Egyptian Sugar Journal (2022), 18: 68 - 77
https:// doi.org/10.21608/ESUGJ. 2022.152681.1016

RESEARCH ARTICLE

Production of Bio-ethanol from sugar beet pulp using recombinant *E. coli and S. cereviceae*

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Received: 17 July / Revised 28 August 2022/Accepted 31 August / Published online: 5 September 2022

Abstract

Ethanol is one of the most important biofuels that can be produced from different renewable sources. Sugar beet pulp (SBP) is used as renewable and cheap raw material for ethanol production. SBP is the by-product of the sugar industry from sugar beet that is used as animal feed after processing (pressing, dehydration, and pelletizing). Ethanol from SBP will be more profitable value than the other uses as animal feed.The two highest cellulases producer isolates S11 and S88 from the previous work were subjected to DNA identification using the 16S rRNA gene. 16S rRNA is tool used to identify the origin, classification, evolutionary and relationship history. The isolates S11 (*Streptomyces* sp. strain FDZH12) and S88 (*Streptococcus mitis* strain FDZH16) had been submitted to EMBL and their accession numbers are OK033363 and OK033364, respectively. Cellulase gene from S11 *Streptomyces* FDZH12 then cloned into *E.coli* to produce superior strain for cellulases production.The recombinant *E. coli* was confirmed by colony PCR using gene-specific primers of cellulases. Ethanol production from SBP is achieved through three steps: first, acid-base treatment for SBP and then the resulting cellulose content hydrolyzed to fermentable sugar using genetically engineered *E.coli* cloned by cellulases enzyme. Finally, the fermentable sugar is fermented to ethanol using *S.cereviciae* FDZH2O The weight of dried SBP after acid-base treatment was 45.5 % of the original dried SBP. Cellulose contents of untreated SBP were 27.95 % and reached 84.22 % after acid-base treatment (842.2g/kg). The maximum yields of glucose by the recombinant *E.coli* after 24 hours of saccharification of treated SBP were 28.36 g/50 g of acid base treated SBP (67.52% of their cellulose content). Each 100 ml saccharified solution has 5.672 g glucose. After fermentation, each 100 ml saccharified solution has 2.83 ethanol (0.5008 g/g sugar 98% of the theoretical value). The maximum yield of ethanol by *S. cerevisiae* FDZH2O (equal to 14.20 g ethanol / 50 g of hydrolyzed SBP which have 42.11 g cellulose) and achieved at pH 6, 30 ºC, and 10% inoculum size after 72 hours of fermentation. According to the mass balance in our study each 6.557 kg, wet beet pulp with the moisture of 86% produces 1 kg dried SBP (DSBP) with moisture of 7.92% then after acid-base treatment produces 455 g treated DSBP that saccharified by recombinant *E. coli* into 258 g glucose and fermented finally by *S. Cerevisiae* into 129.24 g ethanol. This level is relatively low and more experiments are still needed to increase the productivity of this bioprocess.

Keywords: Sugar Beet pulp; Saccharification; *Saccharomyces cerevisiae* FDZH2O; gene cloning.

Introduction

Nowadays, the population increases and faces decreasing in traditional universal energy demand for world energy production from fossil fuels. Ajayo et al. (2022) found that Ethanol from cellulosic biomass could be a promising substitute for petrol and has good octane, which leads to lessened emissions of air pollutants. Berlowska et al. (2016) sugar beet pulp SBP could provide an alternative feedstock for ethanol production. Therefore, there is a need for sustainable and renewable green energy sources that do not affect the environment and ecosystem. Mohd-Azhar et al. (2017) reported that the high 1.carbohydrate make sugar beet pulp an attractive substrate for ethanol production. content of sugar beet pulp (rich in cellulose, hemicelluloses, and pectin) and low lignin content content of sugar beet pulp (rich in cellulose, hemicelluloses, and pectin) and low lignin content make sugar beet pulp an attractive substrate for ethanol production Kamzon et al. (2016).

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reported that sugar beet pulp is particularly rich in cellulose (22–30%), hemicelluloses (24–32%), and low lignin content (around 1%) on a dry weight basis. Moreover, Micard et al. (1996) was recorded that sugar beet pulp is primarily composed of carbohydrate cellulose (21-24%), hemicellulose (26-36%), and pectin (20-22 %) in addition to 10 – 15% protein and 1 – 1.2% lignin, on a dry weight basis. As there is a large amount of beet pulp, which didn't use in the industry and this material has enormous potential for ethanol production, it has been the main target for producing ethanol. The high proportion of glucose that can be obtained from the cellulose fraction of beet pulp and the ability of yeasts to convert these sugars into ethanol are factors that drive the use of this co-product for the production of biofuels as ethanol. Therefore, it is necessary to submit the material to the processes of hydrolysis to release fermentable sugars.

