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ORIGINAL PAPER

Enhanced Bio-ethanol Production from Old Newspapers Waste Through Alkali and Enzymatic Delignification

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Abstract Producing bioethanol from various lignocellulosic wastes is considered as a promising strategy to overcome global energy crisis and waste management in one consolidated step. In this study, we investigated the use of old newspaper waste as potential raw materials for bioethanol production. Two state-of-the-art technologies, alkali (NaOH) and enzymatic (ligninolytic) pre-treatments were applied for the delignification of old newspaper waste. The maximum delignification of 37.7 and 42.2% were achieved with alkali (4.0% after 24 h) and ligninolytic extract (25 mL) treatments, respectively. The delignified residues were then treated with cellulase extract from *Trichoderma harzianum* that resulted in 56.2 and 63.8% cellulose hydrolysis in alkali and enzyme treated substrates, respectively. The enzymatically digested hydrolyzates were submitted for fermentation anaerobically, and up to 11.95 and 12.69 g/L ethanol concentration was recorded, respectively. Effects of several processing parameters such as fermentation duration, substrate level, pH, temperature, and inoculum volumes were optimized that led to significantly higher ethanol production of 17.8 and 20.4 g/L for alkali and enzyme treated substrates, respectively. The results obtained suggested that ligninolytic pre-treatment is a more efficient and environmentally friendlier approach as compared to alkali treatment for economic bio-ethanol production.

Keywords Newspaper waste · Delignification · Enzymatic hydrolysis · Bio-ethanol · Optimization

Introduction

The depletion of fossil fuels diverted the attention of scientific community across the globe toward renewable energy sources and secondly, from last few decades, there are also growing concerns about environmental pollution [8, 15, 51]. Research is underway around the globe on designing environmental friendlier and economically viable processes capable of sustainably producing high amounts of fuel bioethanol, though using different strategies [1–4, 34]. In this context, bio-ethanol production from renewable resources seems a promising approach to combat global warming and also to overcome the energy crises [21, 25, 52]. The first generation fuel ethanol has been derived from starch and sugar-rich crops such as corn, sugar cane, etc. However, the long-term availability and sustainability of these crops are questionable due to direct competition with the world's food and animal feed supply. Thus, the second generation bioethanol from lignocellulosic feedstocks has grown in popularity as an alternative to cope with environmental concerns as well as a desire to reduce oil dependency [20, 23, 26, 29, 54].

Lignocellulose is a major structural component of plant materials and consists of cellulose, hemicellulose, and lignin which accounts for 50–80% of carbohydrates. With

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lower cost and abundant availability, the lignocellulosic raw materials are considered as one of the most attractive feedstocks for bioethanol production [30, 35, 36]. Almost 60% of plant biomass produced on earth comprises of lignocellulosic materials [31]. Among various lignocellulosic, the recycled books, magazines, and newspaper can also be harnessed for ethanol production. The effective utilization of these three components could play a unique role in the economic viability of ethanol production. Nevertheless, the bioconversion of lignocellulosic biomass to ethanol is hindered by the structural and complex nature substrate, which makes these materials a challenging task to be used as feedstocks for ethanol production [7, 9–11, 19].

Biotransformation of lignocellulosic materials to useful products requires multi-step processes including pretreatment, enzymatic digestibility, and fermentation. However, the pretreatment technology is one of the bottlenecks in cellulosic ethanol production. The cost of pretreatment is a significant portion of the total cost of producing fuel ethanol using the cellulosic material. Thus, selecting the most effective and clean pretreatment method will be of great significance to reducing the entire production cost. In the context of cellulose ethanol production, the available chemical-based pretreatments have the disadvantages of high cost, equipment corrosion, and environmental pollution. Use of white-rot fungi (WRF) has been found to be attractive having substantial ability to secrete a unique set of wood-degrading enzymes (WDEs) along with low-molecular-weight effectors. The most prominent enzymes are lignin peroxidases (LiPs, E.C. 1.11.1.14), Mn^{2+}-dependent peroxidases (MnPs, E.C. 1.11.1.13), laccases (Lac, E.C. 1.10.3.2) and H_2O_2 producing oxidases. The concomitant action of these enzymes led to complete lignin deconstruction by generating non-specific highly reactive free radicals. The mild environmental conditions, as well as minimum energy requirement, are the advantages of this enzymatic process [31, 45]. *Ganoderma lucidum* is a medicinal mushroom that has been investigated for the production of lignin-modifying enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). The present study involved the use of ligninolytic enzymes extract from *G. lucidum* IBL-05 for de-lignification of newspaper waste, followed by saccharification using cellulases extract from *Trichoderma harzianum* and bioethanol production by *S. cerevisiea*.

Materials and Methods

Microorganism and Media

G. lucidum IBL-05 strain available in Industrial Biotechnology Laboratory, Department of Biochemistry, University of Agriculture Faisalabad (UAF) Pakistan was cultured and maintained on Potato Dextrose agar (PDA) slants at 28 ± 0.2 °C for 6–8 days and stored at 4 °C.

