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RESEARCH

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Upcycling of tetra pack waste cellulose into reducing sugars for bioethanol production using *Saccharomyces cerevisiae*

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Abstract

Bioethanol production from waste materials offers a promising avenue for sustainable energy and waste management. In this study, fermentable sugars derived from tetra pack waste cellulose were bio-transformed into bioethanol using *Saccharomyces cerevisiae*. Tetra pack waste (180 g) yielded tetra pack cellulosic pulp (TPCP) of 145 g, after removing the different layers representing 80.56 ± 0.32% of the original weight. Cellulase from *Bacillus* sp. RL-07, with a cellulolytic potential of 6.98 ± 0.36 U/ml, released 32.72 ± 0.12 mg/ml of reducing sugars, achieving 44.60 ± 0.56% saccharification of TPCP under optimized conditions. Subsequent fermentation of the broth (1 L) with tetra pack cellulosic pulp hydrolysate (TPCPH) (50% v/v), containing 5.12 g of reducing sugars, by *S. cerevisiae* yielded 1.42 g of bioethanol per g of reducing sugars under optimized conditions, with a volume productivity of 0.24 g/l/h and a purity of 96.42% was confirmed by GC/MS analysis.

The results of this study underscore the viability of utilizing tetra pack waste for bioethanol production, offering a sustainable solution for waste management while alleviating energy deficits and reducing environmental pollution. These findings align with objectives aimed at fostering sustainable progress and development.

Keywords Tetra pack waste, Cellulose, Reducing sugars, Hydrolysis, Biotransformation, Fermentation, Bioethanol

Introduction

Solid waste poses a significant threat to the world's future, driven by rapid population growth, urbanization, and technological progress. Changes in consumption patterns further exacerbate the composition of waste [20]. The generation of waste, particularly solid waste, has surged, contributing to numerous environmental challenges [7]. Municipal waste, comprising a diverse blend of both biodegradable and non-biodegradable elements, encompasses tetra pack, a composite packaging material consisting of 75% cellulose, 20% low-density polyethylene (LDPE), and 5% aluminum, commonly employed for the preservation of liquid food items [3]. Millions of tons of tetra packs are discarded annually, constituting a significant portion of solid municipal waste, leading to environmental problems [18]. Investigations are underway to explore the potential of utilizing this waste for valuable products, notably bioethanol, owing to its abundant availability, renewable nature, and cost-effectiveness [29, 33]. The process of producing bioethanol from tetra pack cellulose encompasses various stages, such as pretreatment, enzymatic hydrolysis, microbial fermentation, and distillation, rendering it a promising alternative biofuel to gasoline [5, 6]. Undoubtedly, this bioethanol has the

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potential to mitigate the depletion of fuel resources and ensure the security of the world's fuel supply. Additionally, it can serve as an alternative renewable energy source to fossil fuels [13]. Cellulases, capable of hydrolyzing cellulose into glucose, are essential for utilizing tetra pack waste to produce bioethanol [19, 32]. The results of this study present a sustainable approach to tetra pack waste management by converting it into bioethanol thereby reducing environmental waste and providing a clean, renewable energy source to safeguard the environment.

Results and discussion

Tetra packs are one of the major parts of municipal solid waste which can be upcycled to bioethanol to serve as a renewable source of biofuel. Technology developments made through this research and the experimental findings are summarized in this section to make it feasible to efficiently upcycle tetra packs waste into renewable and ecofriendly bioethanol.

Characterization of cellulase producing microbial strain

This culture, previously identified for its cellulase activity, was reinvigorated on nutrient agar at 30 °C for 24 h and then stored at 4 °C for future use. Morphological and biochemical examinations of culture RL-07 unveiled rod-shaped cells with creamish-colored mucoid colonies, presenting an oval shape with smooth margins on nutrient agar. Moreover, it was identified as Gram-positive and motile. Biochemical assessments affirmed its positive responses to catalase, Voges-Proskauer, nitrate reduction, oxidase, and glucose tests, while showing negative results for methyl red and indole tests. Based on these analyses, isolate RL-07 was classified as a member of the *Bacillus* genus and named as *Bacillus* sp. RL-07. Initially, the cellulase enzyme activity was recorded at 2.61 ± 0.14 U/ml.

Revival of C-6 fermenting yeast

The C-6 fermenting yeast, *Saccharomyces cerevisiae*, was obtained from Research Lab-5, Department of Biotechnology, Himachal Pradesh University, Shimla. *S. cerevisiae* has demonstrated significant fermentation capabilities for converting C-6 sugars derived from agricultural waste biomass [4]. Therefore, this strain was chosen and employed for fermenting tetra pack cellulosic pulp hydrolysate into bioethanol. The culture was preserved on YPD agar plates and stored at 4 °C for future utilization.

Collection of tetra packs and processing to separate different layers

For this study, 30 tetra packs weighing 180 g were gathered from the garbage bins near Himachal Pradesh University, Summer Hill, Shimla. These packs were then cut into small pieces measuring 40X40 mm. Subsequently, they were treated with 6 mol/L of methanoic acid, acetic acid, hydrochloric acid, and chloroform separately in each case. Among these agents, methanoic acid (6 mol/L) required the least amount of time to separate all the layers (LDPE, Aluminum, and cellulose) of the tetra pack, taking only 18 min, as illustrated in Fig. 1. In contrast, acetic acid and chloroform took 25 and 33 min, respectively. Hydrochloric acid acted rapidly, separating the layers in just 7 min, but it also damaged the cellulosic part of the tetra packs. Therefore, the separation of the different layers of the tetra pack was conducted using methanoic acid as the separating agent instead of hydrochloric acid.

Preparation of tetra pack pulp and its chemical pretreatment

The process of separating the layers of the tetra pack resulted in the production of cellulosic pulp weighing 145 g from the 180 g of tetra pack waste. This cellulosic pulp was ground in a mixer grinder and boiled in 300 ml of distilled water until slurry was obtained, which was then chemically pretreated using 2% (w/v) NaOH to remove inks, fillers, hemicellulose, and lignin. Subsequently, bleaching was performed using 2% (v/v) sodium hypochlorite (NaClO) to further eliminate most of the remaining lignin, which was resistant to alkali treatments, along with a portion of the hemicellulose, resulting in a white cellulose material suitable for further hydrolysis. Following this process, the cellulose content was determined to be 49.76 ± 0.32%, with the release of 03.21 ± 0.24 mg/ml of reducing sugar (data not shown). Comparable findings were reported by Neelamegam et al. [25], demonstrating that NaOH pretreatment yielded 42.34–46.74% cellulose in pretreated office paper. Additionally, a study conducted by Xing et al. [34] demonstrated that over 55% of cellulose fibers can be successfully recycled from tetra packs by separating the paperboards from PE films and purifying the paper.

