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Enzymatic Co-Fermentation of Onion Waste for Bioethanol Production Using *Saccharomyces cerevisiae* and *Pichia pastoris*

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Abstract: This paper evaluates the feasibility of bioethanol production from onion waste by *Saccharomyces cerevisiae* and *Pichia pastoris* and their novel co-culture through fermentation. The process parameters were optimized for each strain and their combination to observe the synergistic effect of co-fermentation. A dinitro salicylic acid (DNS) test was conducted to study the reducing sugar content of samples at different time intervals. Fourier transform infrared (FTIR) spectroscopic analysis was used to compare results for functional groups of samples before and after fermentation, and gas chromatography with flame ionization detection (GC-FID) analysis was performed to measure the bioethanol concentration obtained at different combinations of pH (5, 5.5, 6), temperature (20 °C, 30 °C, 40 °C), and time (24–110 h). The maximum bioethanol concentration was achieved through a monoculture of *Saccharomyces cerevisiae*, i.e., 30.56 g/L. The ethanol productivity was determined based on the ethanol concentration and fermentation time ratio. The energy content was determined using the obtained ethanol value and the specific energy content of ethanol, i.e., 30 kJ/g. The productivity and energy of bioethanol obtained at this maximum concentration were 0.355 g/L h and 916.8 kJ/L, respectively, after 86 h of fermentation at 30 °C and pH 5. *Pichia pastoris* produced a maximum of 21.06 g/L bioethanol concentration with bioethanol productivity and energy of 0.264 g/L h and 631.8 kJ/L, respectively, after 72 h of fermentation at 30 °C and pH 5. The coculture fermentation resulted in 22.72 g/L of bioethanol concentration with bioethanol productivity and energy of 0.264 g/L h and 681.6 kJ/L, respectively, after 86 h of fermentation at 30 °C and pH 5. The results of reducing sugars also supported the same conclusion that monoculture fermentation using *Saccharomyces cerevisiae* was the most effective for bioethanol production compared to *Pichia pastoris* and co-culture fermentation.

Keywords: bioethanol; fermentation; *Saccharomyces cerevisiae*; *Pichia pastoris*; waste-to-energy; co-culture

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1. Introduction

Due to the increase in population and industrialization, fuel and electricity consumption have vastly increased. The need of the hour is to shift consumption patterns towards renewable energy and fuel sources. Sustainable energy production is the only possible way to fulfill the increasing consumer demands to improve the standard of living [1]. Biofuel provides the best solution to overcome all these needs and reduces dependence on carbon-based fuels and GHGs [2,3]. With the ongoing concern of fossil fuel depletion and the development of a clean environment, an increase in the demand for biofuel production from renewable resources has been observed [4–6]. Various sustainable energy resources such as biomethane, biohydrogen, biobutanol, bioethanol, and biomethane have gained

more attention with a focus on production. These green fuels are cheap, renewable, and sustainable [7]. First, second, and third-generation biofuels have previously been produced commonly. The feedstock enriched with sucrose and sugar is considered first-generation biofuel, another feedstock comprising lignocellulosic biomass is the second-generation biofuel, and algal biomass is attributed to third-generation biofuels [8]. These fuel types are more helpful as they are more eco-friendly and the cleanest liquid fuels with low toxicity [9].

As the population increases, and so does the quantity of waste, the need for appropriate and effective waste disposal has gained the utmost importance. Landfilling of food wastes may release offensive odors, contributing to leachate formation and high GHG emissions levels. A significant portion of food waste is commonly treated through biological treatments such as anaerobic digestion and composting [10]. They may also be used as animal feed, incinerating the remainder. The heterogeneous composition of food waste is one of the principal challenges for producing reusable value-added products such as biofuels, methane gas, and biofertilizers [11].

Lignocellulosic materials such as agricultural residues of corn, wheat straw, bagasse, sugarcane, forest residues, industrial wastes, etc., are considered ample renewable resources of biomass [12]. Ideal alternative biomass can be best produced by lignocellulosic biomass instead of grain resources for biofuel [10]. Lignocellulosic biomass generally consists of 25–30% hemicellulose, 40–50% cellulose, and 15–20% lignin [13]. The polysaccharides can be hydrolyzed into pentoses and hexoses [14]. In lignocellulosic feedstocks, xylose is the leading and second most crucial pentose sugar.

Organic (biodegradable) waste fuel production promotes sustainable energy development. Biomass is a promising energy resource for greener transportation fuels [15]. The transformation of biomass into biofuel is the most imperative for producing the cleanest liquid fuel [16].

Microbial fermentation is the best alternative energy resource to overcome the limited availability and resources of fossil fuels. Alternate energy feedstocks such as bagasse, grasses, forest residues, domestic waste, etc., are in demand to be used to overcome food vs. fuel competition [17–19]. The lignocellulosic feedstocks for biofuel production are not fully utilized due to expensive pretreatment techniques such as hydrolysis and the non-utilization of waste residues [20]. Food wastes are rich in lignocellulosic organic content and constitute high energy values appropriate for biofuel production [10,11].

Waste-to-energy technologies offer a synergistic relationship between government and industry to turn various types of waste, such as agricultural, livestock, municipal, food waste, etc., into valuable products while decreasing the quantity of waste being dumped into the environment [21]. This approach significantly promotes sustainability and reduces the adverse effects of the vast scale of anthropogenic activities on public health and the environment. Considering the waste hierarchy, transforming waste into biofuel may yield a higher value than the processes of recycling or composting [22]. According to EPA, the waste-to-energy method can provide up to 10 times more electricity than the landfill-gas-to-energy form.