Chemicals and Substrate

Local suppliers provided analytical grade chemicals and reagents from Sigma-Aldrich (USA) and Merck (Germany). Old newspapers to be used as lignocellulosic substrate lignocellulolytic enzymes production were collected from the offices and Hostels of the UAF, Pakistan. After drying, the substrate was chopped and grounded to powder (4 mm mesh particle size) form in Ashraf Herbal Laboratories, Faisalabad and stored in air tight plastic jars, till experimentation. The percent lignin and cellulose contents of the pristine newspaper materials were 20.06 ± 1.3 and 44.7 ± 0.9%, respectively.

Production of Ligninolytic Enzymes

Cotton plugged triplicate production flasks (500-mL) containing 5 g pre-treated substrate (old newspapers) were moistened with growth (Kirk's) basal nutrient medium (66% w/w) of pH 4.5 and other pre-optimized nutrients. Before inoculation (5 mL seed culture), the flasks were sterilized (121 °C) in an autoclave for 15 min and allowed to ferment in still culture incubator (Sanyo MIR-254, Japan) at 30 °C for 5 days. Ligninolytic enzymes were extracted by adding 100 mL of distilled water under shaking for 30 min at 150 rpm. The flasks contents were filtered, centrifuged (Eppendorf 5415 C) and, clear supernatants were analyzed for enzyme activities [12].

Ligninolytic Enzyme Assays

Lignin Peroxidase

The LiP activity was determined [47] by monitoring the H_2O_2-dependent oxidation of veratryl alcohol to veratryl-aldehyde at 25 °C. Assay mixture comprised of 4 mM veratryl alcohol in 100 mM Na-tartrate buffer (pH 3.0) and 0.2 mM H_2O_2 as a reaction initiator. The absorbance of each sample was taken Spectrophotometrically (HALO DB-20S, UV/Visible double beam spectrophotometer, Dynamica) at 310 nm ($\varepsilon_{310} = 9300$).

Manganese Peroxidase

The activity of MnP was assayed as previously reported [48] at 25 °C following H_2O_2-dependent oxidation of manganic-malonate complex at 270 nm ($\varepsilon_{270} = 11{,}570$). A 100 µL of crude extract was added to a reaction mixture

comprising 1 mM $MnSO_4$, 50 mM Na-malonate buffer (pH 4.5) and 0.1 mM H_2O_2.

Laccase

Laccase was assayed by determining 2, two azinobis (3-ethylbenzthiazoline six sulphonate) oxidation in Na-malonate buffer at 25 °C [49]. Reactive mixture (2.1 mL) consisted of 1 mL of 50 mM Na-malonate buffer (pH 4.5), 1% (w/v) ABTS and 100 µL crude enzyme extract. The change in absorbance was determined at 420 nm (ε_{420} 36,000 M^{-1} cm^{-1}). A mixture containing all reagents excluding enzyme extract was considered as blank. The amount of enzyme oxidizing 1 µmoL/mL of substrate per min was considered as one activity unit (U) of the enzyme.

Cellulase Production

For cellulolytic enzymes production, the *T. harzianum* was cultivated in 500-mL capacity triplicate production flasks comprising 5 g substrate in Vogel's medium (pH 4.5) in submerged fermentation (SmF). The sterilized flasks were inoculated with 2 mL of freshly prepared fungal inoculum followed by incubation in the dark at 28 °C for 5–7 days. Crude cellulase extracts obtained after filtration of flasks contents was employed for subsequent cellulose hydrolysis.

Cellulases Enzyme Assays

Assay of endo 1,4-β glucanase was performed as previously reported [14]. A 100 µL of crude extract was incubated with 1 mL of CMC (1.0%, w/v) and 1 mL of 100 mM citrate–phosphate buffer (pH 4.8) at 50 °C. After 30 min, DNS reagent was added to terminate the reaction, and placed in boiling water bath for 15 min, cooled to room temperature, and change in absorbance was monitored at 540 nm against blank. Exo β-1,4-glucanase was assayed [13] using 1.0% (w/v) salicin as assay substrate with DNS as terminating reagent, and β-glucosidase measurement was carried out as described [17].

Pretreatment of Newspaper for Delignification

Alkali Treatment

The substrate (20 g) was mixed with 100 mL solutions of different concentrations of NaOH (1.0–5.0% w/v) and subjected to thermal treatment at 121 °C for half an hour. Alkali treated substrate was washed with distilled water followed by a dilute acidic solution to get pH 5.5.

Ligninolytic Treatment

For enzymatic delignification, the ligninolytic extract at different dose levels (5–25 mL) was employed for 24 h at room temperature. The pretreated slurry was adjusted to pH 5.5 by washing with distilled water followed by an acidic solution.