Optimization of physicochemical parameters for enhancement of cellulase activity from *Bacillus* sp. RL-07

Inoculum size

Enhancing the cellulolytic potential of *Bacillus* sp. RL-07 involved optimizing various factors, among which inoculum size played a crucial role in increasing enzyme activity. Insufficient inoculum size can lead to delayed microbial growth, consequently prolonging the overall time required for enzyme production. The maximum cellulase activity, 4.94 ± 0.11 U/ml, was observed at an inoculum size of 5% (v/v), while the lowest cellulase activity, 2.68 ± 0.07 U/ml, was recorded at an inoculum size of 1% (v/v), as depicted in Fig. 2a. *Bacillus* sp. RL-07 exhibited

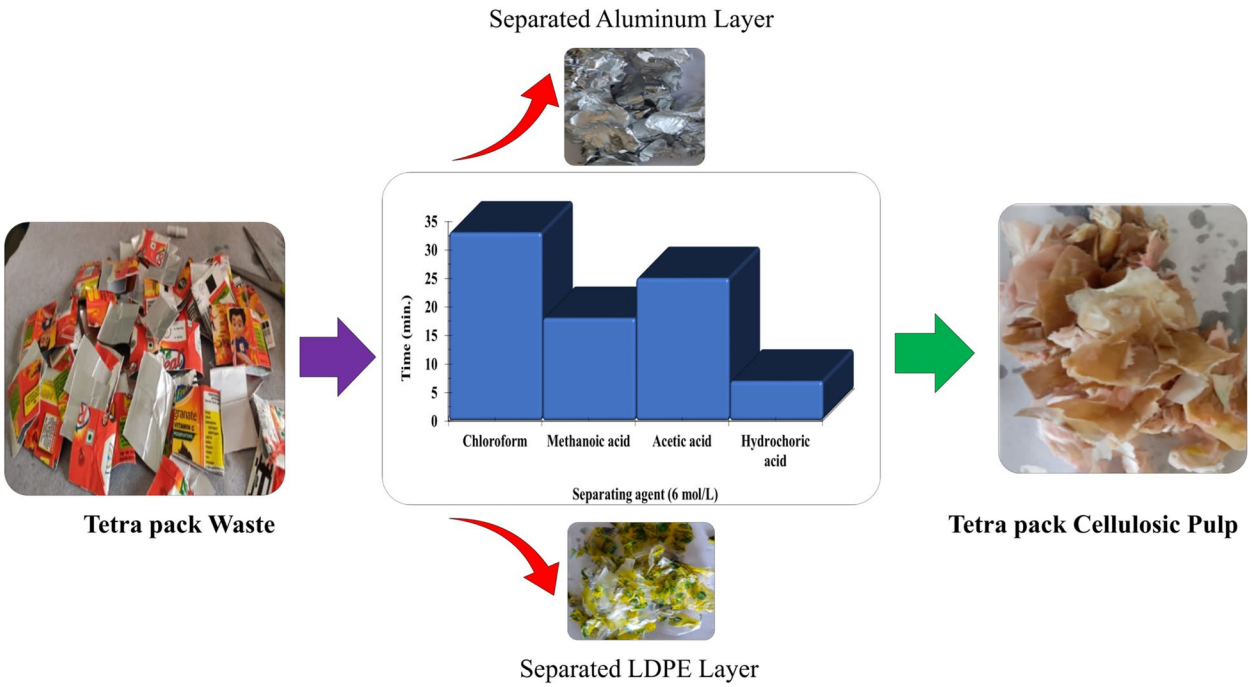

Fig. 1 Effect of different separating agents on tetra pack for removal of different layers to get the TPCP

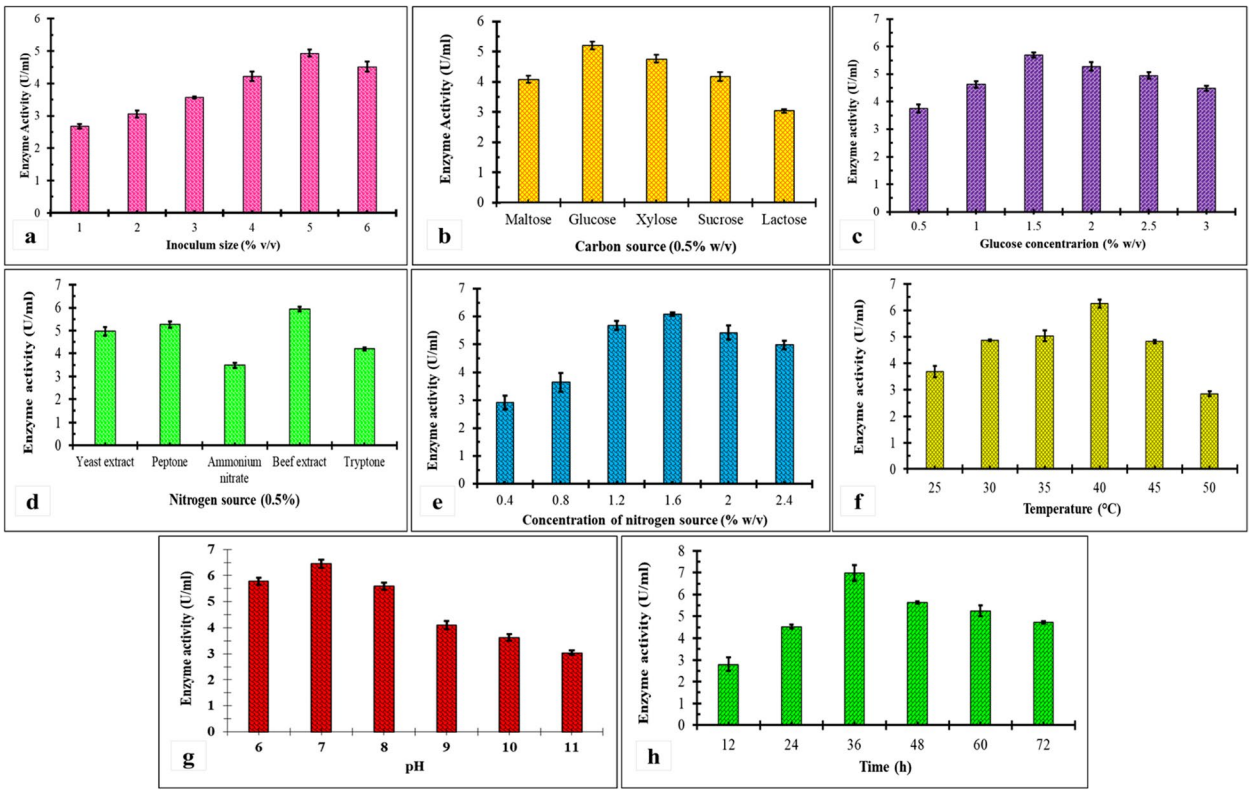

Fig. 2 Optimization of physicochemical parameters (**a** innoculum size; **b** carbon source; **c** carbon source concentration; **d** nitrogen source; **e** nitrogen source concentration; **f** temperature; **g** time; **h** pH) for enhancement of cellulase activity from *Bacillus* sp. RL-07

an increase in cellulase activity up to an inoculum size of 5% (v/v), followed by a decline in activity. However, Behl et al. [4] utilized a 3% inoculum size of 24-h-grown seed culture for the production of cellulase enzyme from *Bacillus* sp. PHS-05.