Ethanol production from beet pulp requires the separation of its fractions through pre-treatment methods. The pretreatment with the acid-base method is one of the widely employed procedures. A second step for obtaining, the fermentable sugar from cellulose is acid or enzymatic hydrolysis in order to make available the monomeric sugars, for fermentation to ethanol. In order to use the cellulose content of sugar beet pulp as a substrate for ethanol production, pretreatment of beet pulp by acid-base method and hydrolysis of the product using genetically engineered E.coli cloned by cellulases enzyme obtained from *Streptomyces sp*. FDZH12 and then fermentation of the hydrolyzed products by *S.cereviciae* FDZH2O were investigated.

Materials and Methods

Sugar beet pulp (SBP)

Wet sugar beet pulp was obtained from the Dakahlia Sugar factories, Belqas, Dakahlia governorate, Egypt. Then dried at 90 °C for 48-72 hours. The dried SBP was then milled and sieved to get particles with sizes ranging between 500 and 1000 µm. After that, it was stored in airtigh containers at room temperature (24 ± 6 °C) until used in both saccharification and fermentation processes.

Microorganisms

Streptomyces sp. was isolated from rhizospheric soil surrounding the beetroot in our laboratory in the previous study. The obtained DNA sequence of *Streptomyces sp*. FDZH12 using 16S rRNA gene deposited in Genbank under the accession number OK033363.The recombinant *E.coli* cloned with cellulases enzyme from *Streptomyces sp*. FDZH12 was also prepared in a previous study by the same authors. Saccharomyces cerevisiae strain was used in this study for fermentation of hydrolyzed SBP obtained from the Plant Protection and Bio molecular Diagnosis Department, the City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt. It is stored on yeast extract malt extract (YM) agar slants at 4°C. The obtained DNA sequence of *S. cerevisiae* FDZH20 using 16S rRNA gene deposited in Genbank under accession no. MZ947164.

Chemical analysis of sugar beet pulp (SBP)

Determination of the carbohydrate content of sugar beet pulp (Rathin data 1981).

This method was adapted to (Chesson1978) the weight loss during each fractionation step gives the weight fraction of each of the major components: water soluble, hemicelluloses, cellulose, and lignin in the lignocellulose raw material. Hot NaOH/KOH (1.25%, w/v) removes protein and saccharides before Sequential Fractionation.

Sequential Fractionation of Lignocellulose Polysaccharides:

A) 1 g of Lignocellulose refluxed for 2 h with 150 ml of H20 at 100°C hot water-soluble (pectins, oligosaccharides). Pectins are an even more diverse set of polysaccharides, sharing the trait of being soluble in oxalic acid and hot water.

B) Dried residue was refluxed for 2 h with 150 ml of 0.5 M H2SO4 at 100 °C to remove hemicelluloses.

C) Dried residue treated with 10 ml of 72% (v/v) H2SO4at room temperature for 4 h, then diluted to 0.5M H2SO4, and refluxed at 100 °C for 2 h to remove cellulose and remain lignin.

Calculations:

- primary weight (S) = 1 g
- Pectin = $\frac{A}{S} \times 100$
- Hemi-cellulose = $\frac{A-B}{S} \times 100$
- Cellulose = $\frac{B-C}{S} \times 100$
- Lignin = $\frac{C}{S} \times 100$

Determination of Non-carbohydrate content of sugar beet pulp(moisture, fat, protein, ashes and lignin).

- **Moisture:** Water content was determined in 10g of SBP sample by drying a representative 2 g sample in an oven with air circulation at 100-105 ºC for 3 h until the weight of samples stabilized. Moisture was calculated by the difference in weights before and after drying. (AOAC2000).0
- **Total lipid:** was extracted from the dried SBP sample (20g) with petroleum ether (60-80 ºC) in a Soxhlet apparatus for about 20 h. The residual solvent was evaporated in a pre-weighted beaker and increased in weight of the beaker giving total lipid. (AOAC 2000).
- **Total nitrogen content:** in 10g of SBP sample was estimated by using micro Kjeldahl method and crude protein was calculated by multiplying the evaluated nitrogen by 6.25(AOAC 2000).
- **Protein:** In 10g of SBP sample was estimated by the Kjeldahl method. Total protein was calculated from the nitrogen content by multiplying with the conversion factor of 6.25(AOAC 2000).
- **Ashes:** Total ash was determined in 10g of SBP sample by incineration of a representative 0.5 g sample in an oven at 450 C for 48 h. (AOAC 2000).

