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Comparative Analysis of Cellulosic Ethanol Production from Lignocellulosic Substrate *Moringa oleifera* Using *Kluyveromyces marxianus* and *Zymomonas mobilis*

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Abstract: In the current investigation, the woody stem of *Moringa oleifera* was processed by chipping and milling and was subsequently exposed to a combination of pretreatments involving a 3% v/v solution of nitric acid and autohydrolysis. The simultaneous saccharification and fermentation (SSF) of the pretreated hydrolysate of *M. oleifera* was conducted using *Zymomonas mobilis* and *Kluyveromyces marxianus* in occurrence of commercial cellulase enzyme, Tween 80, and sodium azide. The fermentation process parameters for *Z. mobilis* were optimized individually, including a substrate concentration of 5% (w/v), concentration of inoculum 5% (v/v), pH 5.4, and temperature 34 °C. Similarly, for *K. marxianus*, the process parameters were optimized individually, with a substrate concentration of 5% (w/v), an inoculum concentration of 3% (v/v), a pH of 5.1, and a temperature of 41 °C. The highest cellulosic ethanol concentration was achieved by the micro-organism *K. marxianus* after a fermentation period of 96 h.

Keywords: thermotolerant yeast; cellulase; *Moringa oleifera*; cellulosic ethanol; Simultaneous Saccharification and Fermentation

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1. Introduction

The depletion of fossil fuels is increasing at an alarming rate, which is an unsustainable situation that is prevailing around the world. Nearly 87% of the world's energy production is solely dependent on fossil fuel resources. The drastic use of fossil fuels will eventually exhaust our supply of non-renewable resources. In order to maintain the projected growth of fuel consumption, scientists are investigating various renewable sources that have the capacity to create maximum-yield biofuels. Biomass seems to be the primary resource available in the production of biofuels, which is presumed to be an important factor in developing countries like India. Biofuels are crucial in the effort to decrease carbon emissions in the transportation sector and represent an excellent option for mitigating the impact of climate change [1–4].

Lignocellulosic biomasses are complex polymers that constitute cellulose, hemicellulose, and lignin. Softwood consists of galactoglucomannan, glucomannan, and arabino glucuronoxylan as hemicelluloses, whereas hardwood contains xylan as the main ingredient. The structure of lignocellulosic material varies from one plant biomass to another.

Cellulosic ethanol from lignocellulosic biomasses is obtained by three major methods: pretreatment to remove lignin content and expose cellulose and hemicellulose for hydrolysis, enzymatic hydrolysis, fermentation, and distillation [5,6]. Though lignocellulosic materials are considered to be a promising alternate renewable resource (to conventional fossil fuels), their complex rigid structure poses challenges to researchers in converting the pentose and hexose sugars to biofuels [7].

Ethanol production, when derived from lignocellulosic biomass, is widely considered an important process due to the alarming rates of ecological and energy crises [8]. Cellulosic ethanol production is mainly obtained from sugar and starch-based raw materials. From a projected view, this is not sustainable, accounting for the enhanced global population food supply. It possesses a high octane number due to the existence of a 35% oxygen content, increased heat of vaporization, engine efficacy, and lower levels of NOx emissions [9,10]. Lignocellulosic biomasses have been processed to produce cellulosic ethanol using sulfuric, hydrochloric, nitric, and phosphoric acids. Acid treatment increases the hydrolysis of hemicelluloses and celluloses, thus yielding the monomers of hemicelluloses in the liquid fraction and consumable celluloses (by enzymes) in the solid fractions. Acid pretreatment can be clubbed together with hydrothermal treatment to obtain a high cellulosic ethanol production yield [11]. Nitric acid was used in the pretreatment of rye straw, which produced glucose and cellulosic ethanol at 324 g/Kg and 96.6 g/Kg, respectively [12]. The fermentation of corn stover was carried out in the presence of nitric acid treatment (0.6% w/w), which yielded 18.58% glucose at 180 °C for 10 min [13]. Studying rice straw xylose synthesis with 0.65 v/v nitric acid pretreatment yielded 86.5% pentose sugar [14]. Acid treatment of lignocellulosic biomass uses sulfuric, hydrochloric, and nitric acids. Unlike nitric acid, sulfuric acid is cheaply available, but it is highly toxic, and hydrochloric acid is highly corrosive and requires corrosive-resistant materials [15].

Horse radish tree, or *Moringa oleifera* Lam., is a widely grown species of the Moringaceae. *Moringa* genus, which consists of 13 species. *M. oleifera* is a slender, softwood tree that branches easily and is a tremendously fast-growing plant. This plant is a native plantation to the South Asian region, which grows from the Himalayan foothills to the northern part of West Bengal. Since the wood is moderately soft in nature, it is not used for construction purposes, and the tree is vulnerable to being disrupted during windy seasons. *M. oleifera*, as a profitable crop, is grown comprehensively in the Indian subcontinent. It is grown in tropical and subtropical regions, especially as a backyard tree for the consumption of its leaves and drumstick pod [16]. The lignocellulosic content of *M. oleifera* was found to be 43% cellulose, 20% hemicellulose, 27% lignin, 2% ash, 2% extractives, and 5% others [17]. Ultrasonication is a physiochemical treatment process that harnesses energy. High-frequency ultrasound proves effective in preparing biomass for further processing. Additionally, utilizing low-intensity sonication shows promise in improving the conversion of fermentable sugars into cellulosic ethanol during fermentation [18].