Carbon source and its concentration

The selection of a carbon source in a growth medium plays a crucial role in influencing the growth, metabolism, and behavior of microorganisms, as it serves as their primary energy source. In this study, glucose, lactose, xylose, maltose, and sucrose were investigated as carbon sources. Among these, glucose exhibited the highest activity, with a value of 5.21 ± 0.13 U/ml, while lactose showed the lowest activity at 3.04 ± 0.06 U/ml, as illustrated in Fig. 2b. Similar results were reported by Shanmugapriya et al. [31] in the case of cellulase activity with CMC as a substrate. When the substrate concentration exceeds the required level, enzyme units may become saturated and fail to function efficiently. The maximum cellulase activity was achieved at a glucose concentration of 1.5% (w/v), reaching 5.69 ± 0.09 U/ml, whereas the lowest activity (3.75 ± 0.14 U/ml) was observed at a glucose concentration of 0.5% (w/v), as depicted in Fig. 2c. A sharp decline in activity was noted after a certain increase in concentration, which could be attributed to elevated viscosity leading to reduced nutrient circulation and disrupted oxygen transfer [30].

Nitrogen sources and its concentration

Nitrogen plays a vital role in the physiology and metabolism of enzyme production in bacteria. Different nitrogen sources can significantly influence enzyme production rates. Beef extract, as the primary nitrogen source, exhibited a positive impact on cellulase activity, reaching 5.94 ± 0.09 U/ml. Conversely, the lowest cellulase activity of 3.48 ± 0.11 U/ml was observed with ammonium nitrate, as depicted in Fig. 2d. A study by Iram et al. [16] reported a similar trend in cellulase activity during the optimization of various nitrogen sources for cellulase production by *Aspergillus niger*. Beef extract as a nitrogen source demonstrated maximum cellulase activity of 6.08 ± 0.06 U/ml at a concentration of 1.6% (w/v). Conversely, the lowest cellulase activity was recorded at 0.4% (w/v), yielding 2.91 ± 0.24 U/ml, as illustrated in Fig. 2e. At lower nitrogen source concentrations, enzyme activity remained low as the media failed to meet microbial demands for vitamins and minerals. However, beyond a optimum concentration, activity levels began to decline, possibly due to reduced nutrient consumption and accumulation, resulting in a constrained environment for microbial growth and the production of essential enzymes, including cellulase [4].

Optimization of temperature, pH and incubation time

These various factors play a crucial role in the growth, production, and activity of enzymes by stabilizing the protein structure and accelerating kinetic energy. Cellulase exhibited maximum activity at 40 °C, reaching 6.25 ± 0.15 U/ml. Initially, as temperature increased, the rate of reaction also rose due to increased kinetic energy. However, beyond optimum temperature, both activity and reaction rate began to decline, likely due to bond breakage and changes in enzyme structure. The lowest cellulase activity was recorded at 50 °C, with a value of 2.83 ± 0.09 U/ml (Fig. 2f). This finding aligns with a study by Pramanik et al. [27], reported maximum cellulase activity at 40 °C for *B. pseudomycoides*.

Enzyme activity is highly dependent on pH, as changes in hydrogen ion concentration can affect intra- and intermolecular bonds, altering the enzyme's shape and catalytic site effectiveness Chakraborty et al. [10]. In our study, maximum cellulase activity of 6.45 ± 0.16 U/ml was observed at pH 7.0. However, activity declined beyond the optimum pH, with the lowest cellulase activity recorded at pH 11.0, reaching 3.05 ± 0.08 U/ml (Fig. 2g). Acharya and Chaudhary [2] reported similar findings for *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3, with maximum cellulase activity observed at pH 6.5 and 7.0, respectively.

Incubation time also significantly influences enzyme production, with protein denaturation occurring with prolonged incubation, leading to loss of catalytic activity over time. In our study, maximum cellulase activity of 6.98 ± 0.36 U/ml was achieved after 36 h, whereas the lowest activity was observed after 12 h incubation with a value of 2.80 ± 0.31 U/ml (Fig. 2h). This underscores the importance of optimizing incubation time to maximize enzyme productivity while mitigating the risk of denaturation and reduced activity over time [24].

Optimization of reaction parameters for saccharification of tetra pack cellulosic pulp (TPCP)

Volume of tetra pack cellulosic pulp

To investigate the optimal volume of tetra pack cellulosic pulp (TPCP) for maximal reducing sugar production, saccharification experiments were conducted using varying volumes of TPCP (5–30% w/v). The production of reducing sugars was evaluated for each volume, revealing that the highest production (15.32 ± 0.06 mg/ml) occurred with a TPCP volume of 15% (w/v) (Fig. 3a), employing cellulase enzyme (6.98 ± 0.36 U/ml) derived from *Bacillus* sp. RL-07. Beyond the 15% volume, reducing sugar yield declined, possibly due to feedback inhibition mechanisms. In a study by Behl et al. [4], cellulase

