Effects of Fractionation Methods on the Isolation of Fiber-rich Cake from Alfalfa and Ethanol Production from the Cake

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Freshly harvested alfalfa was fractionated using centrifugation and filtration, whereby alfalfa was separated into a fiber-rich cake and a nutrient-rich juice. The solid cakes from the above separation processes were used as the feedstock for ethanol production using separate hydrolysis and fermentation. The filtration process proved to be more efficient at reducing the solids mass transfer to the juice than the centrifuge process. Glucose from filtered alfalfa solid cake can be efficiently fermented to ethanol with 75% of the theoretical yield. In conclusion, centrifugation was not as effective as filtration in removing particulates and colloidal matter from alfalfa. The filtration process resulted in a solid cake with a higher cellulose digestibility, which leads to a higher ethanol production.

Keywords: Biorefinery; Alfalfa; Ethanol

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INTRODUCTION

Plant biomass presents an attractive source for the production of fuels and chemicals due to its versatility, renewable nature, and low environmental impacts. Considerable attention has been given to lignocellulosic biomass such as agricultural residues and herbaceous energy crops for biofuel production. Alfalfa is the most cultivated leguminous plant in the world (Mahmoud *et al.* 2011). Production of perennial legume crops offer many advantages, such as low expenditure of nonrenewable resources for tillage and fertilizer, high per acre yield of protein and energy, and excellent soil and water conservation characteristics (Koegel *et al.* 1999).

Utilization of perennial legumes, such as alfalfa, has been limited to ruminant rations by the intimate association of large quantities of fiber with the more easily digested nutrients (Koegel *et al.* 1999). "Green Biorefinery" represents an innovative approach to alternative applications of perennial legumes (Mandl *et al.* 2006; Grass and Hansen 1999; O'Keeffe *et al.* 2011). In a green biorefinery, careful wet or green fractionation technology is used as the first step (primary refining) to isolate the green biomass substances in their natural form. Thus, green biomass is separated into a fiber-rich press cake and a nutrient-rich press juice (Kamm and Kamm 2007). In addition to cellulose and starch, the press cake contains valuable dyes and pigments, crude drugs, and other organics. The green juice contains proteins, free amino acids, organic acids, dyes, enzymes, hormones, other organic substances, and minerals (Kamm and Kamm 2007). Both fractions have an economic value. The press cake has been primarily used for production of fodder pellet and biogas (Richter *et al.* 2009). Little attention has yet

been focused on the utilization of the press cake for biofuel purpose, especially as raw materials for bioethanol production. Koegel *et al.* (1999) investigated the ethanol and organic acids production from alfalfa fiber with or without a liquid hot water pretreatment. However, their publication did not describe the wet fractionation process in detail.

The efficiency of conversion of biomass to ethanol depends upon feedstock characteristics and composition, pretreatment processes, and the fermentation technologies (Xiu *et al.* 2011). Feedstock quality for herbaceous energy crops has been extensively studied for use as livestock feed but not for ethanol conversion (Dien *et al.* 2006). The two important factors affecting herbage quality are herbage species and stage of maturity at harvest. Herbage harvested at an early vegetative stage rather than a later growth stage generally has a lower fiber content, and a higher digestibility and crude protein concentration (Buxton and O'Kiely 2003).

The initial fractionation of the green biomass remains an essential operation for green biorefinery processes. Machinery such as a screw press has been primarily used to press the green juice out of the green biomass. Andersen and Kiel (2000), for example, used a screw press to fractionate a range of grasses. For vegetative biomass such as alfalfa, clover and grass, screw presses remove approximately 55 to 60% of the inherent liquid (Kamm *et al.* 2009). Nevertheless, other means of preprocessing have also been applied, such as the thermal mechanical dewatering method, filter presses, belt presses, centrifuges, Hammer mill, simultaneous application of a pulsed electric field, and superimposition of ultrasounds (Mahmoud *et al.* 2011; Arlabosse *et al.* 2011). Mandel *et al.* (2006) stated that the overall goal of pressing grass is to transfer as much of the soluble components as feasible into the press juice fraction. Although a number of other authors have investigated the fractionation of plant biomass, their publications omitted a detailed description of the fractionation process, such as equipment operation and recovery rates.