Biofuel has been produced from various biomass by adopting several processes in the past. Some critical processes are pretreatment, saccharification, fermentation, and pyrolysis. However, pretreatments are sometimes expensive, and pyrolysis and saccharification require high energy input, hence are energy intensive [23]. Similarly, the processing cost may increase if co-products are produced while simultaneously causing a reduction in the reaction speed. The previously used feedstocks could lead to price hiking and food insecurity [24]. To reduce the need for expensive, commercially used enzymes, many microorganisms have been grown and identified that produce xylanases and cellulases. *Saccharomyces cerevisiae* is the most popular microorganism for industrial biofuel production owing to its capability to deliver an enhanced amount of biofuel and its high-level forbearance [7].

The use of agro-industrial wastes to generate bioenergy via hydrolysis and fermentation processes has recently garnered increased attention. Although these kinds of garbage are non-competitive with foodstuff, they also boast lower costs and are relatively found in abundance. Discarded/rotten onions, an abundantly generated food waste, were selected as a feedstock in the present study. The chosen strains, i.e., *S. cerevisiae* and *P. pastoris*, have not been investigated previously with the rotten/discarded onion feedstock. Hence, the monoculture of these individual strains was investigated for bioethanol production through the fermentation of rotten/discarded onions. The combination of two different yeast strains, not previously explored, with onion feedstock was also examined to analyze the synergistic effect and the maximum extent of bioethanol production that can be achieved with optimized temperature, time, and pH conditions.

2. Methodology

The overall methodology adopted to achieve the study objectives is shown in Figure 1. The details of each of the steps in the methodology shown in Figure 1 are described in the following sections.

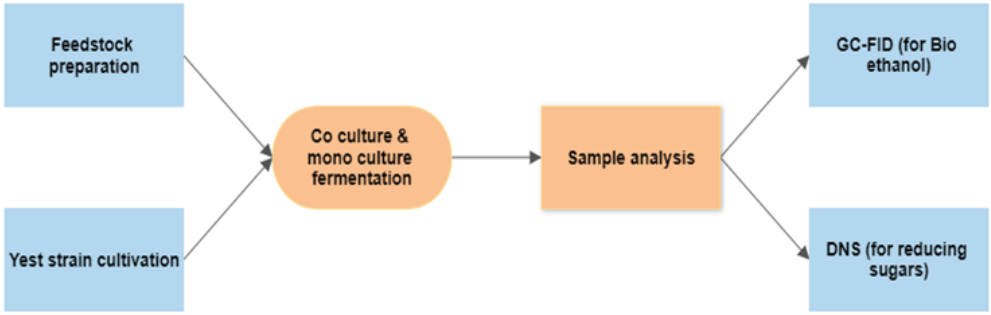

Figure 1. Overall methodology of the study.

2.1. Feedstock Preparation

Feedstock samples (discarded/rotten onions that are usually disposed of) were collected from a local vegetable market in Lahore. The samples were adequately washed with tap water and refrigerated for 2 h to remove the teary compounds (refrigerated and used as needed). The samples were then subjected to mechanical pretreatment, where they were cut and pressed in a mechanical juicer. Glass vials of 1000 mL were used to store the extract generated from the pretreatment. As the Allicin compound in onion extract has an antimicrobial function that could inhibit yeast growth, it was removed by keeping the extract in the oven at 60–70 °C for 30 min before fermentation. The disinfection was performed by autoclaving the onion extract at 110 °C for 10 min [12]. The extract was then allowed to cool down to room temperature before being filtered by Whatman filter paper no. 1. The coarse particles were removed by filtration twice. Finally, the processed onion extract was stored in the refrigerator at 4 °C to be used as feedstock for fermentation.

2.2. Cultivation of Yeast Strains

The strains of *Saccharomyces cerevisiae* and *Pichia pastoris* were obtained from the Centre of Excellence in Microbiology (CEMB), the University of Punjab, Lahore. These strain cultures were preserved at 4 °C during transportation. The *Saccharomyces cerevisiae* and *Pichia pastoris* were maintained on agar slants with YPD medium (1 g glucose/dextrose, 1 g yeast extract, 2 g peptone dissolved in 100 mL distilled water). The cultures were incubated in a shaker incubator for 3–5 days at 30 °C and 100 rpm to allow maximum culture reaction with the substrate during fermentation. Figure 2 shows the cultured strains of *S. cerevisiae* and *P. pastoris*.

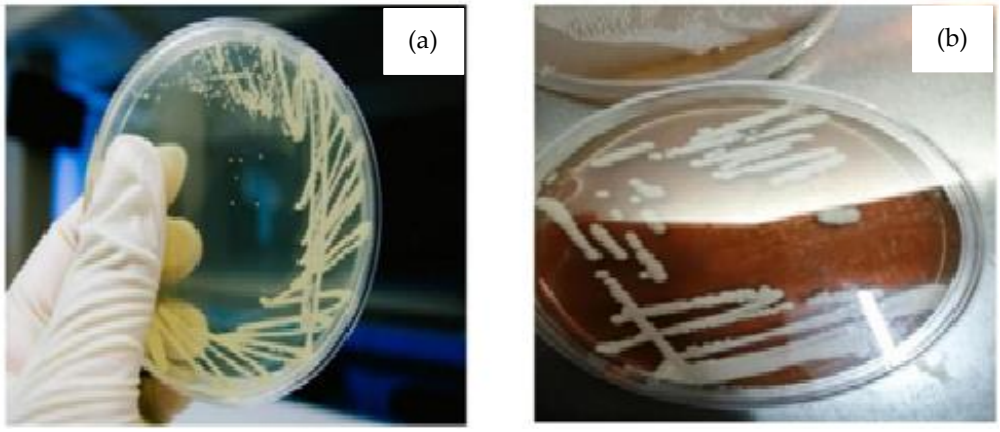

Figure 2. Cultivated strains of (**a**). *S. cerevisiae* (**b**). *P. pastoris*.