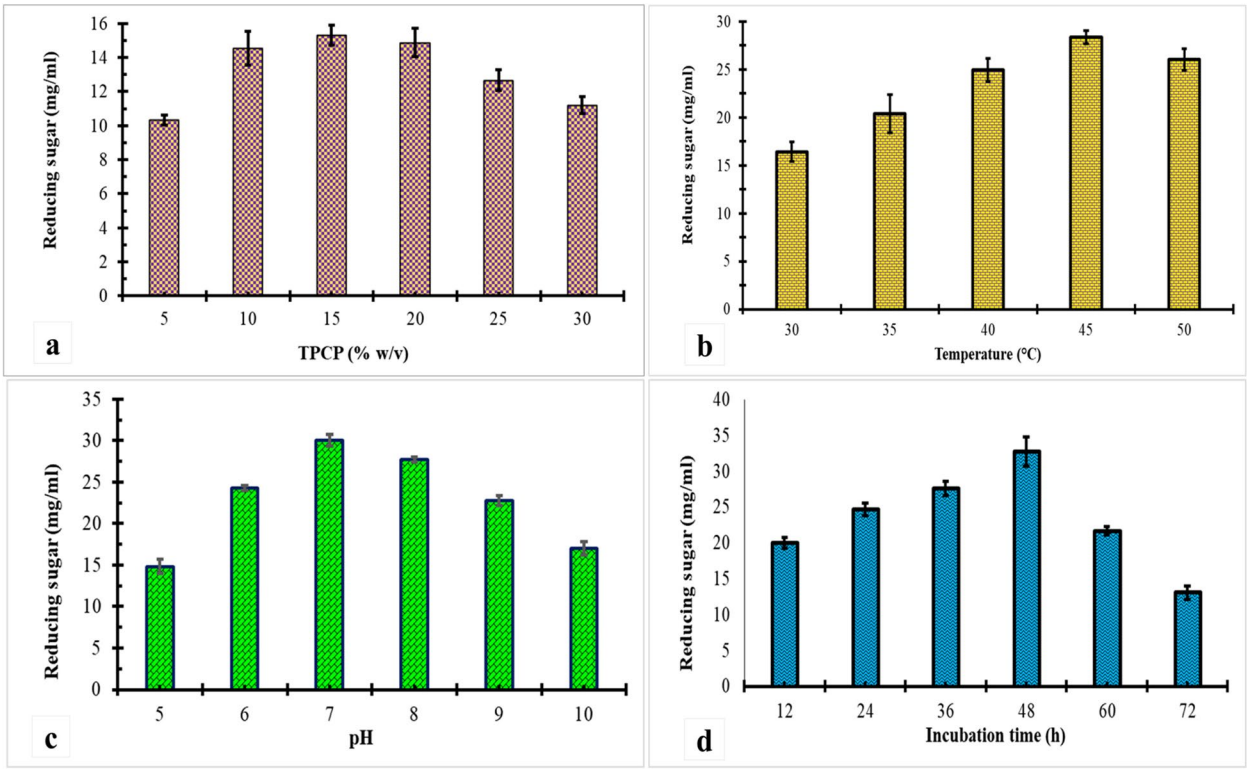

Fig. 3 Optimization of various parameters (**a** volume of TPCP; **b** temperature; **c** pH; incubation time) for the release of reducing sugars from tetra pack cellulosic pulp

treatment of corn stalk pulp resulted in a 77.43 ± 2.44% extraction of reducing sugars.

Effect of incubation temperature

Cellulases are typically most effective for saccharification within the temperature range of 40 °C to 50 °C. In our study, optimal saccharification of TPCP (15%) occurred at 45 °C, resulting in a total reducing sugar content of 28.35 ± 0.07 mg/ml, as depicted in Fig. 3b. Above the optimal temperature range, there was no notable increase in sugar yield. Generally, there was little variation in sugar yield observed within the 40–50 °C temperature range, although saccharification efficiency decreased at 50 °C. Beyond 45 °C, the concentration of total reducing sugars decreased, likely due to enzyme inactivation at higher temperatures. This could result from the breakdown of intermolecular interactions between polar groups (such as H-bonding and dipole-dipole attraction) and the disruption of hydrophobic forces between non-polar groups within the protein structure as temperature fluctuates [17]. Disturbances in these forces can lead to alterations in the secondary and tertiary structure of proteins [26].

Effect of pH

In our study, the maximum saccharification of tetra pack pulp occurred at pH 7.0, resulting in a total reducing sugar content of 30.05 ± 0.11 mg/ml (Fig. 3c). Above the optimal pH, there was no further increase in sugar yield observed, as enzyme efficiency declined below pH 6.0 or above pH 8.0. This trend could be attributed to pH-induced alterations in the shape and structure of the enzyme. Changes in pH impact the ionization state of acidic or basic amino acids, consequently modifying the ionized state of amino acids within the protein structure. This, in turn, disrupts ionic bonds and alters the three-dimensional structure of the protein. In a study conducted by Behl et al. [4], the maximum production of reducing sugars from corn stalk pulp was noted at pH 8.0. Similarly, Ezeilo et al. [14] observed that biomass treated with hydrolytic enzymes of *B. subtilis* and *Rhizopus oryzae* PR exhibited the highest saccharification rate at pH 7.0.

Effect of incubation time

The duration of the saccharification process significantly influences cellulose breakdown. Enzymatic saccharification was conducted for 72 h at 35 °C, with samples collected every 12 h to measure the hydrolyzed reducing

sugars. The optimal saccharification time was determined to be 48 h, during which 32.72 ± 0.16 mg/ml of reducing sugars were obtained (Fig. 3d). Following the 48 h mark, the sugar concentration gradually declined. In a similar study by Rana et al. [28], an optimum saccharification time of 48 h was reported for corn cob, resulting in a yield of 52.17 mg/ml of reducing sugars. The decrease in total reducing sugar concentration after optimum duration may be attributed to enzyme denaturation with prolonged incubation, as well as the accumulation of toxic substances and secondary metabolites [8].

Optimization of fermentation process using *S. cerevisiae*

Impact of inoculum size on bioethanol production

To assess the impact of varying inoculum size on bioethanol production, a 24-h-old *S. cerevisiae* inoculum ranging from 1 to 6% (v/v) was added into the fermentation medium. Bioethanol production was then measured using the potassium dichromate method for each inoculum size. The results indicated that the highest bioethanol yield (0.38 ± 0.02 g/g), was achieved with a 5% (v/v) inoculum of *S. cerevisiae*, as illustrated in Fig. 4a. However, an increase in inoculum size beyond 5% (v/v) led to a decrease in bioethanol production. This decline could be attributed to nutrient depletion and the accumulation of secondary metabolites in the medium. Abdulla et al. [1] similarly reported maximum ethanol production when utilizing starchy food wastes inoculated with a 5% (v/v) inoculum of *S. cerevisiae*.

Effect of tetra pack cellulosic pulp hydrolysate (TPCPH) volume

To determine the optimal volume of TPCPH for maximal bioethanol production, fermentations were conducted using hydrolysate volumes ranging from 10 to 60% (v/v). Bioethanol production was assessed after 48 h of fermentation with 5% (v/v) *S. cerevisiae*. The results revealed that a 50% volume of hydrolysate yielded the highest bioethanol production, reaching 0.42 ± 0.02 g/g. Beyond a 50% hydrolysate volume, bioethanol yields began to decline (Fig. 4b), possibly due to feedback inhibition. Previous studies have shown that bioethanol yields increase with rising glucose concentrations, ranging from 15 to 60 g/L, when utilizing immobilized *S. cerevisiae* [15].