In addition, limited information is available on the impact of the separation processes on the biofuel value of the separated fiber-rich cake fraction. It would be expected that the process of fractionation would have a great effect on the cake composition and its subsequent conversion to biofuel. Thus, the objective of this study was to explore various options for the separation of green juice from alfalfa harvested at different stages of maturity and investigate the effect of the separation method on alfalfa solid cakes with regards to ethanol yield. In this study, freshly harvested alfalfa from the North Carolina A&T State University farm was fractionated using centrifugation and filtration, whereby alfalfa was separated into a fiber-rich cake and a nutrient-rich juice. Two processing methods (following the harvest of fresh alfalfa) were compared. In addition, the effects of grass maturity on the separation efficiency were also investigated. The resulting solid cakes from the two processing methods were collected and characterized for their potential in bioethanol production.

EXPERIMENTAL

Grass Harvest and Processing

Alfalfa (*Medicago sativa* L.) was grown at existing fields on the NC A&T State University farm and harvested from two separate replicate plots (each 5.24 acres) at two dates [May 12 (harvest 1) and June 9 (Harvest 2)] in the primary growth in 2013. On each

of these dates, grass was harvested as direct-cut material using a shear to an average stubble height of 6 cm for immature (late vegetative stage, stem length>31cm, no buds, flowers or seed pods) and mature (early flower stage, open flowers on all stem shoots) alfalfa. Following harvest, the alfalfa samples were immediately transported to the laboratory, and sub-samples were taken for assessment of dry matter content. Freshly harvested alfalfa stems were reduced in size using scissors. 200 g of demonized water was added to representative 100 g sample. These 2:1 (water: alfalfa) mixtures were thoroughly mixed and chopped in a Waring 2 Speed Laboratory Blender (Model# 7010G) for 3 min at high speed (RPM = 22,000). Subsequent juice separation was conducted with either a centrifuge (Centra-GP8R Centrifuge, ThermoIEC) or normal filtration (Whatman 2V Qualitative Filter Paper, Manufacture No. 1202-125, Particle Retention > 8 μm). The centrifugation was carried out at a rotational speed of 2600 RCF for 10 min at 25 ºC. The resulting juice from the two operations was collected and characterized for its potential in value-added processing and co-products generation. The solid cake was also dried at 105 °C for 24 h for chemical analysis. Subsamples of the juice and solid cake were used fresh or kept in a freezer at -80 °C for downstream processing.

To determine how well each separation method would work and to answer the question of which separation method would best fit the process for extracting green juice from fresh alfalfa, the performance of centrifugation and normal filtration was evaluated using the ethanol yield from the solids fermentation. A high ethanol yield from the solid cakes is desirable. All the experiments and analyses were performed in duplicate.

Chemical Analyses and Mass Flow Calculation

The alfalfa pre-fractionation and the solid cake after extracting the juice were analyzed for minerals (*e.g.*, K, Mg, Ca, Cl, S, P), elemental composition (*e.g.*, C, H, O, N), total mineral compounds content, solids content, volatile content, and carbohydrates (cellulose, hemicellulose, lignin). Two stages of acid hydrolysis were performed for determining the structural carbohydrates and lignin content on the alfalfa samples according to NREL Ethanol Project Laboratory Analytical Procedure (Ruiz and Ehrman 1996). The concentrations of total mineral compounds and carbohydrates in the juice can be calculated from the proportions of solid cake and juice in the alfalfa after separation. In addition to chemical analyses, the mass flow of dry matter from the alfalfa into the juice and solid cake were calculated. The dry matter of all subsamples of the alfalfa, the solid cake, and the juice was determined by oven-drying at 105 ºC for 24 h.

The elemental composition (C, H, O, N) of the alfalfa pre-fractionation and the solid cake samples was determined using a PE 2400 II CHNS/O analyzer (Perkin Elmer Japan Co., Ltd.). The alfalfa and the solid cakes were digested with HNO_3/HCl in a microwave oven (200 ºC, 2 MPa) and analyzed by inductively coupled plasma–optical emission spectroscopy (ICP–OES) (ARL 3560, Waltham, MA, US) for minerals.

The solids content analysis was determined using the APHA-AWWA-WPCF Standard Method 2540, which includes total solids (TS), volatile solids (VS), and fixed solids (FS).

Enzymatic Hydrolysis

Enzymatic hydrolysis tests were carried out under the same conditions for both centrifuged solids and filtered solids. The conditions of the enzymatic hydrolysis were as follows: about 4.5 g of wet alfalfa was mixed with 0.05 M citrate buffer (pH 4.8) to a

total volume of 50 mL. Screw-capped 250-mL Erlenmeyer flasks were used as reaction vessels and were agitated at 180 rpm in a constant temperature rotary shaker at 50 °C for 96 h.

