Bioethanol Production from Enzymatic Hydrolyzates of Pretreated Flaxseed Meals by Baker's Yeast

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Research Article

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Title page

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Bioethanol production from enzymatic hydrolyzates of pretreated flaxseed meals by Baker's yeast

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Abstract

The present study investigated the usefulness of flaxseed meals as a novel feedstock for the production of bioethanol. The proximate composition of the flaxseed meal was carried out before the pretreatment of the flaxseed meal. In this study, flaxseed meal was pretreated with dilute acid, alkali, and aqueous for disruption of lignocellulosic compounds. The acid pretreated flaxseed meal was used for enzymatic hydrolysis by different enzymes (cellulase, α-amylase, and cellulase combined with α-amylase) for better release of reducing sugar. The cellulose conversion to reducing sugar was significantly higher for acid pretreated flaxseed meals. After enzymatic hydrolysis with cellulase, cellulose conversions to reducing sugars were found to be significantly higher than those of α-amylase and cellulase combined with α-amylase. The bioethanol production was also investigated. The fermentation process was carried out by using baker's yeast (*Saccharomyces cerevisiae*) with the acid pretreated flaxseed meal enzymatic hydrolyzate. Maximum ethanol production (0.11 g/l) was achieved from the fermented medium obtained from the acid pretreated flaxseed meal followed by enzymatic hydrolysis by using cellulase enzyme. The structural analysis of bioethanol was also investigated by FTIR.

Keywords: Flaxseed meals, reducing sugar, bioethanol, FTIR

1. Introduction

Climate change and energy security considerations have motivated interest in developing fuels from renewable resources [*1*]. Bioethanol is a biofuel produced through the fermentation of simple sugars by yeast [*2*]. Bioethanol is widely used as a partial gasoline replacement in several countries on the planet [*2*]. Ethanol production in the world has reached about 25,930 million gallons in 2020 [*3*]. USA and Brazil are the primary producers and India stands fifth among the highest ethanol producers [*3*]. Bioethanol produced from lignocellulosic biomass has given considerable attention in recent years [*4*].

Flaxseeds (*Linum usitatissimum* L.) are known as a functional food due to their high content of oil rich in α-linolenic acid and dietary fiber and their pharmaceutical properties like anti-inflammatory and anti-tumor activities [*5*]. The average flaxseeds

productions in 2020 were 103 thousand metric tons in India [*6*] Flaxseeds oil is the main component of these seeds and flaxseed meals are the byproduct of the flaxseeds oil industry which is used as cattle feed [*7*]. These meals are good sources of fibers and carbohydrates [*8*]. Flaxseeds also contain insoluble fibers like cellulose, hemicelluloses, and lignin [*9*]. These fibers can be bio-converted into bioethanol through the fermentation method.

To produce ethanol from the lignocellulosic biomass, there is a need for technological improvement and cost reduction in all stages of production [*4*]. The different pretreatment stages are the important steps to the production of ethanol. Hydrolysis is a pretreatment process, occurred by enzyme or acid or alkali, to produce ethanol through the breakdown of cellulose into cellobiose and glucose [*4*]. Dilute acid hydrolysis and enzymatic hydrolysis have been the most popular ones out of these pretreatment methods [*4*]. However, dilute sulphuric acid pretreatment combined with enzymatic hydrolysis is considered a promising method for the production of fermentable sugars [*10*]. Enzymatic saccharification promotes hydrolysis of hemicelluloses and celluloses which helps in the high recovery of monomers [*10*]. Enzymatic saccharification is an important treatment for the production of fermentable sugars [*11*] and has several advantages like less energy requirement, less toxic compound production, high product yield, and less production of fermentation inhibitory compounds [*12*].

Fruit wastes like damaged fruits, peels, and seeds are used as feedstocks for biofuel production [*13*]. A few research articles deal with the production of ethanol from sunflower seed [*14*]; cassava and durian seed [*13*]; jatropha seed cake [*15*] and abrus seed flour [*16*], no literature was found to describing the production of bioethanol from flaxseed meal. To the best of our knowledge, the present study reported for the first time the production of bioethanol from flaxseed meals.

The present work aimed to determine the effect of aqueous, acid, and alkali pretreatment on the lignocellulosic composition and reducing sugar content of flaxseed meals. Determination of the optimal conditions of the most effective pretreatment process before enzymatic hydrolysis was carried out. The present work also aimed to produce bioethanol by fermentation of flaxseed meal hydrolyzate and also determined the reducing sugar content of the fermented sample. The structural features of flaxseed meals, as well as

pretreated flaxseed meals, were investigated by Fourier transform infrared (FTIR) analysis, and the bioethanol produced from flaxseed meals was also investigated by FTIR.

2. Materials and methods

Flaxseeds were collected from the local market of Kolkata, West Bengal, India. All chemicals and solvents used in this study were of analytical grade and procured from Merck (India). Dinitrosalicylic acid and potassium dichromate were obtained from Merck (India). Cellulase (from *Aspergillus niger*, 0.3 U / mg), α-amylase (from porcine pancreas, 5 U /mg) were purchased from Sigma-Aldrich Corp (St. Louis, Missouri, USA). Baker's yeast (R.B Product) was purchase from the local market of Kolkata.

2.1. Preparation of Flaxseed meal

Flaxseeds (50 gm) were dried in a hot air oven (Lasin, LSI 002Ds, Labsoul, India) at 50°C for 1 h. After drying, the flaxseeds were ground to fine powder using a mixer grinder (Bajaj), and oil extraction was done by the Solvent extraction method [*17*]. The flaxseed powder was soaked in n-hexane (1:10 w/v) for 24 h to extract the oil and the extract was filtered by the Whatman No.1 filter paper. After filtration, the residue was collected from the Whatman No.1 filter paper to a Petri plate and the residue was subjected to solvent evaporation in a hot air oven (Lasin, LSI 002Ds) at 30°C for 24 h to obtain the deoiled flaxseed powder. The dried deoiled flaxseed powder was kept in an airtight container labeled as flaxseed meal and stored at desiccator (Photron-PHKJ-50S) until required to be used.

2.2. Proximate Composition of flaxseed meal

The carbohydrate content of the flaxseed meal was determined by the Anthron Method [*18*]. Protein content was carried out by the Folin-Lowry method [*19*]. Fat content was determined according to Kates, 1991 [*20*]. Moisture content and ash content were carried out using the standard AOAC (Association of Official Analytical Chemists) method [*21*]. Crude fiber content was determined by using the standard AOAC method [*22*]. The amount of reducing sugar was determined using the DNSA method [*23*].