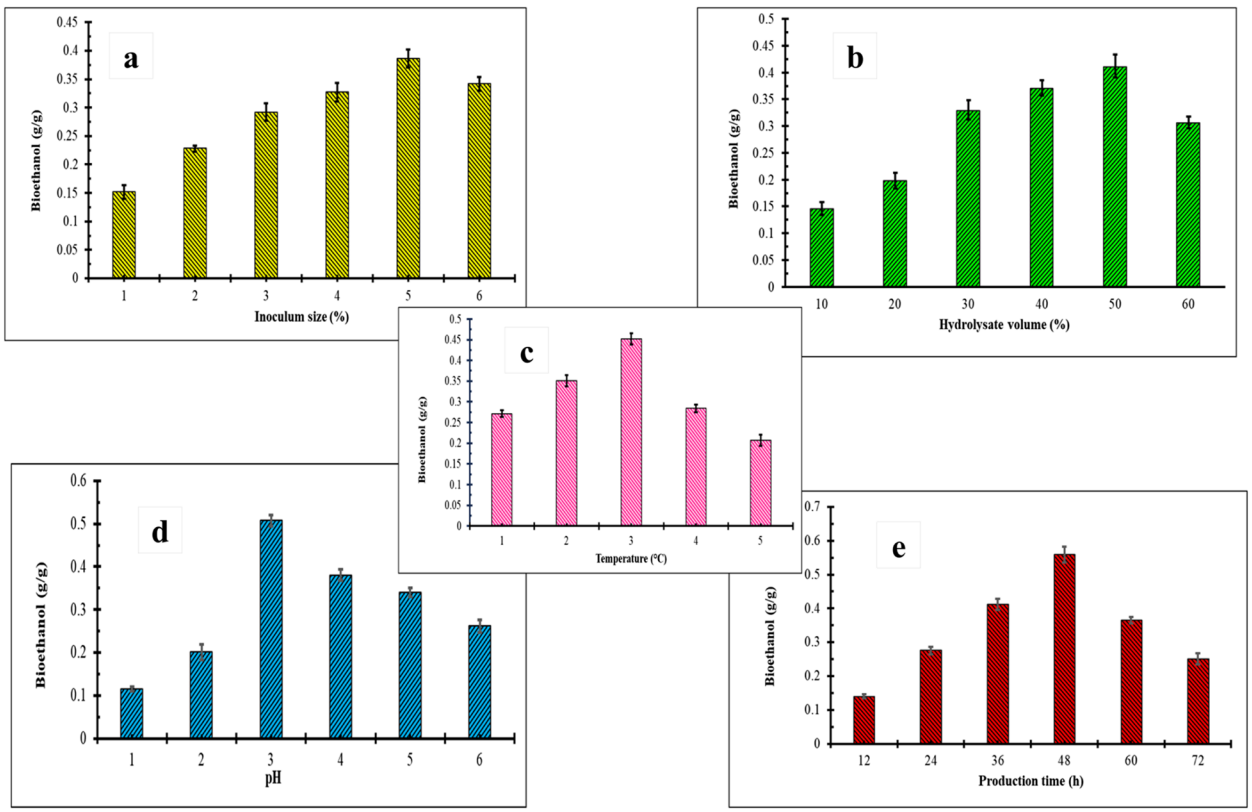

Fig. 4 Optimization of fermentation process parameters (**a** inoculum size; **b** TPCPH volume; **c** temperature; **d** pH; **e** incubation time) for the production of bioethanol from tetra pack cellulosic pulp hydrolysate (TPCPH) using *S. cerevisiae*

Effect of temperature

Temperature plays a crucial role in yeast fermentation performance, impacting both enzymatic activity and yeast cell membrane integrity. In this study, the temperature was optimized for *S. cerevisiae* culture in 50 ml of fermentation media within the range of 25 °C to 45 °C. Maximum bioethanol production was observed at 35 °C, yielding 0.45 ± 0.014 g/g of bioethanol (Fig. 4c). Beyond 35 °C, there was a sharp decline in production, possibly due to yeast inactivation at elevated temperatures. This finding aligns with the results of a study conducted by Caspeta et al. [9] that demonstrated 40 °C as optimal for maximum alcohol production with *S. cerevisiae*.

Effect of pH

The pH of the growth medium also significantly influences yeast activity by inducing morphological changes in the organism. For this experiment, *S. cerevisiae* was inoculated into 50 ml of fermentation media with pH ranging from 3.5 to 5.5 in different 250 ml Erlenmeyer flasks. An optimal pH of 4.0 was observed, resulting in a bioethanol yield of 0.51 ± 0.03 g/g (Fig. 4d). Similar findings were reported in a study on bioethanol production from the starchy component of potatoes using *S. cerevisiae* MTCC-170 by Duhan et al. [12].

Effect of production time

Time plays a critical role in bioethanol production, significantly influencing the overall economics of the process, even at the commercial scale. Optimizing production time is essential to achieve maximum yield and efficiency. In this study, the effect of time on bioethanol production was evaluated by culturing *S. cerevisiae* at 35 °C in a predefined medium using a 5% (v/v) inoculum of 24-h-old cells. Bioethanol production was monitored after every 12 h up to 72 h. he highest bioethanol production of 0.55 ± 0.04 g/g was observed at 48 h. Subsequently, the bioethanol yield began to decline, possibly due to the culture entering a decline phase and experiencing reduced metabolism (Fig. 4e). This finding is consistent with observations reported by Manmai et al. [23].

Production of bioethanol from tetra pack cellulosic pulp hydrolysate (TPCPH) at bench scale

Laboratory-scale production of bioethanol using 1 L of fermentation media was conducted under pre-optimized conditions employing *S. cerevisiae*. The fermentation process was performed under pre-optimized conditions, incorporating 50% (v/v) of TPCPH, equivalent to 5.125 g of reducing sugars. After 48 h, the total bioethanol yield in 1 L of hydrolysate was determined to be 1.42 g/g of reducing sugars (Fig. 5). The residual sugar content in the broth was calculated to be 0.21 g, with a volume

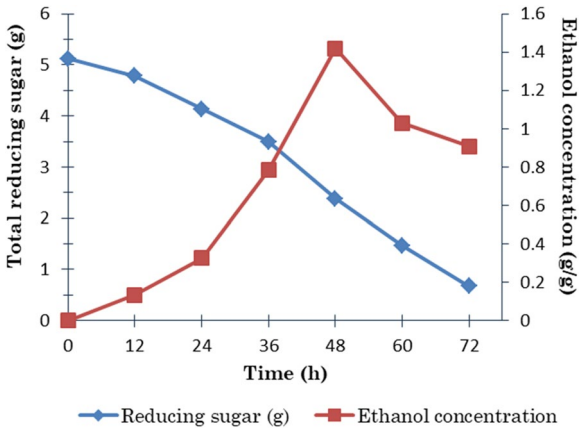

Fig. 5 Biotransforamtion of TPCPH into bioethanol at bench scale (1L)

productivity of 0.24 g/L/h. Following GC analysis of the sample, the purity of bioethanol was found to be 96.47%. In a separate study, Tura et al. [33] reported the highest ethanol production from pre-treated lignocellulosic biomass by *Schizosaccharomyces pombe* at 35 °C. Similarly, Dwivedi et al. [13] recorded a maximum ethanol production of 0.32 g/g after 48 h of incubation using *Schizosaccharomyces* sp. EF-3 and *K marxianus* MTCC. Therefore, this study effectively showcases the sustainable management of tetra pack paper pulp as a feedstock for successful bioethanol production, offering an alternative energy source to mitigate environmental pollution resulting from tetra pack mismanagement.

Conclusion

Bioethanol production from waste materials presents a promising avenue for sustainable energy production and waste management. Converting tetra pack waste cellulose into bioethanol offers a viable method for recycling and generating value from waste. Optimization of cellulase enzyme production by *Bacillus* sp. RL-07 resulted in a threefold increase in its cellulolytic potential. Chemical pretreatment of 180 g of tetra pack waste yielded 145 g of tetra pack cellulosic pulp (TPCP), which, upon saccharification using cellulase (6.98 ± 0.36 U/ml) from *Bacillus* sp. RL-07, released 32.72 ± 0.12 mg/ml of fermentable sugars. Subsequent fermentation on a 1-L scale containing TPCPH (50% v/v) with 5.12 g of reducing sugars and 5% *S. cerevisiae* resulted in the production of 1.42 g of bioethanol per g of reducing sugars under optimized conditions. The process exhibited a volume productivity of 0.24 g/l/h and a purity of 96.42%. The findings of this research conducted on bioethanol production from tetra pack waste at the bench scale can contribute to the development of a prototype for sustainable waste

management. Implementing such action-oriented solutions for upcycling tetra pack waste into valuable products like bioethanol can address not only energy crisis and environmental pollution issues but also promote sustainable growth and development. However, there is still room for improvement in the efficiency and cost-effectiveness of pretreatment methods for cellulosic biomass, which is essential to pave the way for a sustainable biofuel industry.