The centrifuged solid cake and filtered solid cake samples were hydrolyzed using a cocktail of enzymes, which included cellulase loading (Novozyme, NS50013) of 25 FPU(Filter Paper Unit) g/glucan, β-glucosidase (Novozyme, NS50010) at 4.5 CBU/g-glucan, and hemicellulase (Novozyme, NS22002) at 2.5 FBG/g-glucan. After 96 h, the hydrolyzed slurry was cooled and filtered for sugar determination using HPLC.

Fermentation

The bacteria *Escherichia coli* (*E. coli*) were used to ferment the enzymatically released sugars. For ethanol production, 1 mL of seed culture was used to inoculate 4 mL of Luria-Bertani Broth (LB) medium in a 250-mL Erlenmeyer flask. These were incubated in a shaker at 32 °C and 180 rpm and grown aerobically for 24 h. After 24 h, the cultures were transferred into 50 mL of the medium and the cells was harvested by centrifugation at 2600 RCF for 15 min and washed with peptone solution 3 times. The supernatant was discarded, and the cells were transferred into 250-mL Erlenmeyer flasks containing 50 mL of the hydrolysate. The flasks were tightly closed to allow for the fermentation to occur under anaerobic conditions. The cultures were placed in a shaker and incubated at 30 °C for 72 h. Samples were taken at predetermined intervals (0, 3, 24, 72, and 96 h) and collected by filtering through 0.45-μm nylon membranes for ethanol and sugars analysis by HPLC. The ethanol yield was expressed as the percentage of the theoretical yield using the following formula,

$$\boldsymbol{Yield_{ethanol}} = \left[\frac{\boldsymbol{C_{ethanol,f} - C_{ethanol,i}}}{\boldsymbol{0.568 f \cdot C_{biomass}}}\right] \times \mathbf{100}\% \quad (1)$$

where $C_{ethanol,\ f}$ is the ethanol concentration at the end of the fermentation (g/L), $C_{ethanol,\ i}$ is the ethanol concentration at the beginning of the fermentation (g/L), $C_{biomass}$ is the dry biomass concentration at the beginning of the fermentation (g/L), f is the cellulose fraction of the dry biomass (g/g), and 0.568 is the conversion factor from cellulose to ethanol.

Statistical Analysis

All experiments were carried out in duplicate, and data were expressed as average values. Enzymatic hydrolysis and fermentation data were statistically analyzed using the GLM procedure (SAS Institute, Cary, NC). Multiple comparisons among different treatment methods were performed with Tukey's test with a significance level of 0.05.

RESULTS AND DISCUSSION

Mass Flows into Juice and Solid Cake

Approximately 18 to 27% of the dry matter contained in the alfalfa pre-fractionation was directed into the juice during the centrifuge separation, while 73 to 82% was left in the solid cake (Fig. 1). For the filtration process, the mass flow of the dry matter into the juice was between 7 and 16%, depending on the maturity of the alfalfa.

Mature alfalfa results in better separation results with both separation methods than the immature alfalfa.

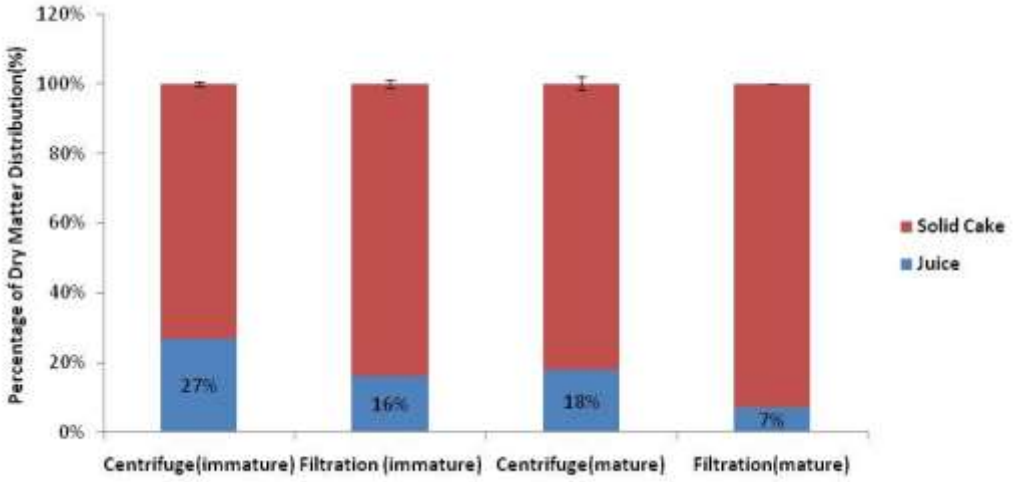

Fig. 1. Percentage of dry matter distribution in the juice and solid cake for mature and immature alfalfa with different separation methods