Lignin Determination

For lignin determination, 5 mg sample was mixed with 5 mL of 25% acetyl bromide and heated at 70 °C for 30 min in a water bath, and the mixture was then transferred to a 100-mL volumetric flask containing 10 mL of 2 M NaOH and 25 mL of glacial acetic acid (GAA). Finally, 1 mL of 7.5 M hydroxylamine hydrochloride was added in it and diluted to 100 mL with GAA, and absorbance was measured at 280 nm [7].

Cellulose Determination

The cellulose content of delignified and cellulase treated material was determined using the reported methodology of Asgher et al. [7]. Delignified sample was centrifuged (5 min at 3000×*g*) and 3 mL acetic/nitric reagent was added to the pellets and kept in boiling water bath for 30 min. After brief centrifugation, 10 mL of 67% H_2SO_4 was added to the residue, mixed and allowed to stand for 1 h. Finally, 10 mL of anthrone reagent was added with the help of pipette, kept in boiling water bath for 15 min, and cooled in ice bath for 2–3 min. After standing the tubes at room temperature for 5–10 min, the absorbance was measured at 620 nm.

Glucose and Xylose Determination

The reported method of Gadgil et al. [14] was adopted for the determination glucose, while xylose was measured using relation given below, where XN and XH represent the xylose concentration (g) in treated and untreated samples, respectively.

$$\text{Xylose} = \frac{\text{XN}}{\text{XH}} \times 100$$

Ethanol Production

A pure culture of *S. cerevisiae* provided by Shakar Ganj Mills (Pvt) Limited, Jhang, Pakistan has used for ethanol production anaerobically in triplicate Erlenmeyer flasks (500 mL) containing 5 g/100 mL hydrolyzate and fermented at 37 °C, pH 4.5 for 72 h in shaking incubator (150 rpm). After 72 h, the contents were filtered, centrifuged at 3000×*g* and supernatants were analyzed for ethanol production.

Optimization of Ethanol Fermentation

Five fermentation parameters, including fermentation period, substrate level, hydrolyzate pH and temperature, and inoculum sizes were optimized at their different levels to enhance bio-ethanol production.

Effect of Fermentation Time

Triplicate shake flasks (500 mL) containing hydrolysates from alkali and enzymes pretreated newspapers waste were sterilized, inoculated and fermented at 37 °C for different incubation periods (24, 48, 72, 96 and 120 h) in a rotary shaker (150 rpm). After, each fermentation time interval, flasks were harvested to screen out the best fermentation time for ethanol production.

Effect of Substrate Level

To study the effect of substrate concentration, the experiment was set up by varying the substrate level from 2.0 to 10 g for fermentation at the optimum time of 72 h with 5 mL inoculum size in 500-mL capacity triplicate flasks.

Effect of pH

To investigate the effect of pH on fermentation performance, fermentation was carried out at different pH values (pH 3.0–7.0), that were adjusted with 0.1 M HCl/NaOH using optimized substrate level for 72 h.

Effect of Temperature

Effect of incubating temperature on ethanol productivity was studied by carrying out submerged fermentation at pH 5.5 for 72 h using different temperatures from 25 to 45 °C.

Effect of Inoculum Level

Determination of optimum inoculum level was made by inoculating triplicate flasks with varying sizes of yeast inoculums (1.0–5.0 mL) followed by fermentation using all above optimized conditions.

Results and Discussion

Lignocellulolytic Enzyme Production

G. lucidum IBL-05 was cultured on newspaper waste for the production of ligninolytic enzyme extract (MnP, LiP, and Lac). The maximum production of MnP (698 U/mL), LiP (546 U/mL) and Lac (53.71 U/mL) were achieved after 5 days of solid-state fermentation (SSF) using pre-optimized conditions (Table 1). On the other hand, *T. harzianum* secreted the highest endoglucanase (6.23 U/mL), exoglucanase (4.61 U/mL), and β-glucosidase (0.73 U/mL) in submerged fermentation (SmF) after 5 days (Table 1). It is not improbable that the simultaneous production pattern of LiP, MnP and laccase activities indicates synergistic catalytic functions of these powerful oxidoreductases on lignocellulose. Since the carbon sources utilized by basidiomycetes are usually of a lignocellulosic character, fungi during vegetative growth produce a range of enzymes to degrade the lignocellulosics substrates. It has been demonstrated that type and composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced during fungal growth [12]. SSF has been recognized as an efficient technology to be applied in the valorization of agro-industrial residues for elevated production of ligninases due to its potential advantages and high yield. The WRF strains, including *G. lucidum*, produce poor ligninolytic enzyme activities in submerged fermentation (SmF) as compared to SSF. Further, the smaller fermenter volume, easier aeration, reduced or eliminated costs for stirring, higher product stability, unfavorable bacterial growth environment, and lower contamination risk are the additional benefits of the SSF process. Different studies have shown that ligninolytic enzyme production is regulated by gene expression induction or through translational or posttranslational regulation. Some enzyme-encoding genes are expressed specifically in solid-state cultures that give rise to the high-level production of extracellular enzymes [9–11].