2.3. Determination of the amount of extractive present in flaxseed meal

Extractive is not a part of lignocellulosic compounds which may interfere with the analysis of lignocellulosic compounds [*24*]. These substances are mainly waxes, fat, resins, gums, sterols, flavonoids, tannin, etc [*24*]. For this reason, extractive present in

flaxseed meal has been removed first and the amount of extractive present in flaxseed meal was determined by the method described by [*25*]. Briefly, 5 g of flaxseed meal was taken in a thimble, which was subjected to extractive extraction in the Soxhlet extractor set up by using 300ml acetone as the solvent. The boiling temperature of the solvent was carefully adjusted to 90°C for 30min on the heating mantle. After 6 cycles, the sample containing thimble was dried in a hot air oven (Lasin, LSI 002Ds) at 110°C until a constant weight was obtained. The amount of extractive was measured by the following equation

$$Extractive\ (\%) = (W1 - W2) \times 100$$

Where W1= Initial weight of flaxseed meal (g).

W2 = Weight of extractive free dried flaxseed meal (g).

2.4. Quantitative estimation of lignocellulosic material present in extractive free dried flaxseed meal

Lignocellulose is mainly contained cellulose, hemicellulose, and lignin and formed directly from plant photosynthesis [*26*]. Lignocellulose is a carbon resource that is used as an alternative to fossil fuels via conversion into liquid and gaseous fuels [*26*]. So the estimation of lignocellulosic material in extractive free dried flaxseed meal was important to the production of bioethanol.

2.4.1. Determination of the amount of hemicellulose present in extractive free dried flaxseed meal

The amount of hemicellulose present in extractive free dried flaxseed meal was measured by the method described by Yang et al. [*27*]. Briefly, 1gm of extractive free dried flaxseed meal was mixed with 150 ml of NaOH solution (0.5mol/L) and heated at 80°C for 3.5 h on a hot plate. Then the mixture was washed with deionized water and filtered until it was free from Na^+. The Na^+ was detected by using pH paper and the reading should be closed to 7. The residue was dried in a hot air oven (Lasin, LSI 002D) at 110°C until a constant weight was obtained. The amount of hemicellulose was estimated by the following equation

$$Hemicellulose(\%) = (W2 - W3) \times 100$$

Where W2 = Weight of extractive free dried flaxseed meal (g).

W3 = Weight of the dried residue (g).

2.4.2. Determination of the amount of acid-insoluble lignin and acid-soluble lignin present in extractive free dried flaxseed meal

The acid-insoluble lignin and acid-soluble lignin of extractive free dried flaxseed meal were determined by the method described in Ayeni et al. [*25*]. Briefly, 0.5 g of extractive free dried flaxseed meal was taken in an Erlenmeyer flask, and 5 ml of 72% H_2SO_4 was added and mixed well. The mixture was kept at room temperature (37°C) for 2 h with carefully shaking to allow for complete hydrolysis. After the hydrolysis, 84 ml of distilled water was added to the mixture and autoclaved for 1 h at 121°C. The hydrolysate was then cooled at room temperature (37°C). The hydrolysate was filtered through a vacuum filter. The acid-insoluble lignin was measured by drying the residue at 110°C.

The acid-soluble lignin was measured by measuring the absorbance of the acid hydrolyzed samples at 320 nm.

$$Acid\ insoluble\ lignin(\%) = \frac{W}{W2} \times 100$$

Where W= Weight of lignin (g).

W2= Weight of extractive-free dried flaxseed meal (g).

2.4.3. Determination of the amount of cellulose present in extractive free dried flaxseed meal

The cellulose content was determined according to the method described by Galiwango et al. [*28*]. 1 g of the extractives free dried flaxseed meal was weighed into a 250 ml beaker and 200 ml of 0.1 M HCl was added to it. The mixture was heated at 100°C and stirred for 2 h. The mixture was then filtered and the residue was washed with deionized water and dried at 40°C overnight in the hot air oven (Lasin, LSI 002Ds). The

residue was then treated with 200 ml of 0.1 N NaOH with constant stirring and heating at 100°C for 2 h. The mixtures were filtered, and the residue was washed with 20 ml of 0.1 N NaOH. After that, the residue was dried at 40°C overnight in the hot air oven (Lasin, LSI 002Ds). The dried isolated residue was treated with 15, 20, and 10 % acetic acid, hydrogen peroxide, and sulfuric acid, respectively, at 75°C for bleaching. The weight of the bleached residue was expressed as the amount of cellulose content present in 1 g of extractive free dried flaxseed meal.

$$Cellulose\ (\%) = \frac{A}{W2} \times 100$$

Where A= Weight of cellulose (g).

W2= Weight of extractive-free dried flaxseed meal (g).

2.5. Pretreatment of flaxseed meal

Flaxseed meal was being pretreated with three different pretreatment methods including aqueous pretreatment, acid pretreatment, and alkali pretreatment.

2.5.1. Aqueous pretreatment of flaxseed meal

Aqueous pretreatment was done according to the method described by Limbu and Sibi, [*29*]. 10 g of flaxseed meal was dissolved in 100 ml of distilled water and autoclaved at 121℃ for 15 min. After autoclaved the pretreated flaxseed meal was cooled at room temperature and filtered by using Whatman's No.1 filter paper. The residue was collected and washed with deionized water followed by drying at 65°C and was labeled as aqueous pretreated flaxseed meal and stored at desiccator (Photron-PHKJ-50S) until required to be used. A portion of aqueous pretreated flaxseed meal was used for the analysis of lignocellulosic composition. After pretreatment, the liquid fraction was subjected to determine the total reducing sugar content by the DNSA method.

2.5.2. Acid Pretreatment of flaxseed meal

Acid pretreatment was determined according to the method described by Limbu and Sibi, [*29*] with little modification.10 g flaxseed meal was dissolved in 100 ml of 6% H_2SO_4 acid solution in Erlenmeyer flask and autoclaved at 121°C for 15 min. The resulting solution was immediately cooled and neutralized using 0.1M NaOH. The neutralized pretreated sample was centrifuged (R-24 Research centrifuge, REMI) at 5000 rpm for 10

min. The supernatant was collected and subjected to determine the total reducing sugar content by the DNSA method. The residue was collected and washed by deionized water and dried at 65°C. The residue was kept in an air-tight container designated as acid pretreated flaxseed meal and stored at desiccator (Photron-PHKJ-50S) before enzymatic hydrolysis. A portion of acid pretreated flaxseed meal was used for lignocellulosic compositional analysis.

2.5.3. Alkali Pretreatment of flaxseed meal

Alkali pretreatment was determined according to the method described by Grover et al. with some modifications [*30*]. 10 g flaxseed meal was mixed with 100 ml of 2% sodium hydroxide solution and autoclaved at 121°C for 15 min. The mixture was centrifuged (R-24 Research centrifuge, REMI) at 7000 rpm for 10 min. The supernatant was collected and determined the total reducing sugar content by the DNSA method. The residue was collected and washed with deionized water followed by drying at 65°C and the residue was kept in an air-tight container labeled as alkali pretreated flaxseed meal and stored at desiccator (Photron-PHKJ-50S) until required to be used. A portion of alkali pretreated flaxseed meal was utilized for the analysis of lignocellulosic composition.