Solids Analysis

For comparison of the solids content of alfalfa juice and solid cake samples after the centrifugal and filtration processes, the total solids (TS) of each fraction are shown in Fig. 2. The TS of juice after the filtration process was lower than that of the juice from centrifugal separation for both immature and mature alfalfa, with a value of 2.1 to 2.2%. Mature alfalfa produced solid cakes with higher solids contents than immature alfalfa, regardless of the separation method. Overall, centrifugation was not as effective as filtration in removing particulate and colloidal matter from both mature and immature alfalfa. Normal filtration of the alfalfa yielded a relatively stable, particulate-free, permeate that was consistent in color. However, the juice after centrifugation had lots of suspended particles, and this mixture was separated into two layers after 24 h.

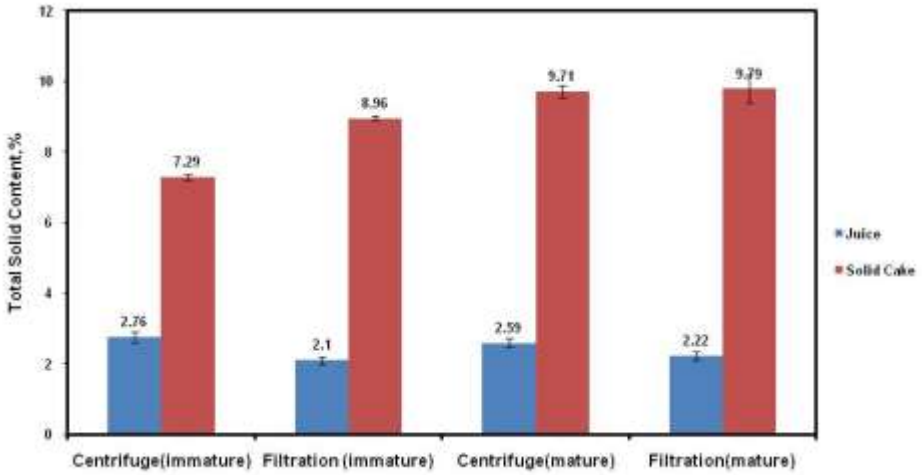

Fig. 2. Total solids (TS) content for alfalfa juice and solid cake after the centrifuge and filtration separation processes

The compositions of the separated alfalfa solid cakes are listed in Table 1. The composition analysis results for fresh harvest alfalfa are also reported in Table 1 for comparative purposes. One of the most notable differences between the mature alfalfa and immature alfalfa was the significantly higher total solids content and lignin content of the mature alfalfa. Differences also existed in the carbohydrates group among these samples. For example, the hemicelluloses content was much lower in immature alfalfa solids with filtration, compared to the other alfalfa, with a value of 6.8%. The elemental composition is very similar for all of the alfalfa samples. The K and Cu concentrations of immature alfalfa were much higher than those of the mature alfalfa, while the Al, Mn, and Ar concentrations of mature alfalfa were significantly higher than those of immature alfalfa.

Table 1. Characteristics of Alfalfa Pre-fractionation and Solid Cakes after Fractionation

Group/specific	Mature Alfalfa	Immature Alfalfa	Immature Alfalfa	Immature Alfalfa
Separation method	None	None	Centrifuge	Filtration
Solids content				
Total solids, %wt	21.74	16.28	7.29	8.96
Volatile solids, % dry matter	93.01	89.19	91.89	94.28
Ash, % dry matter	6.99	10.81	8.11	5.72
H_2O, %wt	78.26	83.72	82.08	82.30
Composition of dry matter %wt				
Hemicelluloses	17.87	15.59	17.49	6.80
Cellulose	36.00	35.29	29.30	20.26
Klason Lignin	21.59	14.35	17.78	15.62
Element group (%)				
C	45.06	45.41	45.00	44.30
H	6.26	6.09	6.39	6.18
O	41.53	42.48	39.9	43.53
N	6.18	5.29	7.76	5.11
S	0.97	0.73	0.95	0.88
Minerals (mg/kg)				
Al	74.03	32.33	171.35	187.26
B	BDL	BDL	BDL	BDL
Ba	22.75	17.43	21.86	25.11
Ca	15,566.12	12,440.91	12,610.57	13,463.30
Cu	0.31	24.41	24.17	18.27
Fe	141.18	76.34	189.38	181.66
K	9,597.23	23,222.44	18,413.84	8,818.52
Mg	3,145.54	3,057.97	2,617.36	1,990.18
Mn	37.49	14.08	23.31	15.19
P	2,865.96	4,137.52	3,741.51	1,680.2
Sr	48.24	7.69	12.36	10.69
Zn	16.66	28.10	35.93	21.08
*BDL=below detection limit				