Molecular identification of the high cellulase producer isolates

Two isolates (S11 and S88) were recorded as the highest cellulases producers. Both of them were subjected for DNA identification. Total genomic DNA of selected two isolates was extracted and purified using InstaGene™ Matrix (Bio-Rad, CA) according to the supplier's recommendations. Two synthetic oligonucleotide primers at the 5' and 3' end of the 16S rRNA, described by (Kumar et al.2010) 9F GAGTTTGATCCTGGCTCAG and 1541R AAGGAGGTGATCCAACC were used to amplify the 16S rRNA gene.The PCR amplification fragment was purified by 1.5% agarose (wt/vol) gel electrophoresis using a gel extraction kit (Qiagen USA) and sequenced the purified PCR products. The sequences were analyzed by MEGA 10 and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov) in order to determine their closest phylogenetic relatives. The partial 16S rRNA sequence of the isolates S11 (*Streptomyces sp*. strain FDZH12) and S88 (*Streptococcus mitis* strain FDZH16). numbers for them are OK033363 and OK033364, respectively.

Cloning of cellulase gene

DNA from the most potent cellulytic isolate *Streptomyces sp*. StrainFDZH12 was prepared as described by (Raeder and Broda1988).To synthesize gene coding for the enzyme CelStrep from this strain, PCR was performed using genomic DNA as a template and degenerate and specific oligonucleotide primers, Forward 5′GCCACCGACTCSGGCTTC 3′ and the reverse primer 5 CKGTTGAACCAGATCAT 3′. The resultant cel strip gene amplicon of 1500 bp was checked on 1% agarose gel. It was then purified using a PCR purification kit and digested with 'SmaI restriction. After purification of the digested amplicon, the gene was cloned (ligated) into the expression vector pET28a (Novagen, Inc.) double digested with the same enzymes, SmaI restriction sites to generate celstrep-pET28a, and then expressed in *E. coli* (DH5α) competent cells. The recombinant *E. coli* was confirmed by colony PCR using gene-specific primers of cellulase enzyme. Recombinant *E. coli* (DH5α) competent cells transformed with a plasmid containing cellulase gene from *Streptomyces sp*. strain FDZH12 inserted in an expression vector pET28a (Novagen, Inc.) Which was used as a source for cellulase enzyme then developed and maintained. The cells were maintained as glycerol stock at −80°C. 200 µL of the glycerol stock was transferred to 5 mL of Lauri–Bertanis medium according to (Bertani Giuseppe 1951) which containing 100 µg/mL ampicillin then incubated at 36-37°C, 180 rpm for 16 hours. 1 mL of this culture was inoculated to 100 mL of LB medium containing 100 µg/mL ampicillin then was incubated at 37°C, 180 rpm for 12 hours.

Acid-base pretreatment

The pretreatment of SBP was performed according to the method described by (Dussan et al.2014). In a 2000 ml flask, 200 g of SBP fine powder was taken employing H2SO41 % (w/v), 1:10 solid-liquid ratio, autoclaved at 121 °C for 20 min. After hydrolysis, the resulting solid material (cellulose-lignin) was removed by filtration, washed, and dried. Afterward, cellulose-lignin was soaked in a solution of 1.5 % w/v NaOH, 1:20 solid-liquid ratio, and temperature at 90°C for 1 h. afterward, the mixture was filtered to collect the cellulose, washed thoroughly with water, and dried. Cellulose, hemicelluloses, and lignin remain analyzed by (Rathin Data 1981) as described.

Saccharification

In 1000 ml flask containing 500 ml yeast extract-malt extract broth medium with 10% pre-treated SBP (50 g/l) at solid: liquid (w/v) ratio 1:10 inoculated with 10 ml of inoculum of the recombinant *E. coli*. and *Streptomyces sp*. FDZH12 individually, the mash mixture was incubated in a shaking at 50 °C and the pH value was adjusted to approximately 7.0 according to temperature and PH optimization in our study and according to (Sheikh et al. 2016) with an agitation speed of 140 rpm for 3 days. After termination of enzymatic hydrolysis by DNS in a sample every (2, 4, 6, 12, 24, 48, 72) h, the samples were centrifuged at 10,000 rpm for 10 min. The supernatant was removed for the determination of glucose content.

Reducing sugars estimation

The reducing sugars in the hydrolysate of SBP were determined by the method described by (Ghose 1987) the dinitrosalicylic acid colorimetric methodology.