2.6. Determination of reducing sugar of pretreated flaxseed meal

The amount of reducing sugar present in each pretreated flaxseed meal was determined using the DNSA method [*23*]. 1 ml (5mg/ml) of each pretreated flaxseed meal was taken into a separate test tube and the volume was made up to 3 ml with distilled water. To all the test tubes 1 ml of DNSA reagent was added and the test tubes were placed in a water bath (RWB 6, REMI) at 90°C for 5 min. After this, 1 ml of 40%, potassium sodium tartrate (Rochelle salt solution) was added to all the test tubes and then allowed to cool at room temperature. The absorbance of each sample was measured at 540 nm using a spectrophotometer (Jasco V-630 UV-VIS). 1 ml of distilled water was used as the blank sample. Glucose was used as standard.

2.7. Determination of the amount of extractive present in pretreated flaxseed meal

Removal and determination of the amount of extractive present in flaxseed meals after different pretreatments were determined by the method described in the previous section (**Section-2.3**).

2.8. Changes in the lignocellulosic composition of pretreated flaxseed meal

The lignocellulosic (cellulose, hemicellulose, and lignin) compositions of flaxseed meal after different pretreatments were determined by the method described in the previous section (**Section-2.4**).

2.9. Enzymatic saccharification of pretreated flaxseed meal

The acid pretreated flaxseed meal was subjected to enzymatic saccharification according to Hamid and Ismail, **[***10*]. 10 g of acid pretreated flaxseed meal residue was taken separately into three 250 ml Erlenmeyer flask and 100 ml of distilled water was added to each flask. After that cellulase enzyme (2% w/w), α-amylase enzyme (2% w/w), and the enzyme cellulase (1% w/w) combined with α-amylase enzyme (1% w/w) were added to each flask separately. Each sample was agitated at 200 rpm at 50ºC for 2 h and was immediately cooled on an ice bath to deactivate the enzyme and centrifuged (R-24 Research centrifuge REMI) at 5000 rpm for 10 min. After centrifugation, each supernatant (enzyme pretreated hydrolyzate) was collected and analyzed for reducing sugar content by the DNSA method described in the previous section (Section-2.8). The most effective enzyme for saccharification, which released the maximum amount of total reducing sugar, was chosen for ethanol production.

2.10. Functional group analysis of pretreated flaxseed meal

Functional group changes during pretreatment of raw and pretreated flaxseed meal substrates were carried out by Fourier Transform Infrared (FTIR) spectroscopic analysis (Jasco-4700) with a scan range of 4000 to 450 cm^{-1} with a detector of 4 cm^{-1} resolution [*31*].

2.11. Production of bioethanol

Bioethanol production was done according to the method described by Arumugam and Manikandan [*32*]. The fermentation process was carried out using baker's yeast (*Saccharomyces cerevisiae*) in hydrolyzate obtained from the enzymatic saccharification of acid pretreated flaxseed meal. The hydrolyzate was autoclaved at 121ºC for 15 min. Then the hydrolyzate was immediately cooled at room temperature and pH was adjusted to 6 using HCl or NaOH and used as a fermentation medium. The baker's yeast was added to the fermentation broth at a rate of 1g yeast/ 1000ml of fermentation broth. Fermentation was allowed for 72 h at 35ºC for ethanol production and sample from the fermented broth was collected periodically at 24 h interval to determine the growth of yeast cell, reducing sugar content, bioethanol estimation, bioethanol yield, and fermentation efficiency.

2.12. Determination of yeast cell growth in the fermented broth

Determination of yeast cell growth in fermented broth was followed by the method described by Yap and Trau, [*33*]. Each fermented broth was directly measured at 600nm using the spectrophotometer (Jasco V-630 UV-VIS Spectrophotometer, Maryland, USA) and plot the calibration curve of yeast cell growth by using the OD values.

2.13. Determination of reducing sugar in the fermented broth

The amount of reducing sugars present in each fermented broth was determined using the DNSA method described in the previous section (**Section-2.6**).

2.14. Estimation of bioethanol by spectrophotometer

The concentration of bioethanol produced from fermented flaxseed meal enzymatic hydrolysate was determined using the method described by Obeta et al. [*16*]. 10 mg of each fermented broth was taken into separate test tubes and diluted with 10 ml distilled water to give a concentration of 1% (v/v). From these, 0.5 ml of diluted medium was taken into different test tubes and was made up to 1 ml with distilled water. Seven milliliters of acidified chromium reagent were added to each of the test tubes and were allowed to stand for 1 h for color development. The absorbance of each sample was measured at 600 nm using the spectrophotometer (Jasco V-630 UV-VIS

Spectrophotometer). Standard ethanol with a known concentration was used for the standard curve. The bioethanol content of each sample was estimated from the ethanol standard curve.

2.15. Determination of bioethanol yield

The bioethanol yield of fermented flaxseed meal enzymatic hydrolysate was determined according to the method of Obeta et al. [*16*]. Bioethanol content (C1) was determined by a spectrophotometer (**Section 2.14**). The initial and final reducing sugar content of the fermented broth was determined. The difference between the initial and final reducing sugar content was noted as reducing sugars used (C2) during the fermentation process. The bioethanol yield was then calculated using the following relation:

$$Bioethanol\ yield\ (\%) = \frac{C1}{C2} \times \frac{100}{1}$$

Where C1 = Bioethanol content.

C2 = Reducing sugar used.

2.16. Determination of fermentation efficiency

The fermentation efficiency (%) calculation was done according to Arumugam and Manikandan **[***32***]** as follows:

$$Fermentation\ efficiency = (Bioethanol\ yield / Theoritical\ maximum\ ethanol\ yield\ from\ sugar\ substrate) \times 100$$

2.17. Distillation of fermented broth to collect the bioethanol

Distillation of fermented broth to collect the bioethanol was done by the method described by Obeta et al. [*16*]. The fermented broth was taken into a round bottom flask and the distillation setup was joined with the flask. The heat was supplied to fermented broth using a heating mantle with the temperature regulator and the distillate bioethanol was collected at 78^{o}C.

2.18. FT-IR Spectroscopic Analysis of distillate ethanol

The functional group of distillate bioethanol was carried out by Fourier Transform Infrared (FTIR) spectroscopic analysis (Jasco-4700) with a scan range from 4000 to 450 cm^{-1} at a nominal resolution of 4 cm^{-1} [*34*].