Pretreatment for Delignification of Newspapers

The crucial step in the production of bioethanol from lignocellulosic is pretreatment, which enables the separation of major components of biomass i.e. cellulose, hemicellulose, and lignin and thus, increased the bio-digestibility and accessibility of enzymes to the materials for cellulose

Table 1 Activities of ligninolytic and cellulolytic enzymes produced by *G. lucidum* IBL-05 and *T. harzianum*, respectively under pre-optimized conditions

Ligninocellulolytic enzymes (U/mL)		
Ligninolytic		
MnP	LiP	Laccase
698.95 ± 6.2	546.23 ± 7.6	53.71 ± 6.3
Cellulolytic		
Endoglucanase	Exoglucanase	β-Glucosidase
6.23 ± 0.044	4.61 ± 0.057	0.73 ± 0.047

hydrolysis [23]. Here, alkali and enzymatic pretreatments were used for the delignification of newspapers waste.

It is well known that alkali treatment breaks the linkages (benzyl ether, glycosidic ether, benzyl ester, acetal groups) present in lignin-cellulose complexes both from softwood and hardwood. A high concentration of sodium hydroxide significantly affects the delignification rate and facilitates the rapid dissolution of the cellulose and hemicelluloses [54]. For alkali pretreatment, different concentrations of NaOH (1.0–5.0%) were used for different time periods (24 and 48 h) for lignin degradation, and results thus obtained are shown in Table 2. The maximum 37.7% lignin was removed in 4.0% alkali treated sample after 24 h treatment. Further increasing the concentration of NaOH, the delignification rate also increased which enhanced up to 4.0% alkali treatment, and beyond that, the delignification rate decreased. The treatment time was found insignificant for delignification since 24 h treatment was found better as compared to 48 h (Table 2). The lignin degradation trend with sodium hydroxide was found similar to previous studies, for example, Zhao and coworkers [53] reported that sodium hydroxide dose beyond 7.0% did not affect the delignification rate. Similarly, Alvarez et al. [6] revealed high delignification rate by using 2.5–3.5% NaOH, and beyond this concentration, the delignification rate decreased. Silverstein et al. [40] found that NaOH (2.0%) treated sample furnished higher delignification of 65% in 90 min at 121 °C using cotton stalks as a substrate and Asgher et al. [7] reported 48.7% of maximum delignification of sugarcane bagasse with 5.0% NaOH treatment for 24 h. Sun et al. [44] obtained maximum delignification by using 1.5% (w/v) NaOH for 144 h at 20 °C, which caused 60 and 80% release of lignin and hemicelluloses, respectively. Zhao and coworkers, [53] treated hardwoods, wheat straw, switchgrass and softwoods with NaOH and resultantly, 26% lignin was released. However, the degradation rate of the newspaper was low as compared to cotton stalks and sugarcane bagasse which may be due to high lignin content in the newspaper.

Lignin depolymerization could be accomplished by lignin-modifying enzymes secreted by WRF as secondary metabolites. Various ligninolytic extract concentrations (5, 10, 15, 20 and 25 mL) were used for different time periods (5 and 10 days) for the delignification of the newspaper, and maximum delignification of 42.2% was achieved when 25 mL ligninolytic extract was used for 5 days. By increasing the time up to 10 days, the reduction in delignification rate was observed (Table 3). The delignification trend by ligninolytic is corroborated with previous studies e.g. Taniguchi et al. [46] reported 41% klason lignin degradation from rice straw by *Pleurotus ostreatus*, and Singh et al. [42] reported 26.9% lignin degradation from oil palm biomass by *Pycnoporus coccineus*. Similarly, Nazarpour et al. [32] reported 45.06, 34.40 and 37.68% lignin degradation from rubber wood using *C. subvermispora, T. versicolor*, and co-culture, respectively. Asgher et al. [7] observed 33.5% reduction in lignin content of sugarcane bagasse treated with 25 mL ligninolytic enzymes extract. Wulandari et al. [50] revealed that *Penicillium* derived ligninolytic enzymes caused lignin degradation as much as 66.3% at optimum conditions. Recently, Asgher et al. [8] recorded 39.6% lignin reduction of wheat straw with 25 mL ligninolytic extract treatment. It is reported that WRF is the well-studied basidiomycetes for producing enzymes responsible for lignocellulose degradation. Pretreatment of lignocellulosic material with ligninolytic enzymes is

Table 2 Lignin and cellulose content of alkali pretreated newspaper

Alkali treatment NaoH (%)	% Lignin reduction			Cellulose (%)		
	Control	24 h	48 h	Before	24 h	48 h
1	20.06 ± 1.3	8.57	11.2	44.7 ± 0.9	46.3 ± 0.7	47.4 ± 0.4
2	20.06 ± 1.3	17.3	23.5	44.7 ± 0.9	49.4 ± 0.9	47.7 ± 0.6
3	20.06 ± 1.3	34.2	35.7	44.7 ± 0.9	49.7 ± 2.1	53.9 ± 0.3
4	20.06 ± 1.3	37.6	35.1	44.7 ± 0.9	55.5 ± 0.4	51.2 ± 0.5
5	20.06 ± 1.3	31.7	31.4	44.7 ± 0.9	48.3 ± 0.6	48.5 ± 0.2.1