Saccharification percentage is calculated using the following formulae according to Irfan et al. (2016).

$$\text{saccharification \%} = \frac{\text{released reducing sugars (mg/ml)}}{\text{substrate content (mg/ml)}} \times 100$$

The SBP hydrolysate from the *E.coli* flask after 24hours of saccharification was filtered under sterile conditions to remove any solid substances and then transferred into another flask. 300 mL of the filtered hydrolysate was evaporated to the 2 third until reach 100 mL in order to raise the glucose content 3 times up to 17 % to be fermented easily by *S. cerevisiae.*

Fermentation of beet pulp hydrolysate

Preparation of Saccharomyces cerevisiae inoculum

Saccharomyces cerevisiae inoculum FDZH20 was first revitalized on yeast extract malt extract agar (YMA) plates contains (g/L): yeast extract, 3; malt extract, 3; glucose, 10; peptone, 5; and agar, 20. Per one liter of distilled water, as was described by (Wickerham 1951) for 24 h at 30°C and inoculated into YM broth (pH 5-6) at 30°C for 24 h. The vegetative cells obtained after 24 h were used as an inoculum source. The inoculum cell suspensions were adjusted and quantified in the broth to 1.5×106 cells/ml using a heamacytometer (Pridham et al. 1957). Ethanol fermentation was performed in a 500 mL Erlenmeyer flask with 100 mL of the evaporated SBP hydrolysate at pH 6 and supplemented with following additional nutrients (g/l): yeast extract, 1; KH2PO4,5; (NH4)2SO4,2; andMgSO4.7H2O,1, as it was described by(Akaracharanya et al. 2011). Then it was sterilized at 121°C for 30 min, cooled and inoculated by 10% of the prepared inoculum *S. cerevisiae* FDZH2O. The fermentation operated at 30°C under static conditions for 72 h. A sample of the fermented broth was collected to determine the yield at the end of the fermentation period.

Ethanol evaluation

Ethanol yield was determined on High-Performance Liquid Chromatography (HPLC) system, the fermented broth was centrifugated and 5µl of the supernatant was injected into an Agilent 1100 HPLC system for quantitative analysis. The phenolic compounds were separated using an ODS HYPERSIL 250x4.6mm 5µm (Thermo scientific, UK) HPLC Column. The compounds were detected using a DAD 1100 diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA). Mobile phase: demineralized water with a constant used flow rate of 0.5 mL/min at the temperature of 80°C. Ethanol – in fermentation broth and thereby the ethanol yield was determined together with sugars on HPLC under the above-mentioned conditions. Ethanol concentration in the distillate was measured by a specific-gravity bottle. Ethanol yield – expressed as a percentage of theoretical yield that would be achieved if all present sugars were absolutely transferred to ethanol and carbon dioxide.

Statistical analysis.

Data obtained from the study was statistically analyzed according to the procedures outlined by Gomez and Gomez (1984) using the M-STAT-C computer program.

Results and Discussion

Chemical composition of native dried sugar beet pulp

Chemical analysis of native dried SBP appeared that the total carbohydrates were 85.05% included (27.95 % cellulose, 34.7 % hemicellulose and 22.4 % pectin). Other contents were 7.92 % moisture, 0.64 % fat, 10.6 % total protein, 1.8 % lignin and Ashes content was 12.6 % (Table1) These results are harmony with those recorded by other researchers.Kamzon et al. (2016) found that sugar beet pulp had (22 – 30 % cellulose, 24- 32 % hemicelluloses, and 1 % lignin) on a dry weight basis. Micard et al. (1996). recorded(21-24% cellulose, 26-36% hemicelluloses, 20-22 % pectin, 10 – 15% protei,n, and 1 – 1.2% lignin). as chemical

components of dry sugar beet pulp. Berłowska et al. (2016) reported that the cellulose, hemicellulose, saccharose, raffinose, and reducing sugar in dry sugar beet pulp were 33.68, 40.55, 14.48, 0.24, and 0.98 %, respectively. Pińkowska et al. (2019) recorded that the total carbohydrate, ashes and protein in dry sugar beet pulp was423±5.9, 23.7±1.2, and 88.4±0.5 g/kg, respectively.

All of these reports are nearly similar to those recorded in our study (Table1). Our results and those were recorded by several authors indicated that the sugar beet pulp is very rich of carbohydrates, which represent an important source in the production of bio-ethanol. On the other hand, the differences in the chemical composition of sugar beet pulp may relate to the varieties of sugar beet, different conditions of sugar beet cultivation and processes in sugar factories.

Table 1. Analysis of native dried SBP

Carbohydrate (Total = 85.05%)			Non-carbohydrate				
Cellulose%	Hemi-cellulose %	Petin%	Moist ure %	Fat %	Protein %	Lig nin %	Ashes %
27.95 ± 0.4	34.7 ±0.1	22.4 ± 0.1	7.92 ± 0.03	0.6 4 ± 0.0 2	10.6 ± 0.6	1.8 ± 0.0 5	1.26 ± 0.05

Molecular identification of cellulase producer isolates

The two highest cellulase-producing isolates S11 and S88 were subjected for DNA identification. Polymerase Chain Reaction (PCR) performed using 16S rRNA gene. The PCR amplification fragment (1.5 kbp) was visualized on 1.5% agarose (wt./vol.) gel electrophoresis (Figure, 1) the amplicon was purified from agarose using gel extraction kit (Qiagen USA) and then obtained purified PCR product were sequenced. Firstly, sequences of the two positive PCR products were aligned to ensure the correctness of the sequence.