The cultures of inoculum from yeast were inoculated by sterile inoculating loops. For inoculation, glassware was washed, including a Petri dish, pipette, 250 mL flask, and 100 mL cylinder with distilled water, and covered with aluminum foil to avoid contamination and sunlight exposure. Flasks were plugged with cotton tightly. The wrapped glassware was autoclaved at 121 °C temperature to sterilize for 15 min. The required culture media (YPD) and 100 mL of distilled water were added to a sterilized flask and plugged in to prepare the culture media. The prepared media was autoclaved again at 121 °C. After that, 20 mL media was poured into a sterilized Petri dish and waited to convert liquid media into a jelly-like material. The yeast strains were inoculated in a Petri dish with a sterilized inoculation needle (sterilized on the flame). The prepared Petri dish was incubated at 30 °C and 24 h. All inoculum was incubated in 250 mL conical flasks with 50 mL inoculation medium in the shaker incubator at a speed of 120 rpm and 20 °C, 30 °C, and 40 °C for a period of 24–110 h. In the Erlenmeyer flask, 50 mL of onion extract and 5 mL of inoculum were added for fermentation.

2.3. Fermentation for Bioethanol Production

The inocula of mono-strains and mixed strains (ratio of 1:1) with 10% inoculum amounts were inoculated into fermentation media after cooling. For bioethanol production, three conditions were selected for comparative analysis, i.e., temperature, time, and pH, and the experiments were carried out in duplicate, and the mean was reported. The temperature of 20 °C, 30 °C, and 40 °C, times of 24 to 110 h, and pHs of 5, 5.5, and 6 were analyzed for monoculture and co-culture fermentation. In one flask, co-culture fermentation was carried out by both *S. cerevisiae (SC)* and *P. pastoris (PP)* inoculum. In other flasks, only *S. cerevisiae* or *P. pastoris* was added, regarded as monoculture fermentation. Samples were withdrawn at intervals of every 24 h of fermentation. The samples were centrifuged for 10 min, and the supernatant was collected and used for analysis.

2.4. Productivity and Energy Content Estimation

The bioethanol productivity was estimated using the method of Thangavelu et al. [25]. The method based the productivity on the concentration of bioethanol obtained and the fermentation time as shown in Equation (1):

$$Bioethanol\ Productivity = \frac{Ethanol\ obatained\ after\ fermentation\ \left(\frac{g}{l}\right)}{Fermentation\ Time\ (hours)} \quad (1)$$

As 1 g of ethanol could release 30 kJ of energy [25], hence, total energy obtained can be estimated by Equation (2):

$$Total\ Energy\left(\frac{KJ}{L}\right) = 30 \times Cocentration\ of\ Bioethanol\left(\frac{g}{l}\right) \quad (2)$$

2.5. Analytical Methods

2.5.1. Reducing Sugar Test

To detect the presence of reducing sugars, the reducing sugar test was performed by adding 3 mL of glucose and 3 mL of DNS reagent in a test tube containing the sample and mixing vigorously. The red-brown color was developed by heating the mixture at 90 °C for 5–15 min. Adding 1 mL of 40% potassium sodium tartrate solution stabilized the color. The sample was then cooled in a water bath at room temperature. Later, the absorbance was recorded by a UV spectrophotometer at 540 nm [26].

2.5.2. FTIR Analysis

Fourier Transform Infrared (FTIR) Spectrophotometer, make Alpha II Bruker Ettlingen Germany, was used to identify the functional groups in samples before and after fermentation. The prepared samples were analyzed directly on FTIR. The wavenumber range of 1000–4000 cm^{-1} was set for analysis. Firstly, onion extract was analyzed by FTIR, and then the bioethanol samples were analyzed subsequently, and finally, the results of both studies were compared.

2.5.3. GC-FID Analysis

Bioethanol concentration was detected by a gas chromatogram (GC), equipment model Shimadzu GC-2014AOC-20 I Autoinjector. The equipment was configured with an FID detector, temperature 28 °C, the column employed was HP-innowax, the column length was 30 m, the column thickness was 0.25 um, and the internal diameter was 0.25 mm. The conditions for the instrument were adjusted as an injector temperature of 220 °C, a split ratio of 50, a column flow of 75 mL/min, and a column temperature of 50 °C for 2 min. The ramp temperature was 10 °C per min 100 °C, and the total run time was 7 min each.

For the sample preparation to run on GC-FID, 100 µL of the sample was collected and centrifuged for 10 min. The clear brown supernatant was collected and added in equal quantities of 300 µL 1-propanol, 300 µL acetonitrile, and 300 µL isobutanol to form a total volume of 1000 µL. It was again centrifuged for 3 min and analyzed through GC-FID using the standard ASTM D5501 [15]. These chemicals were added to separate the layer of water from organics that would otherwise inhibit the instrument's efficiency or might corrode it. The same methodology was undertaken for all parameters and every sample of different strains.

3. Results and Discussion

3.1. Reducing Sugar Analysis

The results of reducing sugar concentrations by DNS test for various samples are presented in Figure 3. The overall production of reducing sugars was highest for SC. Whereas PP provided higher values than co-culture for up to 48 h, a reversal in the trend was observed after this point which continued up to 110 h. The decrease in the concentration of reduced sugar with the increase in the time indicated that the sugar was being converted into some other products. In the co-culture strain, both SC and PP were present, which would have developed some synergistic relation, leading to the accumulation of the reducing sugar. Figure 3 depicts the result of reducing sugar for all yeast strains.