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed to evaluate the cellulose and xylan digestibility of the solid cakes obtained from the centrifuge and filtration processes. Mature alfalfa pre-fractionation was employed as a control. As shown in Fig. 3, the glucose yield increased with enzymatic hydrolysis time and began to level off after 72 h. There was significant difference between the glucose yields of fractionated and un-fractionated samples ($P<0.05$). More glucose was released from fractionated alfalfa solid cakes than from the control experiment using alfalfa pre-fractionation as a feedstock. The highest glucose yield of 50% was obtained at 96 h from the filtered solids, 80% higher than the glucose yield of alfalfa pre-fractionation. These results suggest that the separation process had a significant impact on the cellulose digestibility of alfalfa. The filtration process resulted in higher ($P<0.05$) glucose yields from enzymatic hydrolysis of the solid cake than the centrifuge process. The glucose yield from filtered solid cake is comparable with the literature (Dien *et al.* 2006).

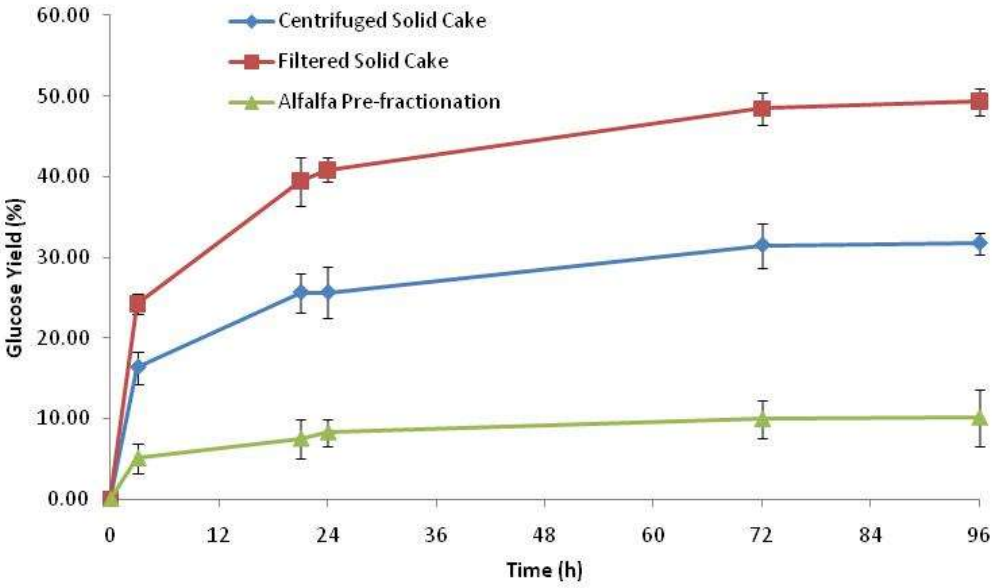

Fig. 3. Glucose yield (% of cellulose from treated alfalfa) after enzymatic hydrolysis of centrifuged solid cake and filtered solid cake

Figure 4 shows the xylose yield from enzymatic hydrolysis of centrifuged solids and filtered solids. As can be seen in Fig. 4, the xylose yield was improved using both fractionation methods ($P<0.05$). The filtered solids produced the highest xylose yield, equivalent to 23% of theoretical yield. Overall, compared to centrifuge separation of alfalfa, the filtration process resulted in higher cellulose and xylose digestibility in the solid cakes ($P<0.05$).