Statistical analysis:

Each experiment was conducted at least in triplicate. All values were means ± standard deviation of three samples. The one-way analysis of variance (ANOVA) was used to compute a statistical decision and the means were compared across groups by the Tukey HSD test. $P \leq 0.05$ and $P \leq 0.01$ were considered statistically significant.

3. Results and Discussion

3.1. Proximate composition of flaxseed meal

The proximate composition of flaxseed meals was shown in **Table-1**. The moisture content of the flaxseed meal was (6.60 ± 0.1 %). This observation was a little higher than the findings of other investigators, who reported that the moisture content of three different varieties (Chandni, LS-20, and LS-1) of defatted flaxseeds was 5.68± 0.10 % to 6.12 ± 0.12 % [*8*].

The carbohydrate content of the flaxseed meal was 50.45 ± 0.45%. According to Aslam et al. the carbohydrate content of three different varieties (Chandni, LS-20, and LS-1) of defatted flaxseeds was 12.13 ± 1.70 %, 18.01±1.23, and 20.45± 0.85 % respectively [*8*] which was lower than the present study.

The amount of protein present in flaxseed meal was 25.75 ± 0.43%. This result was higher than the finding of other investigators who reported that the protein content of three different varieties (Chandni, LS-20, and LS-1) of defatted flaxseeds were 18.67 ± 0.41 % to 20.70 ± 0.55. [8]

The extraction of lipid from the flaxseed to prepare the meal was highly efficient, leaving only 2.35 ± 0.05 % residual lipid. The fat content of three different varieties (Chandni, LS-20, and LS-1) of defatted flaxseeds was 9.50 ± 0.54 % to 11.51 ± 0.62 % studied by Aslam et al. [*8*] which were higher than our resulting value.

Ash content of flaxseed meal was 4.25 ± 0.02 % which was lower than other reported values of defatted flaxseeds (6.21 ± 0.18 % to 7.02 ± 0.22 %) [*8*].

The amount of crude fiber present in flaxseed meal was 10.60 ± 0.05 %. Aslam et al. reported that the fiber content of three different varieties of defatted flaxseeds were 38.51 ± 0.17 % to 42.55± 0.55 % [*8*]. The differences in compositions could be the results of different analytical methods and inherent differences attributed to plant variety and growth conditions.

The total reducing sugar content in flaxseed meal was 1.52±0.1 mg/g. According to Filipovic et al. total reducing sugar content of flaxseed was 1.25 ± 0.08 %. [*35*]

3.2. Quantitative estimation of extractive in flaxseed meal

The extractive is a chemical compound that contains fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, resin acid, rosin, waxes, and many other minor organic compounds [*36*]. The extractive content of flaxseed meal was 10.67±0.29% (**Table 2**). Extractive may interfere with the analysis of lignocellulosic compounds [*24*]. So, extractive present in flaxseed meal was removed before the estimation of lignocellulosic compounds in flaxseed meal.

3.3. Quantitative estimation of lignocellulosic compounds in flaxseed meal

Cellulose, hemicellulose, and lignin composition collectively defined as lignocellulosic compounds [*36*]. Quantitative estimation of lignocellulosic compounds of flaxseed meal was presented in **Table 2** and indicated that flaxseed meal (19.27±0.06%) was a good source of cellulose that could be further converted to fermentable sugars. The high cellulose contents of flaxseed meal made it a suitable substrate for ethanol production. However, the lignin content of flaxseed meals inhibited the bioconversion rate of celluloses and hemicelluloses indicating the requirement of suitable pretreatment of flaxseed meals which degrade lignin and make celluloses and hemicelluloses accessible to hydrolysis. Cellulose and lignin contents of flaxseed meal in this study were higher than the result reported by Shim et al. for flaxseeds (cellulose- 7 to 11% and lignin- 2 to 7%) [*5*].

3.4. Pretreatments of flaxseed meal

The reducing sugar content of each pretreated flaxseed meal was presented in **Table 3**. The concentrations of reducing sugar in the acid pretreated flaxseed meal were slightly increased (8.73±0.07 mg/g) than those present in aqueous (8.27±0.1 mg/g) and alkali (8.54±0.07 mg/g) pretreated flaxseed meal. These results of our study were similar to those obtained by Armugum and Manikandam, who reported the hydrolysis of banana and mango fruit waste with dilute H_2SO_4 acid [*32*]. Limbu and Sibi have reported that the acid pretreatment method was found to be better production of reducing sugars from fruit waste than aqueous hydrolysis [*29*], which was similar to the present study. Sirkar et al. reported that the acid pretreatment method was found to be optimal for better yield of fermentable sugars from fruit peels [*37*].

According to Armugum and Manikandam, the aqueous hydrolysis may be a promising pretreatment method that raised the recovery rates of pentoses and does not generate inhibitors [*32*]. The advantage of aqueous hydrolysis is that solubilized lignin and hemicelluloses which are present in a lower concentration. The limitation of aqueous hydrolysis is higher water demand and high energy requirement [*38*].

Acid hydrolysis is the most common process to extract monomeric sugars from complex carbohydrates [*39*]. The most commonly used acids in hydrolysis are sulphuric acid, hydrochloric acid, phosphoric acid, oxalic acid, and nitric acid [*39*]. In the present study, acid hydrolysis was employed with dilute H_2SO_4 acids (6%) at high temperatures (121◦C). Mukhtam et al. reported that dilute acid hydrolysis is the most suitable option compared to concentrated acid hydrolysis in lignocellulosic compound conversion [*39*]. The dilute acid pretreatment has the advantage of solubilizing hemicelluloses and converting solubilized hemicelluloses to fermentable sugars in wheat straw [*40*]. The major limitation of dilute acid hydrolysis is that sugar degradation occurs at high temperatures and long residence times of reaction [*41*].

Alkaline hydrolysis is more suitable for crops and agricultural residues [*42*]. According to Devi et al. to obtain a high yield of sugar by alkaline hydrolysis, is very difficult because mono and dimeric carbohydrates, such as glucose, fructose, or cellobiose, are severely attacked by alkalis at temperatures below 100 °C [*4*]. Alkaline hydrolysis can be used for the pretreatment of lignocellulosic biomass, being esterification of

intermolecular ester bonds cross-linking xylan, hemicellulose, and other components [*43*]. Dilute NaOH pretreatment of lignocellulosic compound causes an increase in the internal surface area and a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates [*4*]. The major limitation of alkali hydrolysis is the low sugar yield [*4*].

Our result also reported that the extractive present in aqueous (7.53±0.16%), acid (3.73±0.03%), and alkali (5.08±0.1%) pretreated flaxseed meal were lower than the untreated flaxseed meal (10.67±0.29%). According to Nitsos et al. the extractive content in almond shell, olive pruning, and grapevine pruning was 0.55%, 6.04%, and 4.36% respectively when using ethanol as a solvent but they did not calculate the extractive presence in the pretreated sample [*44*].