3.2. Results of FTIR Analysis

FTIR analysis was used to identify the functional group that played a crucial role in fermentation. The results of the FTIR analysis for the onion extract and the samples after fermentation are shown in Figure 4. The first broad peak in Figure 4a shows the O-H group. The other peaks observed in the onion extract spectra are the C=C stretching bond of alkynes molecules, ketones, alkyl amines, halogen compounds (C-Cl), and halogen compounds (C-I), respectively. Similarly, Figure 4b showed a peak of the O-H group at 3319 cm^{-1}. As marked on FTIR spectra, the other groups showed C-N, carbonyl compounds, alkyl amine, C-O (O-H), aromatic compounds, and =CH2, respectively. The spectra of the fermented

sample by PP (Figure 4c) represented the O-H group, C-N, carbonyl compounds, alkyl amine, C-O (O-H), aromatic compounds, =CH2, alkene, and aromatics, respectively.

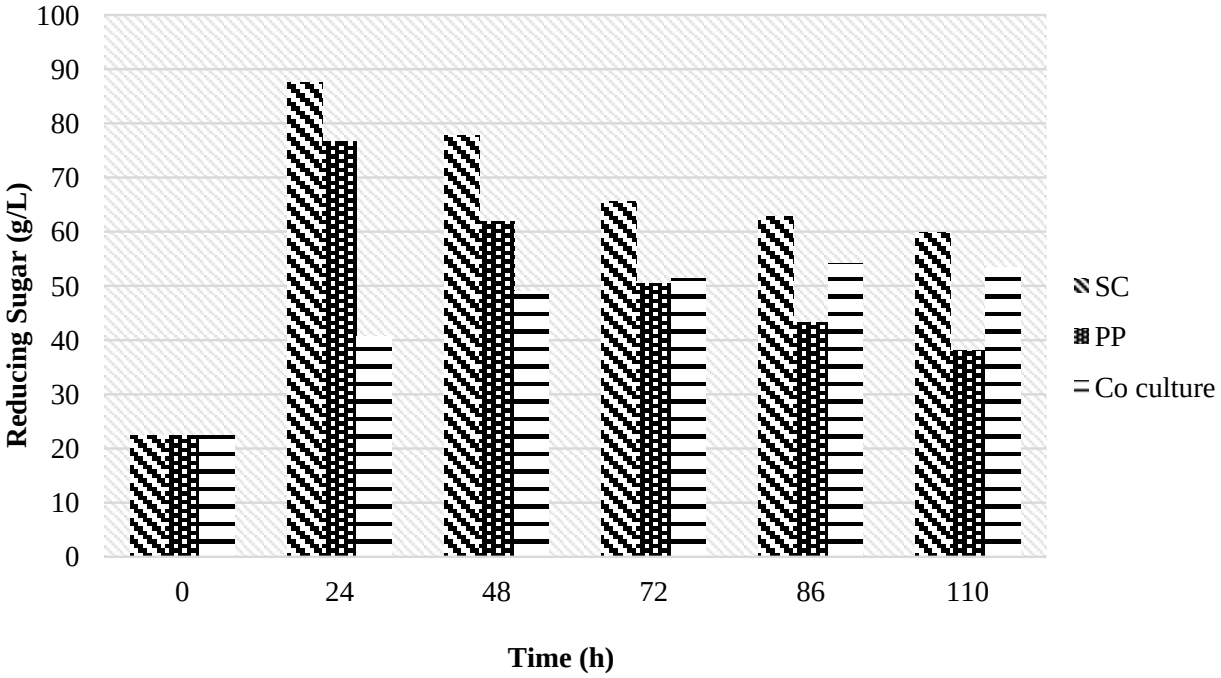

Figure 3. Reducing sugar for all yeast strains.