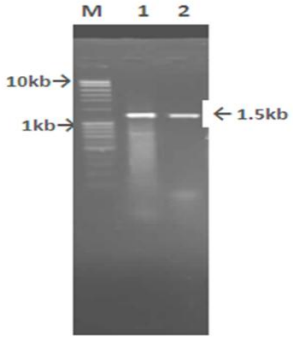

Figure 1. PCR amplification of 16S rRNA gene in cellulytic isolates S11 (Lane 1) and S88 (Lane 2).

The obtained DNA sequences were analyzed by Molecular Evolutionary Genetics Analysis MEGA 6 (Tamura et al. 2011) and compared to the Gen-Bank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website http://blast.ncbi.nlm.nih.gov/Blast.cgi, in order to determine their closest phylogenetic relatives.

Each isolate was then designed to its operational taxonomic unit (OTU) based on measures of sequence similarities, and inferences of phylogenetic trees. Phylogenetic trees were constructed using neighbor Joining (NJ) method and molecular evolutionary analyses conducted using MEGA software. Sequences were then aligned with other similar sequences downloaded from GenBank using ClustalX (Thompson et al. 2003), BioEdit (Hall 1999) and MEGA software. BioEdit (Hall 1999) and Molecular Evolutionary Genetics Analysis (MEGA) software ver. 6.0 (Tamura et al.2011).

Sequence analyses of the identified 16S gene revealed that isolate S11 identified as *Streptomyces sp*. (FDZH12) based on sequence similarity of ≥ 98% of the isolates identified with the other matching homologous Streptomyces sp. The phylogenetic tree of this strain was shown in (Figure, 2). Isolate S88was identified as *Streptococcus mitis*. (FDZH16) based on sequence similarity of ≥ 96% of the isolates identified with the other matching homologous Streptococcus mitis. The phylogenetic tree of the S88 *Streptococcus mitis* strain (FDZH16) shown in (Figure, 3). The obtained DNA sequences were analyzed by MEGA 10 and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov), in order to determine their closest phylogenetic relatives. The partial 16S rDNA sequence of the isolate S11 *Streptomyces sp*. strain (FDZH12) has been submitted to EMBL.

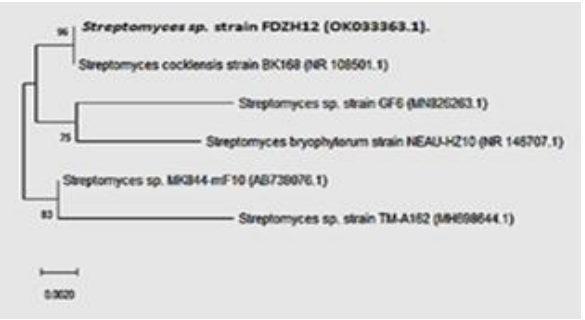

Figure 2. The evolutionary history inferred using the Neighbor-Joining method (Tamura and Nei, 1993) based on partial 16S rRNA gene partial sequences. The Phylogenetic tree showing the phyletic relationships among *Streptomyces sp*. FDZH12 and other 5 matching homologous species and strains from the NCBI Gene bank databas.

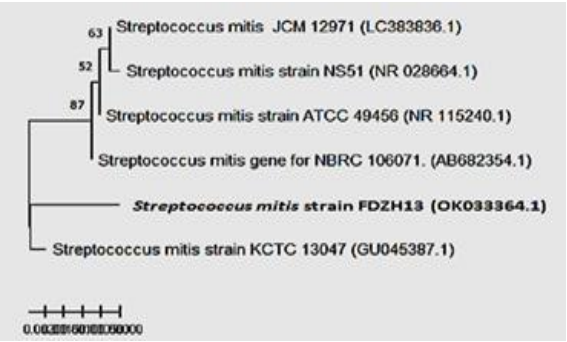

Figure 3. The evolutionary history inferred using the Neighbor-Joining method (Tamura and Nei 1993) based on partial 16S rRNA gene partial sequences. The Phylogenetic tree shows the phyletic relationships among *Streptococcus mitisstrain* FDZH16 and other 5 matching homologous species and strains from NCBI Gene bank database.