Material and methods

Cellulase-producing microbial strain

The cellulase producing bacterial culture RL-07 was earlier isolated and found to be positive for cellulase activity but been had not been characterized for its cellulolytic potential.

The culture was rejuvenated by transferring a small amount of inoculum from the glycerol stock to a nutrient broth, followed by an incubation period of 24 h at 30 °C with agitation at 120 rpm. Subsequently, the culture was preserved by streaking it onto nutrient agar plates and storing them at 4 °C for future applications.

Morphological and biochemical analysis of the isolate RL-07

Morphological characterization

For the identification of the selected isolate RL-07, colony morphology such as colony form, surface, and pigmentation was observed. Bacterial cell suspension from a fresh culture was used for microscopic examination of shape, classification into Gram-positive or Gram-negative, spore formation, and motility. Further identification of the selected isolate RL-07 involved conducting some important tests such as the catalase test, Voges-Proskauer (VP) test, methyl red test, nitrate reduction test, indole production test, oxidase test, and glucose fermentation test as per standard protocols.

Reducing sugars (C-6) fermenting yeast

C-6 fermenting yeast culture of *S. cerevisiae* was also taken from Research Lab-5 of the Department of Biotechnology, Himachal Pradesh University, Shimla. To revive the culture, a small amount of inoculum was transferred from the glycerol stock to YPD broth and then incubated at 30 °C for 24 h with agitation at 120 rpm. Following incubation, the culture was preserved by streaking it onto YPD agar plates and storing them at 4 °C for future use.

Collection of tetra pack waste sample

Tetra pack waste was collected from the garbage dumping bins near Himachal Pradesh University, Summer Hill, Shimla. The collected samples were washed thoroughly and chopped into small pieces of 40X40 mm size.

Processing of tetra pack waste to separate different layers

Tetra pack is a composite packaging material composed of three different layers: paper, aluminum, and LDPE. Different separating agents such as chloroform, acetic acid, methanoic acid, and hydrochloric acid (6 mol/L each, separately) were used to assess their efficiency in removing the various layers, following the modified protocol provided by Zhang et al. [35]. The separated cellulosic layer was then ground in a mixer grinder for 30 min to obtain the cellulosic pulp.

Chemical pretreatment

The tetra pack cellulosic pulp underwent further treatment with NaOH (2% w/v) to remove inks, fillers (such as kaolin, talc, calcium carbonate, and titanium oxide (TiO_2)), hemicellulose, and lignin from the paper matrix, following the procedure outlined by Danial et al. [11]. Following filtration through a Whatman No. 1 filter paper, the resulting mixture underwent washing with distilled water until the pH of the cellulosic pulp reached neutrality. Subsequently, the sample was dried in an oven at 50 °C for a duration of 2 h to complete the process.

Determination of cellulosic content

The separation of cellulose was executed in accordance with the Klason method as detailed by Lv and Wu [21]. The solid residue obtained post-extraction and filtration underwent successive washes with deionized water, followed by three washes with acetone, and subsequently dried in an oven. The cellulose content was determined using the formula provided below:

$$\text{Cellulose}\,\% = \frac{W_{C1} - W_{C2} * 100}{W_{C1}}$$

W_{C1} = Initial weight of cellulose
W_{C2} = Weight of cellulose after treatment

Cellulase production by *Bacillus* sp. RL-07

The production of cellulase from *Bacillus* sp. RL-07 followed the methodology delineated by Suri et al. [32]. Initially, a loopful of *Bacillus* sp. RL-07 culture was inoculated into 50 ml of nutrient broth and then incubated at 30 °C for 24 h in an incubator shaker set at 120 rpm. After the incubation period, 1 ml of the seed culture was transferred to 50 ml of production medium contained in a 250 ml Erlenmeyer flask. The production medium consisted of glucose (1.5% w/v), beef extract (1.6% w/v),

$K_2HPO_4.3H_2O$ (0.15% w/v), KH_2PO_4 (0.1% w/v), $MgCl_2$ (0.05% w/v), and $CaCl_2$ (0.005% w/v), adjusted to pH 7.0. Subsequently, the production medium was incubated at 40 °C for 36 h under continuous shaking at 120 rpm. After completion of the incubation period, the culture was harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The resulting supernatant obtained after centrifugation was utilized for the cellulase assay.

Cellulase assay

Cellulase activity was determined using the Dinitrosalicylic acid assay (DNSA), following the modified method described by Suri et al. [32]. Initially, 0.5 ml of cellulase was diluted with an equal volume of citrate buffer (0.05 M, pH 4.8). To this mixture, 0.5 ml of substrate solution was added, followed by incubation at 40 °C for 30 min. Subsequently, DNS reagent was added, and the resulting mixture was boiled for 15 min. After boiling, the reaction mixture was allowed to cool and then diluted with 10 ml of distilled water. The absorbance of the solution was measured at 540 nm using a UV-Vis spectrophotometer. The concentration of reducing sugars was determined based on a standard curve of glucose, prepared using a 1 mg/ml glucose solution.

Estimation of reducing sugars by DNSA in the tetra pack cellulosic pulp (TPCP)

To determine the amount of reducing sugars in TPCP, the sample was initially centrifuged at 10,000 rpm for 10 min, and the resulting pellet was discarded while retaining the supernatant for further analysis. Subsequently, 0.5 ml of substrate solution was added to the supernatant, and the mixture was incubated at 40 °C for 30 min. Following the incubation period, DNS reagent was added to the mixture, which was then boiled for 15 min. After boiling, the reaction mixture was allowed to cool and then diluted with 10 ml of distilled water. The absorbance of the solution was measured at 540 nm using a UV-Vis spectrophotometer.

Quantitative analysis of bioethanol through dichromate assay

The bioethanol concentration was assessed employing the potassium dichromate method, as described by Dwivedi et al. [13]. Initially, 1 ml of supernatant from the cell-free fermentable tetra pack cellulose pulp hydrolysate was mixed with 2 ml of acidic potassium dichromate reagent and 9 ml of water. Subsequently, the resulting mixture boiled at 100 °C in a preheated water bath for 10 min. Following cooling to room temperature, the absorbance of the solution was measured at 600 nm. Ultimately, the bioethanol concentration in the fermentation broth was determined using a standard bioethanol curve.