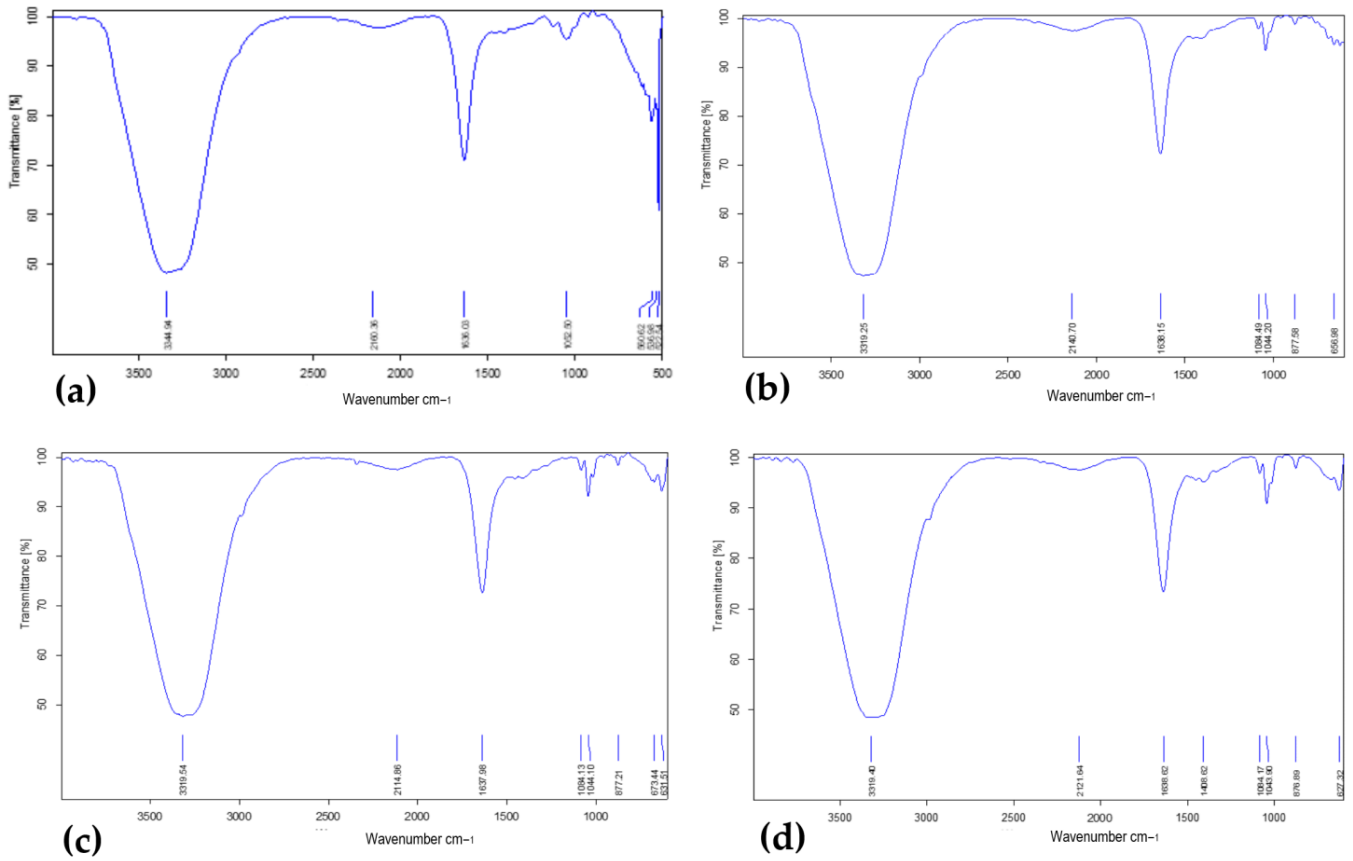

Figure 4. FTIR spectra of (**a**) Onion extract. (**b**) Bioethanol from *S. cerevisiae.* (**c**) Bioethanol from *P. pastoris.* (**d**) Bioethanol from Co-culture strain.

Likewise, the fermented sample of coculture in Figure 4d represents the presence of O-H stretching of the bonded and non-bonded hydroxyl groups. The range of 2500–2000 shows C-H stretching (Aliphatic methylene); 2000–1500 shows O-H bonding, conjugated C-O stretching, and absorbed H_2O carboxylates. The aromatic ring (Lignin) falls in the range of the 1500–1000 wavenumber. Briefly, <1000 Shows C-O-C stretching (Amorphous cellulose). Furthermore, 650–1000 wavenumber represents aromatics C-H [25]. The common functional group in all samples was the hydroxyl group (O-H), which signified the presence of ethanol in all samples. The presence of halogens was observed in *the P. pastoris* and co-culture fermented samples. Whereas alkyl amines were identified in samples of *S. cerevisiae* and *P. pastoris*, as well as the onion extract, ketones were observed only in the onion extract. The ketones could have been converted during the process of fermentation.

3.3. Bioethanol Concentration

The bioethanol concentration was measured by GC-FID. Figure 5 shows a chromatogram of bioethanol obtained for a single sample. Similarly, all samples were analyzed through GC-FID analysis to measure the bioethanol concentration obtained for monoculture and co-culture fermentation at various conditions. The maximum bioethanol concentration obtained by all cultures is discussed in the following sections, and a comparison has been made with other results.

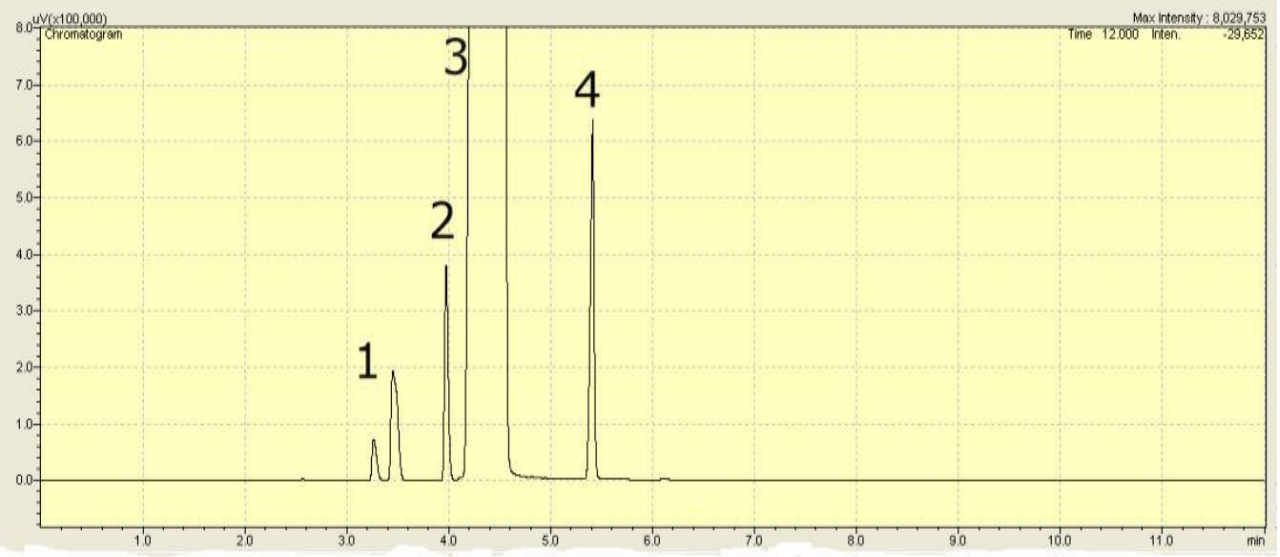

Figure 5. A sample chromatogram of GC-FID analysis with peaks; (1). Bioethanol. (2). Acetonitrile. (3). 1−propanol. (4). Butanol.