The pretreatments are necessary for efficient enzymatic hydrolysis of cellulose by removal of lignin and hemicelluloses [*45*]. Pretreatments also help to disrupt the crystalline structure of cellulose for enhancing enzyme accessibility to the cellulose during the enzymatic hydrolysis step [*45*]. The lignocellulosic composition of flaxseed meal after different pretreatment was very important which controlled the efficiency of enzymatic hydrolysis. The lignocellulosic composition after each pretreatment was shown in **Table 3** and compared with untreated flaxseed meals.

Our results indicated that pretreated flaxseed meal content higher cellulose compared with untreated flaxseed meal. Our results also confirmed that the hemicellulose and lignin in pretreated flaxseed meals were low. The higher cellulose content in the pretreated flaxseed meal may have been due to the decreasing amount of hemicellulose and lignin. This reduction was most effective on acid pretreated flaxseed meals. **Table 3** showed that recovery of cellulose in acid pretreated flaxseed meal was maximum (66.58 ± 0.07 %) followed by alkali and aqueous pretreatment. The better yield of cellulose can be attributed to the removal of glycosidic bond in the hemicellulose and lignin-hemicellulose bond and the lignin bond [*46*].

Hemicellulose inhibited the susceptibility of cellulose surface to enzymatic hydrolysis by creating a barrier by covering the cellulose fiber [*46*]. Pretreatment might have solubilized the hemicellulose fractions, which improve enzymatic hydrolysis by increasing the surface area *47*. Pretreatment of flaxseed meal removed a large amount of

hemicellulose. There was only 7.21 ± 0.10 % hemicellulose present in the flaxseed meal, after the acid pretreatment.

Lignin also inhibited enzymatic hydrolysis by creating a covering effect which results in the prevention of hydrolysis of the substrate [*47*]. The acid-insoluble lignin contents of pretreated flaxseed meal were decreased than untreated flaxseed meal which indicated that pretreatment efficiently removed lignin content by breaking ester bonds. Maximal removal of lignin was observed for acid pretreated flaxseed meal.

The removal of lignin and hemicellulose content before enzymatic hydrolysis of flaxseed meal was highly necessary. The highest removal efficiency of hemicellulose and lignin by acid treatment indicated that it was the optimal method for enzymatic hydrolysis. Devi et al. reported that the acid pretreatment method was efficient for increasing the digestibility of substrates [*4*]. For each pretreatment of flaxseed meal, acid-soluble lignin was found to be higher than acid-insoluble lignin (**Table 3**). Acid hydrolysis processes are matured and preferred technologies for improving morphological features of feedstock [*4*].

However, no literature on aqueous, acid, and alkali pretreatment of flaxseed meal were found. Similar results have been reported by Ji et al.**,** who observed that the cellulose content (72.4%) of acid pretreated hemp biomass was increased than untreated hemp biomass [*48*]. According to Qi et al., chemical pretreatment of bamboo using acid and alkali exhibited the highest cellulose content and lowest lignin and extractive content [*49*].

3.5. Enzymatic saccharification of pretreated flaxseed meal

Enzymatic saccharification is the most important step for the degradation of cellulose to fermentable sugar [*50*]. The pretreated flaxseed meal was subjected to enzymatic hydrolysis for achieving efficient bioconversion for the production of fermentable sugars. In the present study flaxseed meal pretreated with dilute H_2SO_4 was hydrolyzed using cellulase, α-amylase, and a combination of cellulase and α-amylase. The reducing sugar content obtained from enzymatic hydrolyzate was shown in **Table 4**. The enzymatic hydrolysis yield was expressed as the yield of reducing sugar (g/l of the hydrolyzate) released after 2 hr of enzyme hydrolysis. The concentrations of reducing sugar in the flaxseed meal hydrolyzate were increased when using cellulase enzyme for enzymatic

hydrolysis of acid pretreated flaxseed meal (13.72±0.09 g/l) than those present in α-amylase and cellulase combined with α-amylase enzymatic hydrolyzates. These results of our study were similar to those obtained by Armugum and Manikandam, who reported the hydrolysis of banana and mango fruit waste with dilute H_2SO_4 acid with the enzyme [*32*]. Pretreatment of lignocellulosic compounds to remove hemicelluloses and lignin are important because hemicelluloses and lignin are extremely insubordinate to enzymatic hydrolysis. Enzymatic hydrolysis is important to facilitate the accessibility of cellulase to cellulose for maximum recovery of total reducing sugars to produce value-added products [*51*].

3.6. Functional groups characteristics of untreated and pretreated flaxseed meal

The acid pretreatment had an intense effect in the modification of chemical characteristics profile for enhancement of enzymatic hydrolyzability of pretreated flaxseed meal as presented in **Fig 1**. FTIR spectral regions were assigned for untreated and acid pretreated flaxseed meals. The broad range of 3323.71 cm^{-1} was the spectral region characterized to inter and intramolecular hydrogen bonds absorption peaks associated with crystalline cellulose present in acid pretreated flaxseed meal [*52*]. Declined resolution at 1741.41 cm-1, C=O stretching vibration granted the decrease of hemicellulose in pretreated flaxseed meal. Increased resolution at 1043.3 cm^{-1} corresponds to C–O–C β-1, 4 glycosidic linkages was relative to hemicelluloses removal [*53*]. 1624.73cm^{-1} was attributed to C–Ph and C=C bonds, respectively, and was observed in pretreated flaxseed meals.

3.7. Yeast cell growth in the different pretreated fermented medium

In **Fig 2** yeast cell growth in the different enzymatic pretreated fermented medium was observed. Slow microbial growth was observed at 24 hours during which the consumption of substrate and production of ethanol were small. After the slow microbial growth, it was followed by rapid growth at 48 h. The consumption of glucose and ethanol production was increased rapidly during this period. The yeast cell growth dropped at 72 h. The decrease in cell viability could be due to the consumption of substrate and this led to the inhibition of ATP synthesis [*13*]. Another reason for the decrease in cell viability could be due to the higher rate of ethanol formation which metabolically inactivated the yeast cells and this conducted to the leakage of intracellular

metabolites into the growth medium [*13*]. These conditions caused cell death. The maximum cell growth was observed for the fermented medium which was pretreated by acid followed by enzymatic hydrolysis using cellulase enzyme. For this reason, 72 h fermented medium was optimized for ethanol estimation. These current observations were in good agreement with similar results reported by Arumugum and Manikandam, in banana and mango fruit waste [*32*].