Optimization of cellulolytic enzyme production by *Bacillus* sp. RL-07

The One Variable at a Time (OVAT) experimental approach was utilized to optimize cellulase production with *Bacillus* sp. RL-07. Various physicochemical conditions were systematically altered, including inoculum size (1–6% v/v), carbon sources like glucose, sucrose, xylose, lactose, and maltose (0.5% w/v each), carbon source concentration (ranging from 0.5% to 3.0% w/v), nitrogen sources (0.4% w/v) such as meat extract, tryptone, yeast extract, beef extract, peptone, and ammonium nitrate, nitrogen source concentration (varying from 0.4% to 2.4% w/v), temperature (25–50 °C), pH levels (ranging from 6.0 to 11.0), and production duration (12–72 h). Cellulase activity was evaluated using the cellulase assay method as outlined in cellulase assay section. The cellulase enzyme obtained was subsequently utilized for the saccharification of tetra pack cellulose pulp (TPCP) to release C-6 fermentable sugars.

Optimization of reaction parameters for saccharification of tetra pack cellulosic pulp

The lignin-free tetra pack cellulose pulp (TPCP) underwent biological pretreatment through enzymatic hydrolysis using cellulase with an enzyme activity of 6.98 ± 0.36 U/ml, produced by *Bacillus* sp. RL-07, to liberate fermentable (C-6) sugars during saccharification. Various conditions were evaluated for enzymatic hydrolysis of TPCP, comprising temperature ranging from 30 °C to 50 °C, pH levels from 5.0 to 10.0, and incubation times from 12 to 72 h. These conditions aimed to optimize the release of reducing sugars, which were quantified using the DNSA method as discussed earlier in estimation of reducing sugars by DNSA in the tetra pack cellulosic pulp (TPCP) section, and subsequently utilized for fermentation.

Total saccharification

Following lignin removal, the tetra pack cellulosic pulp (TPCP) underwent saccharification employing cellulase enzyme to catalyze the complete breakdown of polysaccharides into monosaccharides. The effect of pretreatment on the TPCP structure was assessed using the DNSA method to detect C-6 sugars, as delineated in estimation of reducing sugars by DNSA in the tetra pack cellulosic pulp (TPCP) section. The degree of saccharification was calculated utilizing the formula established by Mandels and Sternberg [22], expressed as follows:

$$\text{Saccharification \%} = \frac{\textit{Reducing sugar } (mg/ml) * 0.9 * 100}{\textit{Initial substrate concentration } (mg/ml)}$$

Fermentation of tetra pack cellulosic pulp hydrolysate (TPCPH) to bioethanol

Seed culture and media preparation

A loopful of *S. cerevisiae* was employed as the seed culture, which was then inoculated into 50 ml of YPD broth and allowed to incubate at 35 °C for 24 h. Following this incubation period, 3% of the culture was introduced into the fermentation medium containing 10% glucose, 1.6% yeast extract, 2% $CaCl_2$ (w/v), 0.2% KH_2PO_4 (w/v), 0.025% Na_2HPO_4 (w/v), and 0.025% $MgSO_4$ (w/v). The mixture was then subjected to further incubation at 35 °C, following the procedure outlined by Behl et al. [4]. Upon completion of the incubation period, the cell-free fermentation broth was collected and used to determine the bioethanol content using the dichromate assay protocol outlined in quantitative analysis of bioethanol through dichromate assay section.

Optimization of the fermentation process

Fermentation processes are known to be sensitive to various culture conditions. Therefore, it is essential to optimize parameters such as inoculum size (1–6%), TPCP hydrolysate volume (10–60% w/v), temperature (25 °C-45°C), pH (3.0–6.0), and production time (12–72 h) to enhance the efficiency of bioethanol production. Each bioethanol production experiment was conducted in 500 mL Erlenmeyer flasks containing 250 mL of sterilized fermentation medium. The conditions were varied according to the ranges mentioned above. The concentration of ethanol was subsequently estimated using the dichromate method outlined in quantitative analysis of bioethanol through dichromate assay section.

Biotransformation of tetra pack cellulosic pulp hydrolysate (TPCPH) into bioethanol at bench scale (1 L)

To upscale the production of bioethanol from flask to bench scale (1 L), fermentation was conducted using a New Brunswick Scientific Co. BioFlow fermenter with a capacity of 3 L. The fermentation process involved the utilization of a cocktail comprising tetra pack cellulosic pulp hydrolysate (TPCPH) (50%) and *S. cerevisiae* (5% v/v) as the fermentative yeast inoculum. This mixture was transferred to the pre-optimized fermentation medium to facilitate efficient fermentation. Throughout the fermentation process, samples were collected at regular intervals to monitor sugar utilization and bioethanol production. Upon the completion of fermentation, the broth was harvested and subjected to centrifugation at 15,000 rpm for 5 min to separate the *S. cerevisiae* cells and bioethanol. The bioethanol obtained after centrifugation distilled, and the distilled bioethanol was subsequently analyzed for quantitative and qualitative assessments using a Gas Chromatograph triple quadrupole Mass Spectrometer with a mass range of up to 2,1100 amu (Thermo Scientific TSQ 8000).

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During the preparation of this work the author(s) did not used any AI enabled tool/service.

Authors' contributions
1. Rinki and Pooja Yadav = Writing original draft. 2. Aditya Sharma and Pushpak Dahiya = Data curation and formal analysis. 3. Aakash Kashyap and Abhishek Walia = Investigation; Visualization. 4. Arvind Kumar Bhatt, & Ravi Kant Bhatia = Conceptualization, Review & Editing. All authors read and approved the final manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

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The authors declare no competing interests.

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References

1. Abdulla R, Matam NA, Derman E, Sani SA, Jawan R, Sabullah MK. Preliminary Study on Biethanol Production from Starchy Foodwastes by Immobilized Saccharomyces cerevisiae. InJournal of Physics: Conference Series 2022 Aug 1 (Vol. 2314, No. 1, p. 012003). IOP Publishing.
2. Acharya S, Chaudhary A. Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis* MVS1 and *Bacillus* sp. MVS3 isolated from Indian hot spring. Braz Arch Biol Technol. 2012;55(497):503.
3. Baltac I, Kan S, Turan A and Yücel O. Aluminum recycling and recovery of other components from waste tetra pack aseptic packages. In: TMS annual meeting and exhibition. Cham: Springer Nature Switzerland; 2023. pp. 867–872.
4. Behl M, Dahiya P, Kumari D, Thakur K, Devi M, Rathour RK, Bhatt AK, Bhatia RK. Development of tailored bioprocess for pretreatment