Cloning of cellulase gene

The purified resultant celStrep gene amplicon of 1500 bp and digested with 'SmaI restriction' was shown in (Figure, 4). The recombinant *E. coli* was confirmed by colony PCR using gene-specific primers of cellulase. Recombinant *E. coli* (DH5α) competent cells were transformed with plasmid containing cellulase gene from *Streptomyces sp*.

strain FDZH12 were inserted in an expression vector pET28a (Novagen, Inc.) which was used as a source for cellulase then developed and maintained. After inoculating medium with the cloned competent E.coli and incubation there are two different types of colonies appeared after 24 hours. Colonies with the blue color is for the competent *E .coli* and colonies of white color are the recombinant *E.coli* (Figure, 5). Isolates of recombinant E.coli used in saccarification process.

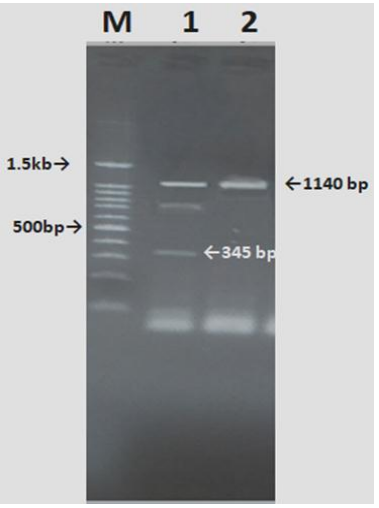

Figure 4. PCR amplification of cellulase: M marker (1.5k), Lane 1 digested PCR product of cellulose gene and Lane 2, undigested PCR product.

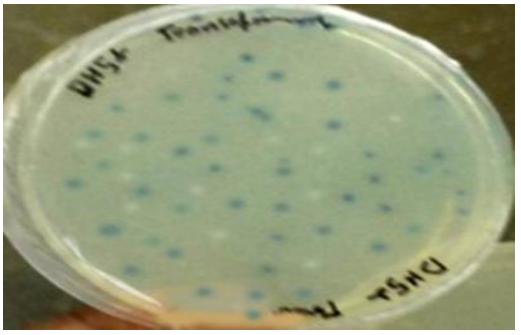

Figure5. Cloning transformation in E. coli (DH5α) competent cell after incubation 24 h.

Acid- Base treatment

Different procedures have been employed, for example, acid hydrolysis, alkali hydrolysis, and steam explosion, among others. The pre-treatment using dilute sulfuric acid (acid hydrolysis) is the most widely used for having high efficiency in the separating process of cell wall components resulting in hemicellulose hydrolysate and cellulignin. Luckily, lignin content in SBP is a maximum of 2% so, compared with lignin content in bagasse and other lignocellulosic materials. A second step for obtaining the cellulose present in the cellulignin, studies have shown a need forthe delignification stage using sodium hydroxide as a catalyst. Bio-ethanol production from lignocellulosic material involves three major steps, including pretreatment, saccharification, and fermentation (Bhuyar et al. 2021 and Chen et al. 2021). The cellulose is submitted to acid or enzymatic hydrolysis to solubilize the glucose (cellulose hydrolyzate).The results of acid-base treatment for native were dried SBP recorded in (Table 2). The results clearly appeared that the content of hemicellulose, pectin, and lignin was highly decreased to 6.3, 5.8 and 0.3 %, respectively. On the other side, the cellulose content was increased to 84.22 % in the treated dry SBP. This result appeared that the acid-base treatment method used in this study was succeeded in dissolving a high percentage of each of hemicellulose, pectin and lignin. the acid treatment has the advantages of low catalytic cost, easy to neutralized by alkali, and highly degrades hemicellulose, while alkaline treatment mainly degrades the lignin in sugar beet pulp, and ultimately leads to the decrease of cellulose crystallinity.

Irfan et al (2017) uses Leptochloa fusca L. Kunth or Kallar grass (KG) was thermochemically (0.625M NaOH solution followed by steam treatment at 121°C for 1 h) pretreated and utilized as a substrate for ethanol production in simultaneous saccharification and fermentation process. Analysis of variance for ethanol production by Kluyveromyces marxianus demonstrated that 10% pretreated KG, 0.6 mg/mL

enzyme concentration, 40°C temperature and 48 h of simultaneous saccharification and fermentation time were the optimized variables. At optimum factor setting, predicted values of ethanol production were 30 g/L. The experiment was in close agreement with the predictive model and the results obtained was 40 g/L. Iram et al (2018) was pretreated sugarcane bagasse (2 mm) with 2.5% NaOH followed by steaming at 121°C for various time periods. The maximum cellulose content of 81% and delignification of 68.5% were achieved by soaking bagasse in 2.5% NaOH with a residence time of 1 h at room temperature followed by steaming at 121°C for 30 min.

Table 2. Analysis of carbohydrate and lignin content of SBP after acid-base treatment.