3.8. Reducing sugar content of fermented medium after fermentation

The reducing sugar content of the fermented medium after fermentation was presented in **Table 5**. There was a significant decrease in the amount of reducing sugar in each fermented medium after 72 h fermentation. A maximum decrease of reducing sugar was observed for cellulase enzyme-treated flaxseed meal hydrolyzate fermented medium. A similar result was reported by Obeta et al.**,** who reported that the release of reducing sugar decreased after fermentation [*16*]. Obeta et al., also reported that the concentration of bioethanol increased due to the concentration of reducing sugar released decreased [*16*]. For this reason, the ethanol estimation was done for the 72 h fermented medium.

3.9. Estimation of bioethanol by spectrophotometer

Alcohol production was estimated after 72 h for all fermented samples which was presented in **Table 6**. *Saccharomyces cerevisiae* has a high capability in alcohol production using less sugar as reported by Janani et al. [*54*]. In this study, maximum bioethanol productions (0.11±0.07 g/l) were observed for the fermented medium obtained from the acid pretreatment followed by enzymatic hydrolysis of flaxseed meals using cellulase enzyme. According to Arumugam and Manikandan, the ethanol productions were high for enzyme-treated banana and mango fruit waste [*32*]. It can also be observed that the ethanol productions were increased rapidly when the reducing sugar content decreased. These observations were similar to the report of Seer et al. which indicated that the amount of released reducing sugar influenced ethanol production in mixed cassava and durian seeds [*13*]. These observations were agreeable with the recent studies reported by Bouaziz et al. **[***55*]. They reported that the sugar content decreased over time while ethanol production increased of the date seed. There was no literature available on bioethanol production from flaxseed meals.

3.10. Bioethanol yield and fermentation efficiency of the *fermented medium after 72 h fermentation*

Bioethanol yield (%) and fermentation efficiency of fermented medium were shown in **Table 6**. It was found that, at a constant fermentation time (72 h), maximum bioethanol yield (%) was obtained from cellulase treated fermented medium followed by α-amylase and cellulase combined with α-amylase treated fermented medium respectively. The fermentation studies on the hydrolyzates of flaxseed meal obtained from enzymatic hydrolysis have shown poor ethanol yield (**Table 6**) and it might be due to the inhibitory effect of high polyphenol content or less availability of fermentable sugar after even saccharification. Fermentation efficiency was higher for cellulase-treated fermented medium after 72 h fermentation than those of α-amylase and cellulase combined with α-amylase treated fermented medium. The fermentation efficiency was also showed poor efficiency.

3.11. FT-IR Spectroscopic Analysis of distillate ethanol

FTIR analysis of the alcohol showed the presence of various functional groups. The chromatograms of ethanol obtained from the different fermentation mediums by fermentation were shown in **Fig 3**. Alcohols have characteristic IR absorptions associated with the region 3500-3200 cm^{-1} with a very broad band indicated the O-H stretch of alcohols, while the region 1260-1050 cm^{-1} confirms the C–O stretch. The bands present in 2880 and 2930 cm^{-1} were indicated the symmetric stretching modes of the –CH2 and–CH3 groups, respectively [*56*; *57*]. Sanchez-Acuna et al., indicated that the band observed in 2950 - 3000 cm^{-1} were C-H bonds *34*. In another study, Bouaziz et al. reported that peaks observed around 2800–3000 cm^{-1} for the respective stretching vibrations of C-H *55*. The products obtained from different fermentation mediums were definitely ethanol due to the confirmation of these regions.

4. Conclusions

The present work concluded that flaxseed meals could be used as raw materials for the production of ethanol. Dilute H_2SO_4 pretreatment of flaxseed meal was a favorable method for the obtained high capability of enzymatic hydrolysis for higher sugar yield. This was mainly due to effective disruption of the lignocellulosic compounds by dilute acid pretreatment which increased cellulose accessible surface area. Bioethanol obtained

from the fermentation of acid pretreated flaxseed meals followed by enzymatic hydrolysis by using cellulase enzyme was highly satisfactory. This work also performed the IR characterization of bioethanol those obtained from the fermented medium by fermentation at 72h.

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Declarations

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Authors' contributions:

Conceptualization: Jayati Bhowal, Saheli Ghosal; Methodology: Jayati Bhowal, Saheli Ghosal; Formal analysis and investigation: Saheli Ghosal; Writing - original draft preparation: Saheli Ghosal; Writing - review and editing: Jayati Bhowal; Supervision: Jayati Bhowal .

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Table-1: Proximate Composition of flaxseed meal

Composition	Flaxseed meal
Moisture (%)	6.60 ± 0.1^{a}
Carbohydrate (%)	50.45 ± 0.45^{b}
Protein (%)	25.75 ± 0.43^{c}
Fat (%)	2.35 ± 0.05^{d}
Ash (%)	4.25 ± 0.02^{e}
Crude fiber (%)	10.60 ± 0.05^{f}
Total reducing sugar content (mg/g)	1.52 ± 0.1^{g}

All values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c, d, e, f, g) letters within the same column indicated significant ($P< 0.05$) differences.

Table-2: Extractive and lignocellulosic composition of flaxseed meal

	Flaxseed meal
Cellulose (%)	19.27±0.06[a]
Hemicellulose (%)	16.03±0.06[b]
Acid soluble lignin (%)	1.15±0.05[c]
Acid insoluble lignin (%)	8.03±0.03[d]
Extractive (%)	10.67±0.29[e]

All values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c, d, e) letters within the same column indicated significant ($P < 0.01$) differences.

Table-3: Reducing sugar content, extractive and lignocellulosic composition of different pretreated flaxseed meal

Substrate	Treatment	Reducing sugar (mg/g)	Extractive (%)	Cellulose (%)	Hemicellulose (%)	Acid soluble lignin (%)	Acid insoluble lignin (%)
Flaxseed meal	Acid	8.73±0.07[a]	3.73±0.03[b]	66.58±0.07[c]	7.21±0.10[d]	2.47±0.06[e]	5.28±0.08[f]
	Alkali	8.54±0.07[a]	5.08±0.1[b]	53.12±0.08[c]	10.44±0.05[d]	2.03±0.05[e]	6.43±0.05[f]
	Aqueous	8.27±0.1[a]	7.53±0.16[b]	48.47±0.05[c]	12.41±0.10[d]	1.84±0.07[e]	6.75±0.09[f]
	Untreated	1.52±0.1[a]	10.67±0.29[b]	19.27±0.06[c]	16.03±0.06[d]	1.15±0.05[e]	8.03±0.03[f]

All values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c, d, e, f) letters within the same row indicated significant ($P < 0.01$) differences.

Table-4: Total reducing sugar content (g/l) of acid pretreated flaxseed meal hydrolyzate with enzyme

Substrate	Enzyme	Total reducing sugar content (g/l)
Acid pretreated flaxseed meal	Cellulase	13.72±0.09 [a]
	α-amylase	12.26±0.08 [b]
	Cellulase with α-amylase	12.87±0.05 [c]

All values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c) letters within the same column indicated significant ($P < 0.01$) differences.