Table 3 Lignin and cellulose content of enzymatically pretreated newspaper

Enzymatic treatment (mL)	% Lignin reduction			Cellulose (%)		
	Control	5 days	10 days	Control	5 days	10 days
5	20.06 ± 0.8	8.7	10.2	44.7 ± 1.0	46.7 ± 1.3	48.8 ± 2.1
10	20.06 ± 0.8	13.3	23.3	44.7 ± 1.0	45.5 ± 1.9	49.1 ± 1.3
15	20.06 ± 0.8	21.7	32.8	44.7 ± 1.0	49.7 ± 1.4	50.2 ± 0.7
20	20.06 ± 0.8	33.2	35.1	44.7 ± 1.0	51.1 ± 2.3	57.3 ± 1.3
25	20.06 ± 0.8	42.3	30.3	44.7 ± 1.0	64.8 ± 1.7	47.7 ± 1.9

the most preferred strategy because of maximum production of desired product, minimum by-product formation, low energy requirements, mild processing conditions and environmentally-friendlier nature. Further, the possibility of enzyme recycling could effectively increase the extent of hydrolysis [45], and enzymatic pretreatment is also comparatively cheaper compared with acid or alkali pretreatment since it is usually conducted at ambient conditions and does not have a corrosion problem [25].

Saccharification and Fermentation

The ethanol production from lignocellulosic waste also depends on cellulose digestibility during the fermentation process, and cellulases have been found to be most effective in this regard. The paper pulp can be saccharified using either acid or enzymes, but enzymes are preferred because enzymes are specific in nature [25]. After alkali and enzymatic pretreatment, the best-treated substrates (4% alkali and 25 mL ligninolytic pretreated samples) were subjected to saccharification. For this purpose, different concentration of concentrated cellulose extract (5, 10, 15, 20, 25 mL) was used for both alkali and enzyme pretreated samples and cellulose, glucose and xylose percentages were determined. The sample pretreated with NaOH (4%) saccharified up to 56.2% and resultantly, 37.2 and 2.1 g/L glucose and xylose, respectively, was obtained from cellulases (25 mL) for 24 h at 50 °C (Table 4). In the ligninolytic pretreated sample, 63.8% saccharification and 41.3 g/L glucose and 2.9 g/L xylose were observed after 24 h of incubation with cellulase extract from *T. harzianum* (Table 4).

For ethanol production, fermentative microorganisms, especially *S. cerevisiae* is regarded as prime species that works optimally at ambient temperature (30–40 °C), resists a high osmotic pressure in addition to its tolerance to low pH (4.0) and inhibitory product as well [18]. *Saccharomyces* yeasts owe their competitiveness to a combination of several desirable properties including fast growth, efficient glucose repression, the good fermentative capacity to furnish high ethanol, and a tolerance for various environmental stresses, such as high osmotic pressure, ethanol concentration, lower pH and stress imposed by the process inhibitors. These characteristics are unevenly dispersed among different yeasts but are inimitably combined, dedicated to perfection, organized and regulated by an efficient network in *S. cerevisiae* and its closest strains. Strikingly, efficient regulation is the most exclusive invention of the Saccharomyces yeasts, providing a key competitive 'advantage' when these strains are selected for industrial fermentations, particularly, in wineries and breweries [33]. The hydrolyzate obtained from newspaper treated with 4% NaOH and 25 mL cellulase extract was submitted to ethanol production, and resultantly, 11.95 g/L ethanol was produced in alkali pretreated samples (Table 4). In enzyme treated sample, the maximum ethanol production of 12.69 g/L was recorded when 25 mL ligninolytic extract pretreated substrate, and 25 mL cellulase extract treatment was used (Table 4). Similar to this study, Asgher et al. [7, 8] worked on *S. cerevisiae* using different lignocellulosic wastes and found that *S. cerevisiae* is efficient for fermentation, and promising yield can be obtained using this organism; however, there is little information regarding ethanol production using pretreated old newspaper by this organism, and yield obtained was promising. The ethanol production from various agricultural wastes and newspaper (few studies) are compared with the present study as shown in Table 5. The Oil palm frond, sugarcane bagasse, paper fiber residue, solid paper, corncob, wheat straw furnished higher ethanol production which might be due to the high content of sugar, and other studies reported ethanol yield comparable with the present study. Sigurbjornsdottir and Orlygsson [39], studied ethanol production from the newspaper (H_2SO_4, NaOH-treated and without treatment) using *Thermoanaerobacterium* AK54 and yield was quite low that may be due to the mildly acidic and alkali treatment. However, Kuhad et al. [25] reported ethanol yield comparable with our study using newspaper by *S. cerevisiae* using pure enzymes.

