{-1})	44.14	0.46	0.37	1.63
YE (5 g L^{-1})	42.50	0.45	0.50	1.35
YE (5 g L^{-1}) + Tryp (5 g L^{-1})	44.10	0.50	0.88	1.94
YE (7 g L^{-1})	34.57	0.45	0.78	1.59
YE (2 g L^{-1}) + Stock sol. (10 mL L^{-1})	36.70	0.41	0.42	1.69
YE (2 g L^{-1}) + Stock sol. (20 mL L^{-1})	33.10	0.33	0.20	1.72
Commercial media				
CSL (Solulys) (10 g L^{-1})	39.50	0.48	0.46	NM
(15g L^{-1})	40.90	0.49	0.59	NM
(25 g L^{-1})	38.10	0.45	0.28	NM
Soy Peptone (10 g L^{-1})	33.40	0.42	0.61	1.89
(15 g L^{-1})	42.20	0.49	0.74	2.30
(20 g L^{-1})	42.30	0.45	0.59	2.25

NM – not measured; 10 g L^{-1} NaCl (Sigma) was also added to all semi-synthetic media.

One approach to reduce the cost of ethanol production is to use industrial nutrient solutions such as CSL and/or SP. For these studies we used CSL at 3 different levels (10, 15, and 25 g L^{-1}). Among the 3 levels, the highest ethanol production (40.90 g L^{-1}) was achieved at 15 g L^{-1} CSL concentration (**Table 1**). The effect of SP was also investigated at 3 different levels (10, 15, 20 g L^{-1}). At a level of 15 g L^{-1} SP, 42.20 g L^{-1} ethanol was produced with a productivity of 0.74 g L^{-1} h^{-1} and specific productivity of 0.32 h^{-1}. It is anticipated that the use of SP at this ethanol concentration, productivity, and yield would be more economical than the use of 5 g L^{-1} yeast extract plus 5 g L^{-1} peptone which resulted in the highest productivity (0.88 g L^{-1} h^{-1}). In case of ethanol production, two of the most important parameters that were identified included productivity, and ethanol concentration and these parameters impact process economics significantly.[3]

In the above experiments, initial xylose concentration was 95±5 g L^{-1} and in most cases no residual sugar was left at the end of fermentation. Hence, lack of sugars was the reason for not producing ethanol in excess of 44 g L^{-1}. Therefore, an attempt was made to observe if more ethanol would be produced if the initial xylose concentration in the medium was increased to approximately 125 g L^{-1}. The medium contained yeast extract 5 g L^{-1} and tryptone 10 g L^{-1}. The fermentation profile for this run is shown in **Fig. 1 (A, B)**. At the end of fermentation 2.86 g L^{-1} residual xylose was measured and in the reactor an ethanol concentration of 49.32 g L^{-1} was recorded suggesting that this is the maximum ethanol tolerance of the culture. In this reactor a maximum cell concentration of 2.15 g L^{-1} was achieved.

Next, ethanol was produced from **CFH** which contained 67.11 g L^{-1} total sugar in the beginning of fermentation (**Fig. 2**). In approximately 31 h, 20.1 g L^{-1} ethanol was produced leaving behind 19.57 g L^{-1} residual sugars. The reasons behind such a low concentration of ethanol were combined toxicity due to hydrolyzate and ethanol. An ethanol productivity of 0.65 g L^{-1} h^{-1} was obtained and its yield was 0.42. It is known that inclusion of betaine in the fermentation medium enhances product (ethanol, lactic acid) concentration by regulating cell's osmotic tolerance. This has been evidenced by Thomas et al.[8] and Underwood et al.[9] for ethanolic fermentations and Xu and Xu[10] for lactic acid fermentation.