Table-5: Reducing sugar content (g/l) of the fermented medium after fermentation

Substrate	Enzyme	Before fermentation	After fermentation		
			24h	48h	72h
Acid pretreated flaxseed meal	Cellulase	13.72±0.09 [a]	5.41±0.08 [b]	4.66±0.05 [c]	4.61±0.06 [c]
	α-amylase	12.26±0.08 [a]	4.87±0.08 [b]	2.91±0.02 [c]	2.81±0.07 [c]
	Cellulase with α-amylase	12.87±0.05 [a]	4.55±0.02 [b]	2.82±0.01 [c]	2.79±0.01 [c]

All values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c) letters within the same row indicated significant ($P < 0.01$) differences.

Table-6: Bioethanol concentration, bioethanol yield, and fermentation efficiency of different enzyme-treated 72h fermented sample

Substrate	Enzyme	Bioethanol concentration (g/l)	Bioethanol yield (%)	Fermentation efficiency (%)
Acid pretreated flaxseed meal	Cellulase	0.11 ± 0.07^{a}	1.20	1.34
	α-amylase	0.095 ± 0.06^{b}	1.01	1.12
	Cellulase with α-amylase	0.10 ± 0.10^{c}	0.99	1.10

Bioethanol concentration values were presented as mean ± standard deviation for triplicate experiments; Mean values with different superscript (a, b, c) letters within the same column indicated significant ($P< 0.01$) differences.

Figures

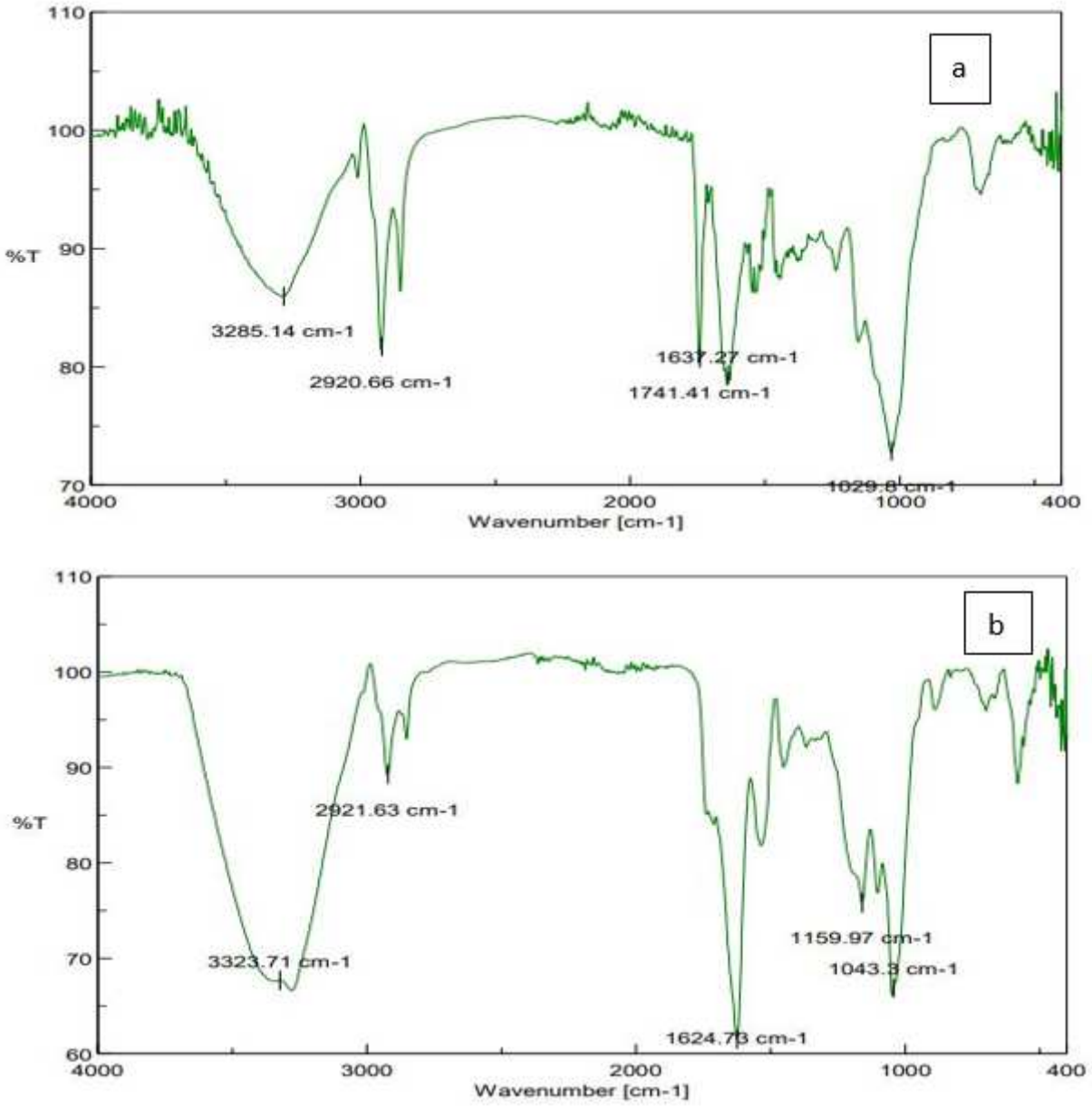

Figure 1

FTIR spectral regions of untreated and acid pretreated flaxseed meal. (a) Untreated flaxseed meal, (b) Acid pretreated flaxseed meal.