Cellulose	hemicellulose	pectin	lignin
84.22 ± 3.5	6.3 ± 0.6	5.8± 1.2	0.3 ± 0.1

Saccharification

Data recorded in (Table 3) & (Figure 6) has shown that the recombinant E.coli and Streptomyces sp. that used in the hydrolysis of the acid-based treated SBP. The recombinant E.coli start to hydrolyze cellulose of the treated SBP for the first 2 hours. Hydrolysis reached its maximum ratio after 24 hours of recorded maximum glucose production (28.36g/50 g of acid-base treated SBP) determined by the dinitrosalicylic acid (DNS) colorimetric method adapted from previous work (Ghose1987).

While Streptomyces sp. hydrolysed cellulose of the treated SBP reached its maximum ratio after 48 hours of recorded maximum glucose production (16.2g/50 g of acid-base treated SBP).

These results indicate that the reducing sugar yield by the recombinant strain was 567.2 g/kg of acid-base was treated with dried SBP, which has 840 g cellulose. Kattab et al. (2020) hydrolyzed the acid-base treated sugar beet waste by different concentrations of H2SO4 and found that the maximum yield of reducing sugars was 124.80 mg/g treated sugar beet waste and obtained by 1% H2SO4.

Irfan et al (2022) used Seed pods of B. ceiba as a novel, cheap, and sustainable feedstock for second-generation bio-ethanol production. B. ceiba waste was pretreated with NaOH under different conditions where they found morphological modifications made by NaOH pretreatment followed by steam was more effective as it offered 60% cellulose and 9% lignin at 10% substrate loading, 5% NaOH conc., and 4 h residence time. Samples with maximum cellulose were employed for ethanol production by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using indigenously produced cellulase as well as commercial cellulase. HPLC analysis revealed the best saccharification (50.9%) at 24 h and the best ethanol yield (54.51 g/L) at 96 h of fermentation in SSF using commercial cellulose *by Saccharomyces cerevisiae*.

Ghazanfar et al (2022) Hydrolysis of biomass was performed using both commercial and indigenous cellulase. Two different fermentation approaches were used, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Findings of the study show that the maximum saccharification (58.6% after 24 h) and highest ethanol titer (57.34 g/L after 96 h).

Table 3. Glucose yield g/50 g of acid- base treated SBP during enzymatic hydrolysis periods by recombinant *E.coli* and *Streptomyces sp*. FDZH12.

hydrolysis	2 h	4h	6h	12h	24h	48h	72h
Recombinant E. *coli*	1.60 ±0.03	5.2±0.04	10.4±0.1	25.6±0.5	28.36±0.2	20.82±0.4	17.2±0.3
Streptomyces sp. FDZH12	0.06 ±0.01	0.72±0.02	2.94±0.03	10.9±0.1	14.60±0.2	16.2±0.3	9.96±0.2
P. valueT.test	0.001**	0.001**	0.001**	0.01**	0.01**	0.01**	0.01**

Independent t-test was used to compare the means **, significant at 1% level.

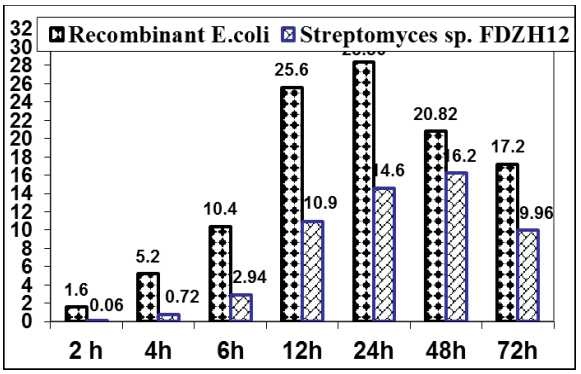

Figure 6. hydrolysis percentage of SBP into monosaccharide during 72 hours using recombinant E coli and *Streptomyces sp*. FDHZ16.

After 24 hours of saccharification the released reduced sugar recorded by the recombinant *E.coli* was 28.36g/50 gram hydrolyzed SBP / 500 ml. 300 ml of the filtered hydrolysate with glucose concentration in the solution of 5.672% has evaporated to its third volume to raise the glucose content of the glucose solution up to 17 % in order to be fermented easily by S. cereviseae. The hydrolyzed solution was filtered

from any residual solids and sterilized at 121ºC and 30 min then enters fermentation process.

Table 4. illustrates the analysis of the hydrolyzed SBP by the recombinant E.coli after 24 hours at a maximum glucose value of 28.36 g/ 50 g hydrolyzed SBP cellulose, hemicellulose, pectin and lignin were 15.3, 6.0, 5.2 and 0.13 respectively on the other hand the analyses of carbohydrate content of the hydrplyzed SBP by the streptomyces sp. after 48 hours at the maximum glucose value 14.6 g/ 50 g hydrolyzed SBP cellulose , hemicelluloses , pectin and lignin were 23.84 , 5.9, 5.2 and 0.3 respectively.