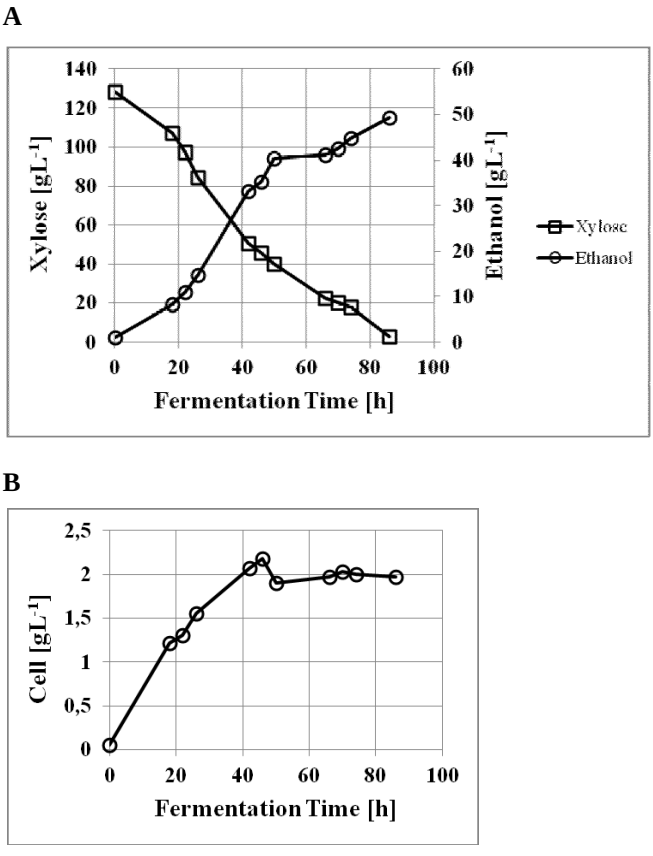

Figure 1. Maximization of ethanol production from xylose (approximately 125 g L^{-1}) in a batch reactor using *E. coli* FBR5. Nutrients were 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 10 g L^{-1} NaCl. A. Xylose and ethanol concentration; and B. Cell concentration at various fermentation times.

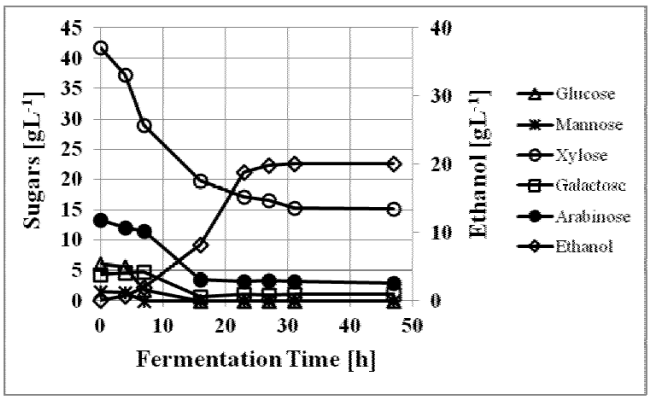

Figure 2. Production of ethanol from CFH in a batch reactor using *E. coli* FBR5. Nutrient sources were yeast extract 5 g L^{-1}, tryptone 10 g L^{-1}, and NaCl 5 g L^{-1}.

For that reason, next fermentation was run in which 2mM betaine was included in the fermentation medium. In the beginning of the fermentation 64.89 g L^{-1} total sugars were present in the reactor. The fermentation lasted for 46 h and during this time period 35.0 g L^{-1} ethanol was produced (**Fig. 3**). At the end of fermentation 0.60 g L^{-1} (arabinose 0.22 and galactose 0.38 g L^{-1}) residual sugars were measured. This system resulted in the production of 74.1 % higher ethanol than the CFH fermentation without betaine. In this reactor, a productivity of 0.76 g L^{-1} h^{-1} and ethanol yield of 0.54 was obtained. This yield is higher than the theoretical yield of ethanol. Since the medium contained 5 g L^{-1} yeast extract and 10 g L^{-1} tryptone (total 15 g L^{-1}) it is possible that the carbon sources present in the nutrients were responsible for the elevated yield. The productivity achieved in this fermentation is comparable to 0.77 g L^{-1} h^{-1} previously reported in our lab[2] under similar hydrolysis and fermentation conditions of CFH.

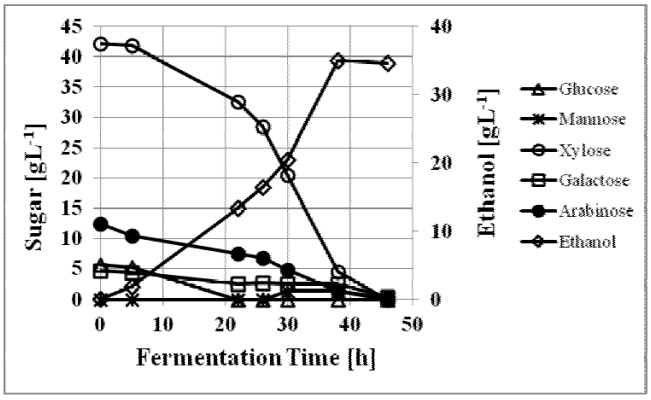

Figure 3. Production of ethanol from CFH supplemented with 2 mM betaine in a batch reactor using *E. coli* FBR5. Nutrient sources: yeast extract 5 g L^{-1}, tryptone 10 g L^{-1}, and NaCl 5 g L^{-1}

In CFH fermentation with betaine, there are four note worthy points: i) achieving a higher concentration of ethanol which would require less energy for product recovery; ii) obtaining a higher yield which would result in the production of more ethanol; iii) improving ethanol productivity which would require a smaller size plant when scaled up; and iv) resulting in reduced level of sugars at the end of fermentation, thus reducing waste water treatment costs.