and saccharification of corn stalk into bioethanol using hydrolytic enzymes cocktail and fermentative yeasts. Biocatal Agric Biotechnol. 2023;53:102896.
5. Bhatt AK, Bhatia RK, Thakur S, Rana N, Sharma V, Rathour RK. Fuel from waste: a review on scientific solution for waste management and environment conservation. Energy Environ Sustain. 2018;205:233. https://doi.org/10.1007/978-981-10-7518-6_10.
6. Bhatia RK, Kumar R, Rathour RK, Kumar V, Sharma V, Rana N, Thakur S, Bhatt AK. Enhancement of cellulose degradation potential of *Bacillus* sp. Hcb-21 through mutagenesis. J Microb Biochem Technol. 2017;9:6. https://doi.org/10.4172/1948-5948.1000374.
7. Bhatia SK, Jagtap SS, Bedekar AA. Recent developments in pretreatment technologies on lignocellulosic biomass: effect of key parameters, technological improvements, and challenges. Bioresour Technol. 2020;300:122724. https://doi.org/10.1016/J.BIORTECH.2019.122724.
8. Brar KK, Raheja Y, Chadha BS, Magdouli S, Brar SK, Yang YH, Bhatia SK, Koubaa A. A paradigm shift towards production of sustainable bioenergy and advanced products from Cannabis/hemp biomass in Canada. Biomass Convers Biorefin. 2024;14(3):3161–82.
9. Caspeta L, Coronel J, Montes de Oca A, Abarca E, González L, Martínez A. Engineering high-gravity fermentations for ethanol production at elevated temperature with *Saccharomyces cerevisiae*. Biotechnol Bioeng. 2019;116(10):2587–97.
10. Chakraborty S, Rao PS, Mishra HN. Effect of pH on enzyme inactivation kinetics in high-pressure processed pineapple (*Ananas comosus* L.) puree using response surface methodology. Food Bioprocess Technol. 2014;7(3629):3645.
11. Danial WH, Majid ZA, Muhid MNM, Triwahyono S, Bakar MB, Ramli Z. The reuse of wastepaper for the extraction of cellulose nanocrystals. Carbohyd Polym. 2015;118(165):169.
12. Duhan JS, Kumar A, Tanwar SK. Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170. Afr J Microbiol Res. 2013;7(46):5253–60.
13. Dwivedi D, Rathour RK, Sharma V, Rana N, Bhatt AK, Bhatia RK. Co-fermentation of forest pine needle waste biomass hydrolysate into bioethanol. Biomass Convers Biorefin. 2022;1:13.
14. Ezeilo UR, Wahab RA, Mahat NA. Optimization studies on cellulase and xylanase production by *Rhizopus oryzae* UC2 using raw oil palm frond leaves as substrate under solid state fermentation. Renew Energy. 2020;156:1301–12. https://doi.org/10.1016/j.renene.2019.11.149.
15. Inal M, Yigitoglu M. Production of bioethanol by immobilized *Saccharomyces cerevisiae* onto modified sodium alginate gel. J Chem Technol Biotechnol. 2011;86(12):1548–54.
16. Iram S, Parveen K, Usman J, Nasir K, Akhtar N, Arouj S, Ahmad I. Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater. Biologija. 2012;58(3):107–16.
17. Joshi SM, Gogate PR. Intensification of dilute acid hydrolysis of spent tea powder using ultrasound for enhanced production of reducing sugars. Ultrason Sonochem. 2020;61:104843.
18. Koh-Dzul JF, Carrillo JG, Guillen-Mallette J, Flores-Johnson EA. Low velocity impact behaviour and mechanical properties of sandwich panels with cores made from tetra pack waste. Compos Struct. 2023;304:116380.
19. Kucharska K, Rybarczyk P, Hołowacz I, Lukajtis R, Glinka M, Kamiński M. Pretreatment of lignocellulosic materials as substrates for fermentation processes. Molecules. 2018;23(11):2937.
20. Lakhouit A, Shaban M, Alatawi A, Abbas SY, Asiri E, Al Juhni T, Elsawy M. Machine-learning approaches in geo-environmental engineering: exploring smart solid waste management. J Environ Manage. 2023;330:117174.
21. Lv G, Wu S. Analytical pyrolysis studies of corn stalk and its three main components by TG-MS and Py-GC/MS. J Anal Appl Pyrol. 2012;11:18.
22. Mandels M, Sternberg D. Recent advances in cellulase technology. Hakko Kogaku Zasshi (Japan). 1976;54:4.
23. Manmai N, Unpaprom Y, Ramaraj R. Bioethanol production from sunflower stalk: application of chemical and biological pretreatments by response surface methodology (RSM). Biomass Convers Biorefin. 2021;11(1759):1773.
24. Nargotra P, Vaid S, Bajaj BK. Cellulase production from *Bacillus subtilis* SV1 and its application potential for saccharification of ionic liquid pretreated pine needle biomass under one pot consolidated bioprocess. Fermentation. 2016;2(4):19.
25. Neelamegam A, Al-Battashi H, Al-Bahry S, Nallusamy S. Biorefinery production of poly-3-hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation. J Biotechnol. 2018;265(25):30.
26. Oguro Y, Nakamura A, Kurahashi A. Effect of temperature on saccharification and oligosaccharide production efficiency in koji amazake. J Biosci Bioeng. 2019;127(5):570–4.
27. Pramanik SK, Mahmud S, Paul GK, Jabin T, Naher K, Uddin MS, Saleh MA. Fermentation optimization of cellulase production from sugarcane bagasse by *Bacillus pseudomycoides* and molecular modeling study of cellulase. Curr Res Microb Sci. 2021;2:100013.
28. Rana N, Sharma V, Rathour RK, et al. Utilization of corn cob waste for xylanase production by newly isolated *Bacillus pumilus* XRL5 and its application in saccharification of lignocellulosic biomass. Trends Carbohydr Res. 2019;20:32.
29. Rathour RK, Behl M, Dhashmana K, Sakhuja D, Ghai H, Sharma N, Meena KR, Bhatt AK, Bhatia RK. Non-food crops derived lignocellulose biorefinery for sustainable production of biomaterials, biochemicals and bioenergy: a review on trends and techniques. J Ind Crops Prod. 2023;204:117220. https://doi.org/10.1016/j.indcrop.2023.117220.
30. Saini M, Anu, Rapoport A, Tiwari SK, Singh D, Malik V, Singh B. Bioacetoin production by *Bacillus subtilis* subsp. subtilis using enzymatic hydrolysate of lignocellulosic biomass. Fermentation. 2023;9(8):698.
31. Shanmugapriya K, Saravana PS, Krishnapriya MM, Mythili A, Joseph S. Isolation, screening and partial purification of cellulase from cellulase producing bacteria. Int J Adv Biotechnol Res. 2012;3(1):509–14.
32. Suri P, Dwivedi D, Rathour RK, Bhatt AK, Bhatia RK. Enhanced C-5 sugar production from pine needle waste biomass using *Bacillus* sp. XPB-11 mutant and its biotransformation to bioethanol. Biomass Convers Biorefin. 2021;1:10. https://doi.org/10.1007/S13399-021-01277-4.
33. Tura A, Montipó S, Fontana RC, Dillon AJ, Camassola M. Ethanol production from sugar liberated from *Pinus* sp. and *Eucalyptus* sp. biomass pretreated by ionic liquids. Braz J Chem Eng. 2018;35(467):476.
34. Xing L, Gu J, Zhang W, Tu D, Hu C. Cellulose I and II nanocrystals produced by sulfuric acid hydrolysis of tetra pack cellulose I. Carbohyd Polym. 2018;192(184):192.
35. Zhang JF, Yan DH, Li ZH. The recycling of the Tetra-pack packages: research on the wet process separation conditions of aluminum and polythene in the Tetra-pack packages. In: 2009 3rd international conference on bioinformatics and biomedical engineering. Beijing: IEEE; 2009. p. 1–6.

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