Table 4. Analysis of hydrolyzed SBP after saccharification by *Streptomyces sp*. FDZH12 after 48 hours and analysis of hydrolyzed SBP after saccharification by recombinant *E.coli* after 24 hours.

hydrolysis	Glucose %	Cellulose %	Hemi-cellulose %	Pectin %	Lignin %
Streptomyces sp. FDZH12	14.60± 0.2	23.84± 0.7	5.98± 0.3	5.27± 0.2	0.31± 0.01
Recombinant E. *coli*	28.36 ± 0.1	15.53 ± 0.2	6.06 ± 0.2	5.22±0.5	0.13±0.3
P. value T. test	0.01**	0.01**	n. s	n. s	0.01**

n's not significant

Ethanol production

The hydrolyzed evaporated SBP 100 ml with 17% initial glucose fermented by *S. cerevisiae* FDZH2O in this experiment. Raud et al. (2016) found glucose from saccharified material is converted into ethanol by the fermentation process. Frias-Sanchez et al. (2017) reported maximum ethanol yield (17.1 g/L) in separate hydrolysis and fermentation of pine sawdust treated with nitric acid followed by sodium hydroxide pretreatment. Trevorah and Othman (2015) pretreated sawdust from Australian timber mills with 7% NaOH and reported a maximum ethanol yield of 30.6% after 24h through simultaneous saccharification and fermentation with commercial enzymes and Saccharomyces cervisae. The result in (Table 5) shows that the ethanol production was recorded after 72 hours and reached to 50.08 % of glucose content in the dilute hydrolyzed SBP. This concentration represented 98 % of the theoretical value based on the initial glucose. Therefore, the maximum yield of ethanol was 0.5008 g/g and equal to 98% of the theoretical value based on reduced sugar in hydrolyzed SBP (14.2 g ethanol/ 28.36g initial glucose / 50 g of hydrolyzed SBP) which was achieved at pH 6, 30 ºC and 10% inoculum size after 72 hour of fermentation.

These results indicate that the ethanol yield reached to 284 g / kg of hydrolyzed SBP. These results are in agreement with Mahmoodi et al. (2018) who obtained 44.6 and 44.4 g ethanol per 100 g of hydrolyzed glucose and acid treatment liquor, respectively. Rorick et al. (2011). used *S. Cerevisiae* with pectinases, cellulases, cellobiases, and following with a second fermentation with *E. coli* KO11 for the production of ethanol from sugar beet pulp and found that the total ethanol yield reached to 0.34 g ethanol g-1 sugar in the treated sugar beet pulp. According to results were recorded in this study, each one ton of dried SBP (with8 % moisture content) gives 129.24 kg ethanol, while 19.35 kg ethanol produced from each one ton of fresh wet SBP (with 86 % moisture content).

Table 5. Ethanol yield and productivity obtained by fermentation of the dilute hydrolyzed SBP.

Parameters / Isolate	Initial glucose (g/50g) hydrolyzed SBP)	Ethanol yield (g /28.36 g) initial glucose	ial glucose g/300ml accharified solution	Ethanol yield g /300ml saccharified solution	Optimum fermentation time (hour)	Maximum ethanol yield (% of initial glucose)
S. cerevisiae	28.36 ± 0.1	14.2	17	8.5	72	50.08

Conclusions

The results recorded in this study appeared that the sugar beet pulp, as an inexpensive byproduct of sugar beet production, could provide alternative raw materials for bio-ethanol production. The pretreatment methods and microorganisms used for the hydrolysis carbohydrate content of sugar beet pulp to fermentable sugar as well as yeast strain used for fermentation are the limiting factors for ethanol yield. Acid-base treatment used in this study is effective and highly decreased hemicellulose, pectin, and lignin contents with increasing cellulose content to 84.22 % in the treated SBP. Reducing sugar yield by the recombinant E. coli strain was 67.52% of cellulose in treated SBP. Ethanol yield reached to 129.24 kg from each one ton of dried SBP (with 8 % moisture content) and19.35 kg from each one ton of fresh SBP (with 86 % moisture content). Increasing the efficiency of cellulose hydrolysis as well as using another suitable microbe able to ferment other released hexose and pentose sugars should be achieves to increase the ethanol yield. These results indicated that glucose yield by the recombinant strain was 567.2 g/kg of acid- base treated dried SBP, which have 840 g cellulose. On the other side, ethanol yield reached to 284 g / kg of hydrolyzed SBP. According to the results recorded in this study,each one ton of dried SBP give 129.24 kg ethanol.

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