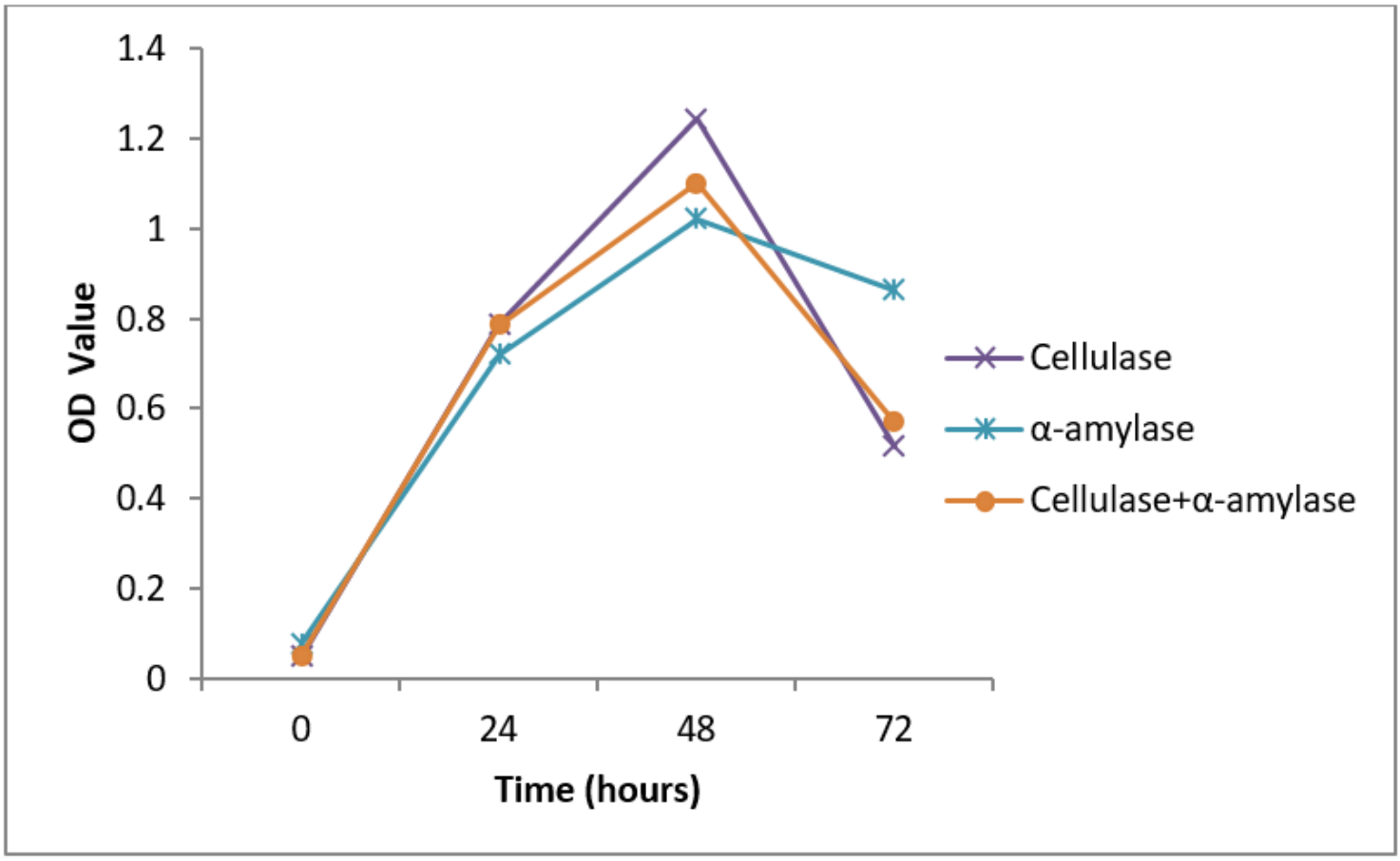

Figure 2

Yeast cell growth in the different enzyme pretreated fermented medium

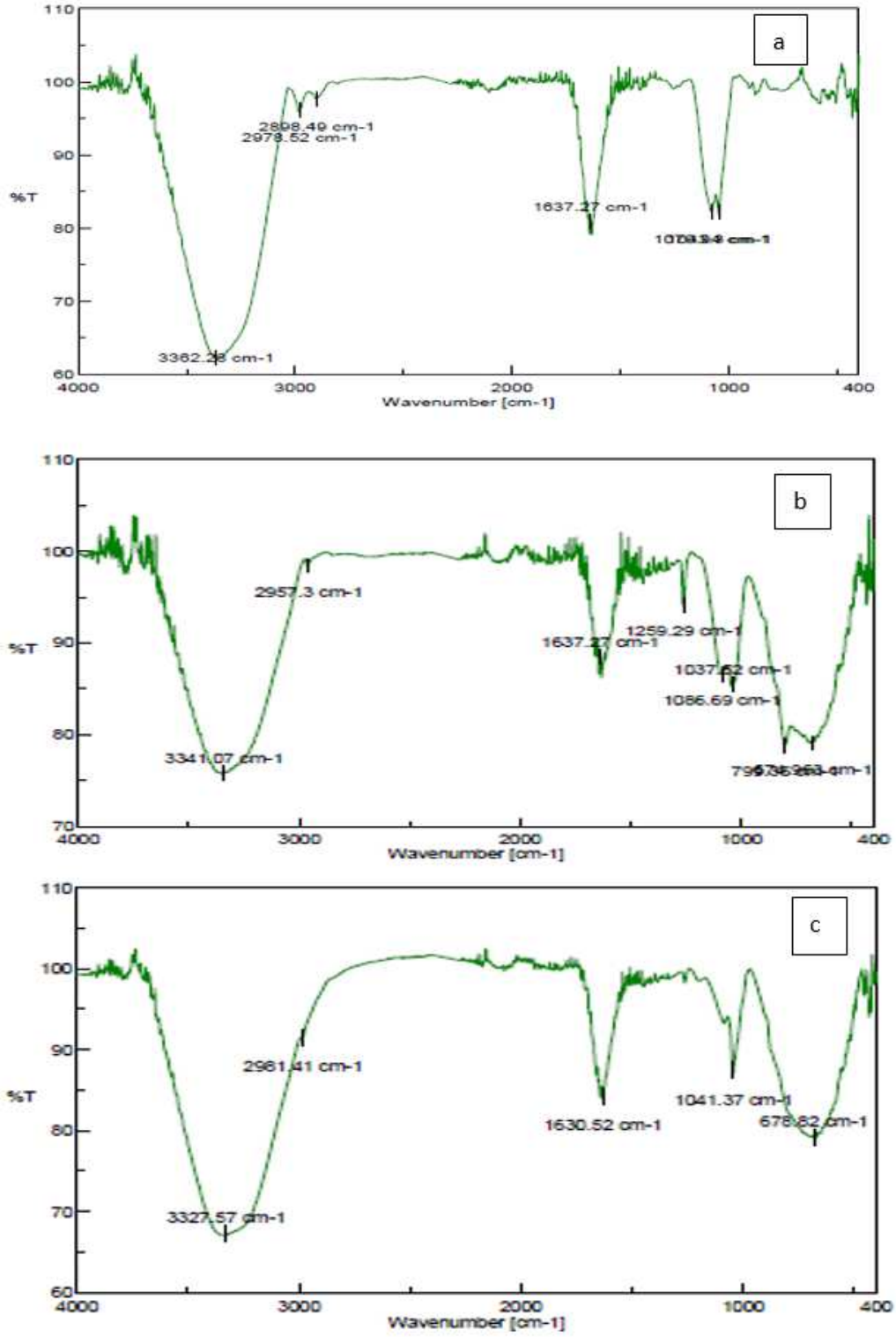

Figure 3

FTIR spectral region of ethanol. (a) Ethanol of cellulase treated fermented medium, (b) Ethanol of α-amylase treated fermented medium, (c) Ethanol of cellulase combined with the α-amylase treated fermented medium.