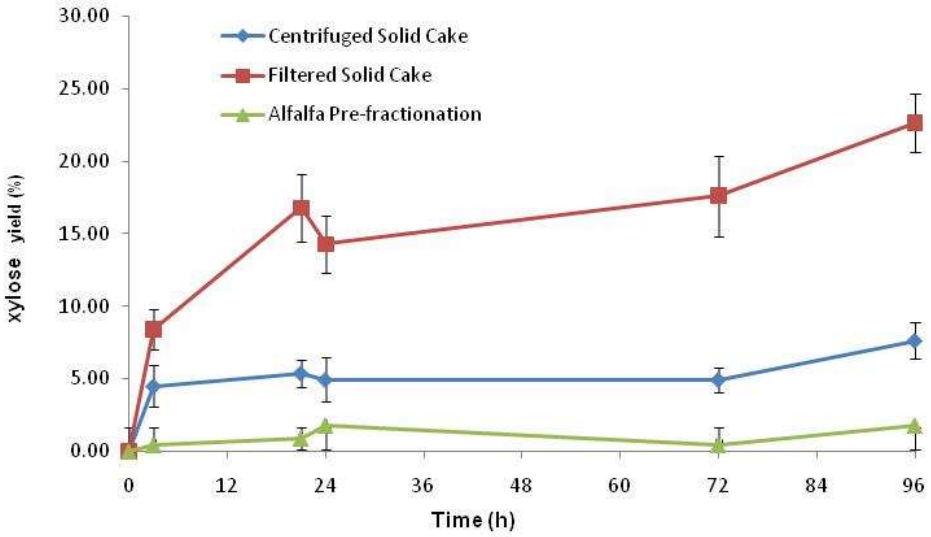

Fig. 4. Xylose yield (% of xylan from treated alfalfa) after enzymatic hydrolysis of centrifuged solid cake and filtered solid cake

Fermentation of Alfalfa Solids for Ethanol Production

The ethanol yield was calculated according to Eq. 1. Figure 5 illustrates the ethanol production over fermentation time for the filtered solid cake and centrifuged solid cake. As illustrated in Fig. 5, ethanol was rapidly produced in the first 24 h, and began to level off in the following 72 h. Filtered solid cake resulted in higher ($P<0.05$) ethanol yields from fermentation than the centrifuged solid cake. The final ethanol yields for filtered solids and centrifuged solids were 75% and 51%, respectively. These results suggest that the glucose and xylose produced from alfalfa-separated solids can be efficiently fermented to ethanol.

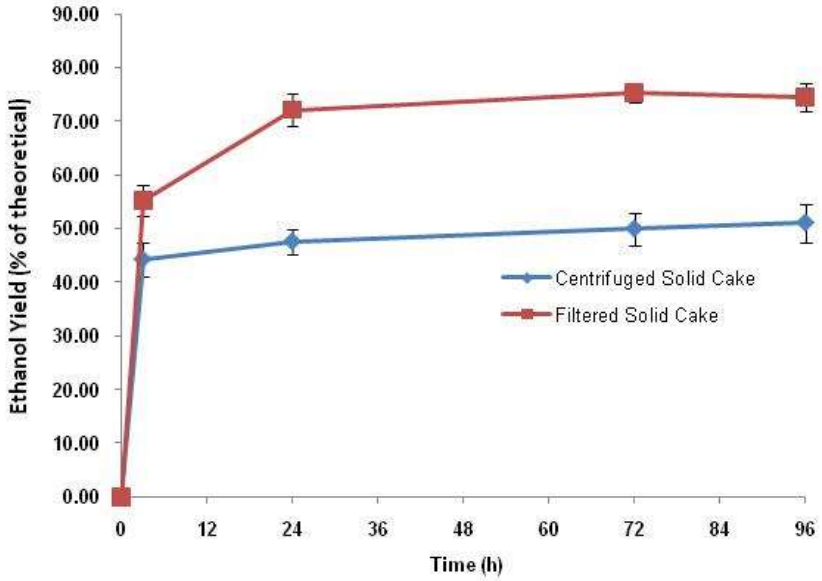

Fig. 5. Ethanol yield (proportions of the theoretical potential) after separate hydrolysis and fermentation of centrifuged solid cake and filtered solid cake

CONCLUSIONS

1. In comparison with the centrifugal process, normal filtration reduced the solids mass transfer to the juice and increased the dry matter concentration of the resulting solid cake.
2. The two fractionation processes employed in the present study were effective in improving the enzymatic convertibility compared to the unfractionated alfalfa. The filtration process resulted in a solid cake with a higher glucose and xylose yield than the centrifuge process.
3. *E. coli* were able to convert the fermentable sugars released from enzymatic hydrolysis of alfalfa solid cakes into ethanol. Approximately 75% and 51% of the theoretical ethanol yield were obtained for filtered solid cake and centrifuged solid cake, respectively.

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