In the above CFH run with betaine, the total concentration of nutrient sources was 15 g L^{-1} which is expected to be uneconomic when used in large scale fermentations. Hence, it was considered to use a commercial nutrient source (SP) at a level of 15 g L^{-1} as it resulted in high productivity, high ethanol concentration and high yield when using xylose as a substrate (**Table 1**). The fermentation was started with 65.0 g L^{-1} total CF sugars in the medium. In approximately 35 h of fermentation (when fermentation ceased) 35.31 g L^{-1} ethanol was produced (**Fig. 4**) thus resulting in a productivity of 1.01 g L^{-1} h^{-1} which is 56 % higher than the CFH fermentation without betaine. Sugar utilization was complete and an ethanol yield of 0.54 was achieved. Further improvement in productivity can be achieved by using continuous membrane cell recycle systems[11-18] or high cell density reactors[19]. The reason for high productivity is cell concentrations in excess of 60-100 g L^{-1} that can be achieved in these advanced culture systems. Based on a specific productivity of 0.32 h^{-1} (15 g L^{-1} SP; Table 1) and cell concentrations in the range of 60-100 g L^{-1}, productivities as high as 19.2-32.0 g $L^{-1}h^{-1}$ can possibly be achieved. Such high productivities would favor economic production of ethanol.[3]

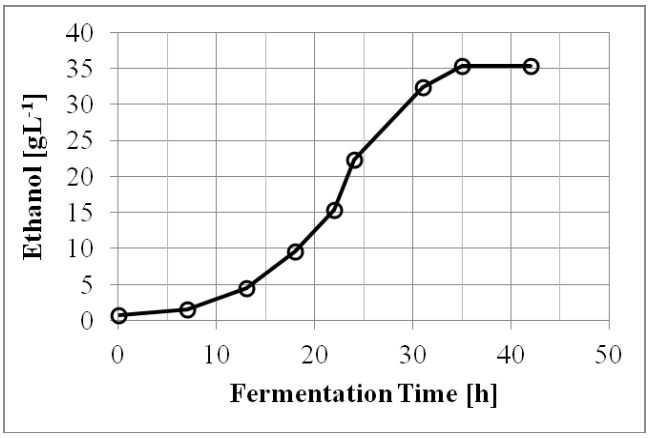

Figure 4. Production of ethanol from CFH supplemented with 2 mM of betaine and 15 g L^{-1} soy peptone. Culture used *E. coli* FBR5. No NaCl was added to the medium in this experiment.

In the three fermentations (control, CSL, and SP), the culture also produced acid by-products including succinic, lactic, acetic, and formic acids (**Table 2)**. The succinic acid concentration was high ranging from 4.10 to 6.72 g L^{-1} while formic acid concentration was low ranging from 0.18 to 0.41 g L^{-1}. Lactic acid concentration ranged from 0.00 to 1.42 g L^{-1} and acetic acid concentration varied from 0.97 to 1.64 g L^{-1}. Production of these acids is undesirable and negatively impacts economics of ethanol production because: i) it requires alkali to be added to control pH during the fermentation which is costly; ii) production of organic acids diverts carbon source away from ethanol; and iii) recovery of these acids from the fermentation broth is difficult and adds to the process costs. The reader is informed that pathway leading to the production of succinic acid has been eliminated in other *E. coli* strains.[20] It is suggested that succinic acid production should be eliminated in FBR5 as it is a potential industrial strain and concentration of this acid is the highest among acids production.

Table 2. Production of by-products during ethanol fermentation from xylose using *E. coli* FBR5

Nutrient Source	By products [g L^{-1}]				
	Succinate	Lactate	Acetate	Formate	Total acids
Control	4.10	1.42	1.33	0.32	7.17
YE 5 + Tryp 5	4.64	1.05	0.97	0.41	7.07
CSL 15 g L^{-1}	6.72	0.77	1.54	0.33	9.36
SP 15 g L^{-1}	4.80	0.00	1.64	0.18	6.62

Control: Yeast extract 5 g L^{-1}, tryptone 10 g L^{-1} and 10 g L^{-1} NaCl, YE 5+Tryp 5: Yeast extract 5 g L^{-1}, tryptone 5 g L^{-1}, and NaCl 10 g L^{-1}

Table 3. A brief summary of ethanol production from CFH using *E. coli* FBR5 and commercial nutrient medium.

Substrate & Media	Ethanol [g L^{-1}]	Ethanol yield [-]	Ethanol productivity [g L^{-1} h^{-1}]
CFH with 5 g L^{-1} YE & 10 g L^{-1} Tryptone	20.10	0.42	0.65
CFH + 2 mM betaine + above nutrients	35.00	0.54	0.76
CFH + 2 mM betaine + 15 g L^{-1} Soy Peptone	35.33	0.54	1.01

In these studies we were able to demonstrate use of commercial fermentation media for ethanol production from cellulosic sugars. A brief summary of the results obtained is presented in **Table 3**. Using SP commercial nutrient medium a productivity of 1.01 g L^{-1} h^{-1} was achieved when using CFH as a substrate. In comparison to the control experiment where a productivity of 0.72 g L^{-1} h^{-1} was obtained, the productivity achieved in the above experiment (CFH + SP medium) is high in addition to the economic potential in using cellulosic substrate (CF) and commercial nutrient medium (SP). Hence, the objectives mentioned in the introduction section of this paper have been achieved.

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**Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

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