3.3.1. Bioethanol Production Using *S. cerevisiae*

The bioethanol concentration produced at different time intervals in relation to pH and temperature is shown in Figure 6. As evident from Figure 6, the bioethanol concentration increases with time providing the maximum production at 86 h for 20 °C and 30 °C, whereas maximum output for 40 °C was attained at 56 h. Afterwards, a decrease in bioethanol concentration was observed with an increase in time. The highest bioethanol concentration (30.56 g/L) was achieved by *S. cerevisiae* at 86 h at 30 °C.

The optimum time to obtain maximum bioethanol concentration was 86 h for all pH values, whereas the highest concentration (30.5 g/L) was attained at pH 5. Hence, the optimum time, temperature, and pH for fermentation with *S. cerevisiae* were 86 h, 30 °C, and pH 5, respectively. The effect of pH remains insignificant up to 60 h for fermentation at 30 °C, whereas it became greatly significant afterwards. The lowest bioethanol concentration (12 g/L) was attained at pH 6, whereas the highest concentration (30.5 g/L) was achieved at pH 5. *S. cerevisiae* grows best under acidic conditions, whereas higher pH may cause

chemical stress on the yeast and inhibit their growth, decreasing the overall fermentation rate. A similar trend of more bioethanol production at acidic pH of 5 was observed at temperatures of 20 °C and 40 °C. In previous studies, comparable bioethanol concentration was obtained from *S. cerevisiae*, i.e., 49.06 g/L using sunflower stalk as feedstock [27] and 35.5 g using watermelon waste as a feedstock [28].

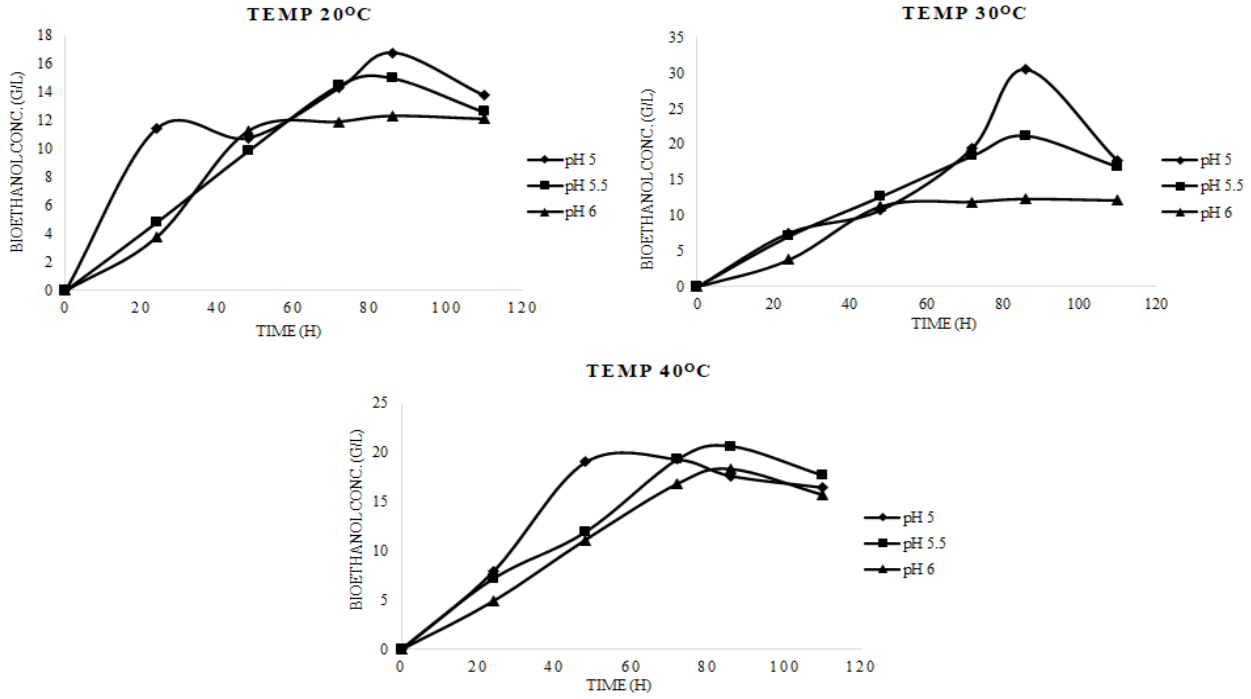

Figure 6. Effect of time, pH, and temperature on bioethanol production using *S. cerevisiae*.