Optimization of Process Parameters for Ethanol Production

Fermentation Duration

The bioethanol production at different time periods (24, 48, 72, 96 and 120 h) as shown in Fig. 1. One clearly indicated

Table 4 Determination of cellulose hydrolysis, glucose and xylose during ethanol production by using different concentrations of cellulases

Cellulase (mL)	Hydrolysis (%)		Glucose (g/L)		Xylose (g/L)		Ethanol (g/L)	
	Alkali	Enzyme	Alkali	Enzyme	Alkali	Enzyme	Alkali	Enzyme
5	11.9	19.9	17.8 ± 0.09	19.7 ± 0.09	1.2 ± 0.9	1.4 ± 0.7	2.24	3.74
10	28.4	32.5	24.7 ± 0.06	27.1 ± 0.06	1.5 ± 0.1	1.5 ± 0.4	4.63	5.32
15	39.6	40.2	28.3 ± 0.07	31.5 ± 0.05	1.8 ± 0.5	1.9 ± 0.1	5.31	7.48
20	45.5	51.5	37.2 ± 0.09	37.3 ± 0.09	2.1 ± 0.9	2.4 ± 0.9	11.95	10.08
25	56.2	63.7	33.5 ± 0.05	41.3 ± 0.07	2.3 ± 0.7	2.9 ± 0.2	9.18	12.69

Table 5 Comparative analysis of ethanol potential results from various lignocellulosic substrates after different pretreatments

Substrates	Pretreatment	Ethanol yield (g/L)	References
Waste newspaper	4% NaOH	17.8	Present study
waste newspaper	Enzymatic	20.4	Present study
Wheat straw	Enzymatic	33.5	[8]
Sugarcane bagasse	4% NaOH	32.45	[7]
Sugarcane bagasse	–	28.15	
Oil palm frond	Biological (*A. niger* USM Al1)	23.1	[29]
Wheat straw	Microwave assisted Alkali treatment	16.4	[41]
Paper sludge	Hot water	24.9	[22]
Horticultural waste (HW)	Organosolv method	11.69	[16]
Newspaper	Without treatment	13.9	[39]
Newspaper	0.75% H_2SO_4	14.4	
Newspaper	0.75% NaOH	12.8	
Paper fibre	Alkali (10%)	34.06	[15]
Residue			
Solid paper	Enzymatic	30.48	[51]
Sludge			
Newspaper	–	14.77	[25]
Corncob	Dilute H_2SO_4	84.7	[52]
Sugarcane bagasse	HCl	4.7	[19]
Wheat straw	1% NaOH	31.1	[54]
Rice straw	H_2SO_4	6.83	[23]

*Used *thermoanaerobacterium* AK54 as fermentative organism

that maximum ethanol was accumulated during the first 72 h and then decreased presumably due to consumption of all available fermentable sugars by yeast, ethanol oxidation, and organic acid formation during the fermentation phase. It has been demonstrated that after sugar exhaustion from the medium, previously produced ethanol was reconsumed, and as a result, new cell material was generated. In this so-called "diauxic shift" or "biphasic growth", the microorganism's grow into two phases; at first phase, glucose is assimilated to ethanol and carbon dioxide via aerobic fermentation. When ethanol is available, but virtually the concentration of sugars is substantially low into the culture medium whereas the dissolved oxygen concentration is above a critical level, ethanol is now the only accessible carbon source and serves as a substrate for further yeast growth. At this point, biosynthesis of the enzymes associated with gluconeogenesis occurs and causes a lag in the yeast growth. The (ethanol) "make-accumulate-consume" strategy relies on the evolution of Saccharomyces against its competitors as ethanol is toxic to most other microbes. Saccharomyces strains eliminate their competitors by producing ethanol, but in a next fermentation step, they consume the previously generated ethanol [5, 33, 37]. The peak value of ethanol concentration of 13.4 and 15.4 g/L was appeared by alkali and ligninolytic pretreated newspaper

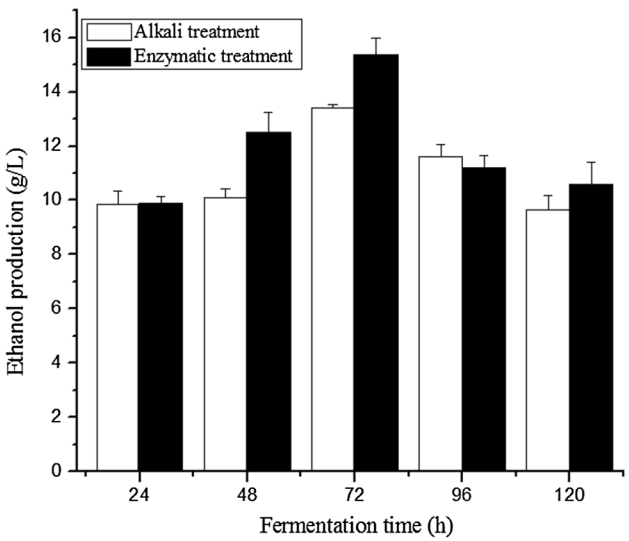

Fig. 1 Effect of fermentation time on ethanol production

waste, after 72 h that was used for all subsequent experiments. In previous studies, Ko et al. [24] demonstrated 96 h as the ideal cultivation time for maximum ethanol yield. Similarly, maximal ethanol concentrations of 27.0, 23.0, and 21.0 g/L (w/v) from corncob residues (CCR) were

recorded by *S. cerevisiae, C. tropicalis* and mixed culture, respectively, after 96 h [28].

Effect of Substrate Concentration

Figure 2 illustrates the effect of different substrate concentrations on ethanol production performance. Alkali and enzyme treated substrates accomplished high ethanol yield of 14.6, and 17.6 g/L. The observed increase in ethanol production with initial increasing substrate concentration is in agreement with previously reported investigations. Nonetheless, ethanol production decreased when the substrate concentration was further elevated most likely due to substrate inhibition. Additionally, Sreela et al. [43] highlighted that higher substrate concentrations are thought to cause high intracellular osmotic pressure, causing cell damage due to the purging of water molecules outside from the cells.

Optimization of pH

pH of the fermentation medium is regarded as an imperative parameter affecting the yeast functioning and product formation [7]. Initially increasing the hydrolyzate pH, the bioethanol concentration increased, reaching its maximum at pH 5 and declined again by a further increment of pH. Recorded ethanol production values at optimum pH were 15.5 and 18.3 g/L with alkali and enzyme pretreated substrate, respectively (Fig. 3). In agreement to present study, Shafaghat et al. [38] and Geetha et al. [15] reported the highest ethanol productivity by *S. cerevisiae* at an optimum pH of 5.3. The majority of the reports presented in literature have used pH 5.5 for fermentation, because, at this pH value, the bacterial contamination during fermentation is negligible and slightly acid pH range also favors the yeast performance [28].

Optimization of Temperature

To decipher the temperature influence on the performance of ethanol production, triplicate shake flasks were incubated in an arbitrary shaker at different temperatures using all above optimized conditions. As can be seen from Fig. 4, that maximum ethanol production was achieved at 35 °C under optimal conditions from alkali and ligninolytic pretreated substrates. Noticeably, at higher temperatures, the yeast replication was ceased that led to marked reduction in final ethanol titers. Ethanol production is significantly reduced at 50 °C, probably due to change in a transport system which might increase toxins accumulation in the cell, including ethanol [29]. The optimal temperature in the range of 30–40 °C for yeast *S. cerevisiae* was also demonstrated by Asgher et al. [7] and Lim et al. [29].

Optimization of Inoculum Level

To optimize the inoculum level, different doses of freshly prepared inoculums (1.0–5.0 mL) were used during ethanol fermentation using above optimized conditions. Maximum production of 17.8 g/L of ethanol was attained using 5 mL inoculums from alkali treated while 20.4 g/L of ethanol was obtained from the ligninolytic enzyme treated substrate (Fig. 5). The presence of sufficient and viable cells community can provide effective fermentation at high substrate concentrations. It was hypothesized that high cell densities up to certain level could improve