3.3.2. Bioethanol Production Using *P. pastoris*

The bioethanol production by PP at different time intervals in relation to pH and temperature is shown in Figure 7. As is apparent from Figure 7, the optimum bioethanol production for 30 °C and 40 °C was attained at 72 h, whereas the optimum production for 20 °C was achieved at 20 h and remained nearly constant afterward. Similarly, the optimum time to obtain maximum bioethanol concentration was 72 h for all pH values, whereas the highest concentration (21.06 g/L) was obtained at pH 5. Hence, the optimum time, temperature, and pH for fermentation with *P. pastoris* are 72 h, 30 °C, and 5, respectively. The effect of pH was significant for fermentation at 20 °C and 30 °C, whereas it was not notable at 40 °C. At 20 °C, up to 20 h, the bioethanol production was high for pH 5 and 5.5. The effect of pH decreased afterwards, with the same trend that pH 5 attained maximum production (14 g/L) and least production (8 g/L) was achieved at pH 6. At a temperature of 30 °C, the effect of pH remained significant up to 70 h, where maximum yield was obtained at pH 5.5 (20 g/L), whereas the minimum was at pH 6 (13 g/L). The bioethanol production achieved in this study was higher than in previous research, in which 11.7 g/L was produced using wheat straw as feedstock [29]. *Pichia pastoris* had a maximum yield of 21.06 g/L bioethanol concentration with bioethanol productivity and energy of 0.264 g/L h and 631.8 kJ/L, respectively, after 72 h of fermentation at 30 °C and pH 5, as followed by the Thangavelu method.

According to the International Energy Agency, alongside other activities, the presented considerations align with Net Zero Emissions according to the 2050 scenario.

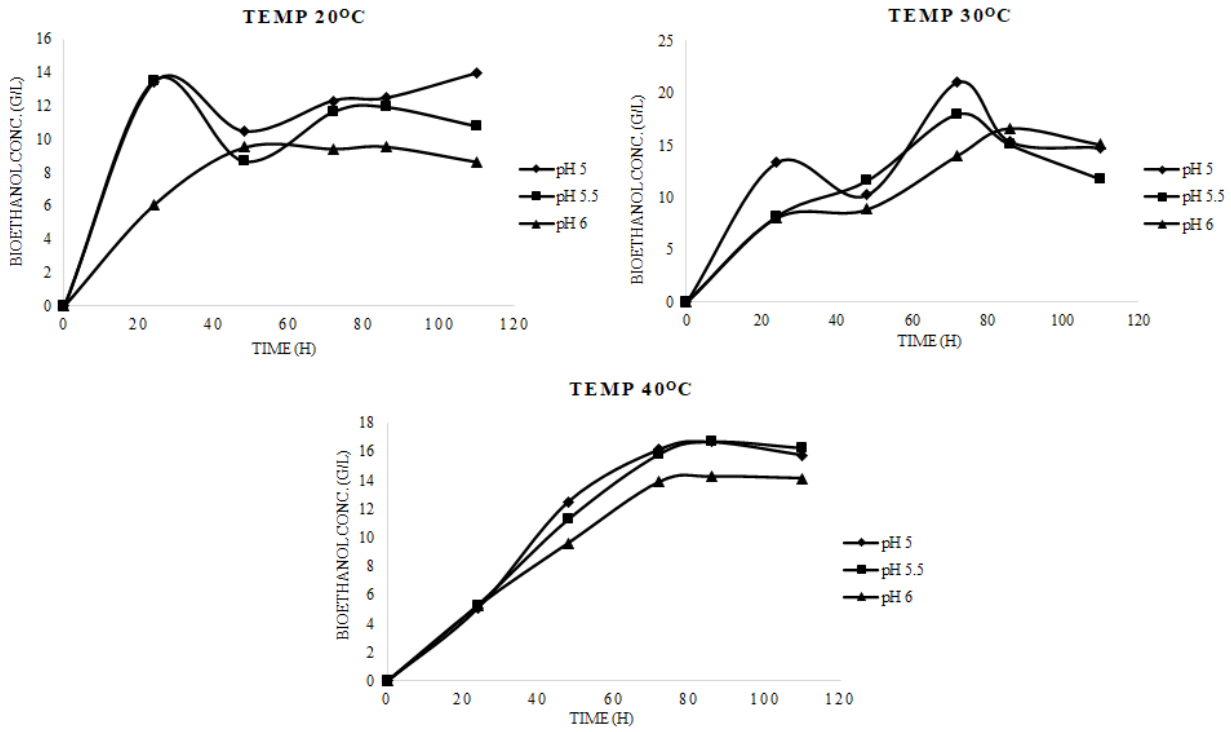

Figure 7. Effect of time, temperature, and pH on bioethanol production using *P. pastoris*.

3.3.3. Bioethanol Production Using Co-Culture Strains

Figure 8 demonstrates bioethanol production with co-culture fermentation as a function of time, temperature, and pH. The highest bioethanol production was observed at 86 h for 30 °C and 40 °C, whereas at 20 °C, the maximum concentration was achieved at 24 h, which showed slight variation afterward. The optimum time to obtain maximum bioethanol concentration was 86 h for all pH values, whereas the highest concentration (22.72 g/L) was attained at pH 5. Hence, the optimum time, temperature, and pH for fermentation with co-culture strain were 86 h, 30 °C, and pH 5, respectively. The effect of pH on bioethanol production is considerable for fermentation at 40 °C, whereas it becomes essential for fermentation at 30 °C. At 30 °C, better bioethanol production was obtained for pH than other pH values. A similar effect was observed during fermentation with *S. cerevisiae* (Figure 6); hence, the same effect may be attributed to the growth of *S. cerevisiae* at pH 5.

Previously, co-culture fermentation by *S. cerevisiae* and *P. pastoris* resulted in 13.3 g/L of bioethanol using rice straw as the feedstock (Mohapatra et al., 2020). Another bioethanol concentration obtained from *S. cerevisiae* and *P. stipitis* was 12 g/L with wheat straw as feedstock by co-culture fermentation [29]. Though co-culture strains in this study produced better results as compared to other feedstocks, the production was lesser than the individual strain of *S. cerevisiae.* It showed that yeast strains competed in co-culture fermentation and could not generate a higher yield of bioethanol synergistically. The coculture fermentation resulted in 22.72 g/L of bioethanol concentration with bioethanol productivity and energy of 0.264 g/L h and 681.6 kJ/L, respectively, after 86 h of fermentation at 30 °C and pH 5.

The presented considerations, similar to the other activities [30–33], are in line with the actions toward the Net Zero Emissions by 2050 scenario listed by the International Energy Agency [34].

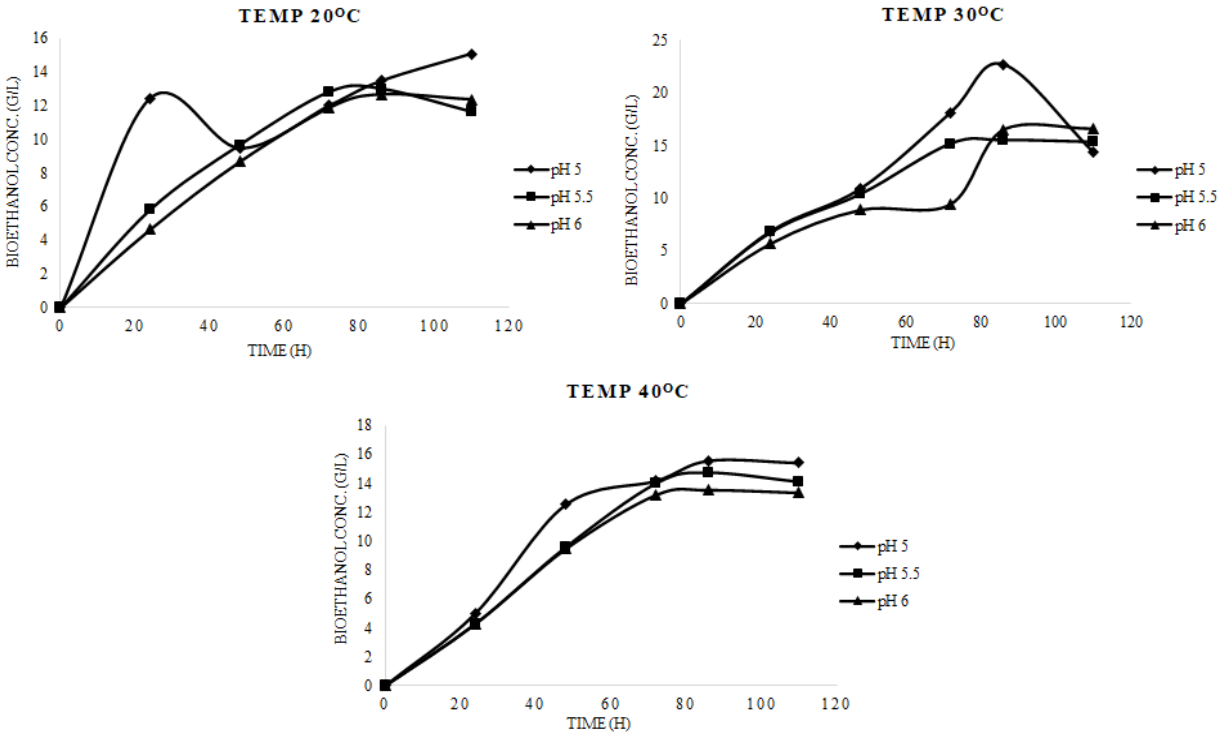

Figure 8. Effect of time, temperature, and pH on bioethanol production using co-culture strain.

4. Conclusions

The bioethanol production using *S. cerevisiae* was observed to be better than *P. pastoris* and co-culture fermentation. The optimum yield of S. *cerevisiae*, *P. pastoris*, and co-culture fermentation was 30.56 g/L, 21.06 g/L, and 22.72 g/L, attained at 86 h, 72 h, and 86 h, respectively. The optimum pH of 5 and 30 °C temperature produced the highest yield. The efficiency of *P. pastoris* and co-culture was found to be almost similar while still being far less than that of *S. cerevisiae*. The strains competed in co-culture and failed to produce a synergistic effect to improve efficiency. The bioethanol production, determined through ASTM D5501, was appreciable compared to similar past studies. Hence, the strains used, the process, and onion waste as the feedstock showed good potential for bioethanol production. The efficient conversion of cellulose and hemicellulose simultaneously into fermentable sugars and bioethanol was obtained from co-fermentation using two different yeast strains. Xylan using *P. pastoris specifically* improved the lignocellulosic utilization by degrading the xylan. The present study may provide a foundation for direct bioethanol production from cellulose and hemicellulose without incorporating additional enzymes. The energy potential of discarded onions (by *S. cerevisiae* at optimal conditions) is calculated to be 916 kJ/L which shows that discarded onions feedstock is a good alternate feedstock for bioethanol production.

Optimization studies of the proposed bioethanol production can be performed in the future, e.g., via a fuzzy logic-based approach [35–37], which belongs to the main artificial intelligence methods.

Author Contributions: Conceptualization: I.S.; Methodology: I.S. and G.H.; investigation: I.S., M.U.F. and M.U.; data analysis: M.A., G.H. and M.U.; original draft preparation, G.H., M.A., M.U.F., M.U. and J.K.; review and editing, Y.F. and J.K.; Data Curation: Y.F.; Visualization: Y.F. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflict of interest.

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