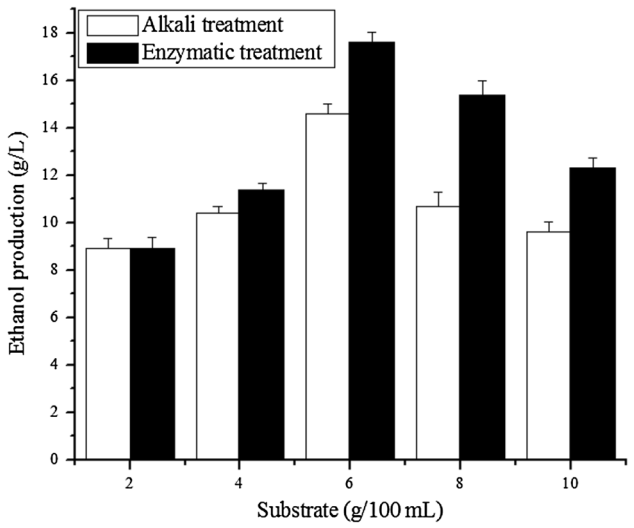

Fig. 2 Effect of substrate concentration on ethanol production

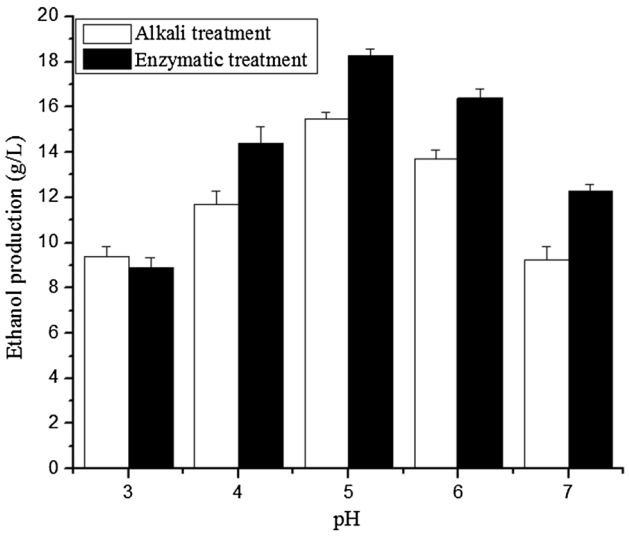

Fig. 3 Effect of medium pH on ethanol production

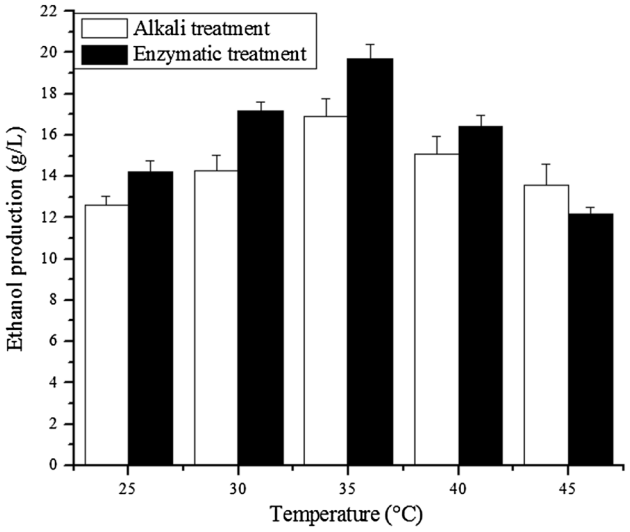

Fig. 4 Effect of incubation temperature on ethanol production

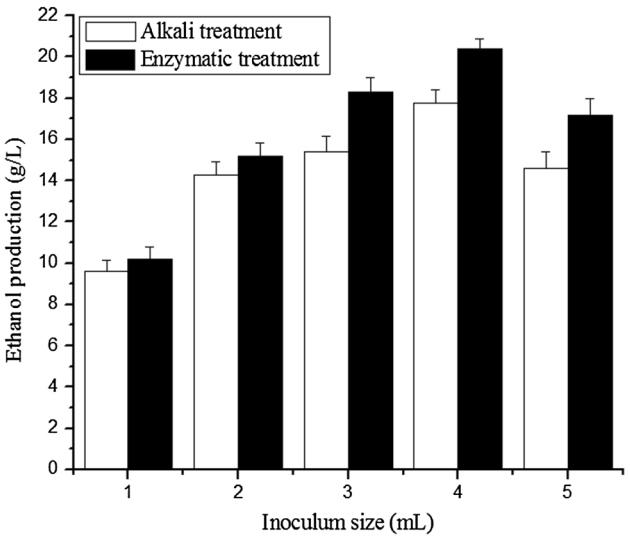

Fig. 5 Effect of inoculum size on ethanol production

the production of ethanol and other commodity products [8]. On the other hand, lower inoculum size reduces the production cost in ethanol fermentation. In an earlier study, Laluce et al. [27] obtained higher ethanol productivities using high inoculum level.

Conclusions

In the present study, a comparative evaluation was performed using two state-of-the-art technologies i.e. alkali (NaOH) and enzymatic (ligninolytic) pre-treatments for the delignification of old newspaper waste. After 24 h pretreatment, a maximal of 42.2% delignification was achieved using 25 mL of ligninolytic extract as compared to the 37.7% delignification with 4.0% alkali solution. Overall, more than 60.0% cellulose hydrolysis was achieved using cellulase extract from *Trichoderma harzianum*. Initially, the fermentation profile of enzymatically digested hydrolyzates revealed 12.69 g/L ethanol concentration. Various parameters including fermentation duration, substrate level, pH, temperature, and inoculum volumes were optimized that led to significantly higher ethanol production from 12.69 to 20.4 g/L. In conclusion, the ligninolytic pre-treatment was found efficient as compared to alkali treatment for high titter bio-ethanol production. In summary, lignocellulosic biomass holds considerable potential to meet the current energy demand of the modern world. Enzyme-based pretreatment processing of lignocellulosic-based biomass and waste materials like newspaper waste could also be of particular interest within the bioethanol concept. Moreover, the enzymatic pretreatment strategies appear more promising, cost-effective and eco-friendly to carrying out waste biomass for bioethanol production. The utilization of newspaper waste for fuel ethanol production seems feasible approach for energy conservation and waste management in one consolidated step. However, the non-utilization of pentose sugars by *S. cerevisiae* limits the overall economics of ethanol production from cellulosic materials.

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