The hydrolysis of lignocellulosic hydrolysate in the presence of an enzyme converts cellulose to hexoses and hemicellulose into pentoses (xylose and arabinose). These enzymes are highly specific in nature and usually carry out this process under mild conditions (pH 4.8–5.0; temperature of 45–50 °C). Three primary enzyme groups of cellulases involved in the hydrolysis are Endoglucanases, Exoglucanases or cellobiohyrolases and β-glucosidases [19].

One bioreactor unit may perform hydrolysis and fermentation during simultaneous saccharification and fermentation (SSF) to manufacture ethanol from cellulosic materials. The monomer generated during enzymatic hydrolysis is immediately consumed by the fermenting micro-organism present in the culture. When compared to separate hydrolysis and fermentation (SHF), SSF provides high yields of cellulosic ethanol with a minimum quantity of enzyme used. Once cellulosic ethanol is produced, due to its presence in the bioreactor, the risk of contamination is totally removed. In the case of SSF, the optimum conditions for enzymatic hydrolysis and fermentation such as pH and temperature would be close enough when thermos-tolerant bacteria and yeast are implemented [20]. SSF

possesses ample advantages, like maintaining a low sugar concentration, thereby reduced the risk of substrate inhibition; the temperature for the fermentation of ethanol is lower compared to the saccharification process, which aids in proper enzymatic hydrolysis and reduces the contamination of risk during cellulosic ethanol fermentation [21].

Zymomonas mobilis is gifted in fermenting hexose sugars via the Entner–Doudoroff pathway, providing minimum energy gains due to the reduced biomass yield, and this is recognized for its capacity to produce cellulosic ethanol rapidly and effectively from pre-treated hydrolysate with low byproduct formation. *Z. mobilis* cannot ferment pentose sugars, like arabinose and xylose, but converts glucose, fructose, and sucrose into cellulosic ethanol. Recently, scientists from NREL have developed a *Z. mobilis* strain that was found to convert pentose sugars to cellulosic ethanol. *Z. mobilis* is resistant to osmotic pressure and ethanol concentration and has evidenced increased glucose uptake and ethanol production [22,23]. *Kluyveromyces marxianus* is utilized to convert monosaccharides into cellulosic ethanol since its operating conditions are compatible with that of enzymatic hydrolysis. The application of *K. marxianus* is widely observed due to its thermal resistance, enhanced growth rate, and ability to metabolize a wide range of sugars, and it is tolerant towards high ethanol and inhibitor concentrations. A heat-resistant strain of *K. marxianus* OFF1 permits fermentation from 40 °C to 50 °C, which is considered a more effective strain in the SSF process. It has an enhanced metabolic rate and quickly produces cellulosic ethanol [24–29].

The present work focuses on cellulosic ethanol production from the woody stem of *M. oleifera*, which was chipped and milled and later subjected to combined pretreatments, followed by a sonication process. The hydrolysate of *M. oleifera*, along with the commercial cellulase enzyme Tween 80 and sodium azide, was subjected to SSF in the presence of *Z. mobilis* and *K. marxianus*, separately. The process parameters, like pH, substrate concentration, temperature, and inoculum concentration, were optimized individually for *Z. mobilis* and *K. marxianus.*

2. Materials and Methodology

2.1. Woody Stem Moringa oleifera

The substrate was dried and grounded for a particle size of 1–2 mm. This improves the surface area of the material for the saccharification process. Further, the substrate was rinsed using raw water, dried at 60 °C overnight and stored until further use.

2.2. Pretreatment Methodologies

2.2.1. Sequential Treatment of Biomass

The lignocellulosic biomass was autoclaved at 15 psi for 1 h at at 121 °C and then dried at 60 °C for 24 h. The auto-hydrolyzed material was subjected to acid saccharification process. A 10% (*w/v*) substrate was placed in a 250 mL Erlenmeyer flask and subjected to individual chemical pretreatments at various temperature levels using a heating mantle. Sulfuric acid, nitric acid, and hydrochloric acid at 3% (v/v) were each used at a temperature range of 60 °C, 70 °C, 80 °C, and 90 °C for 1 h. The mixture was passed through a muslin cloth to separate the contents, and the resulting hydrolysate underwent two rinses with distilled water before being ultimately neutralized. The leftover biomass was dried and ultrasonicated.

2.2.2. Ultrasonication

A sample at a substrate concentration of 10% (w/v) was subjected to ultrasound treatment (individually) via a sonicator (processor: 750 W, power 500 W, VCX series) at 40 kHz and temperature at 60 °C for 5 min [30].

2.2.3. Steam Coupled with Acid Hydrolysis Followed by Ultrasonication: Combination Treatment Method

After treating the lignocellulosic biomass with 3% v/v nitric acid at a solid-to-liquid ratio of 10%, it was autoclaved at 121 °C and 15 psi for 1 h. The mixture was strained

through muslin cloth, and the hydrolysate, which had undergone pretreatment, was thoroughly washed and rinsed multiple times with distilled water. To achieve a neutral pH, an inorganic solvent was used. Afterward, the pretreated biomass was dried and subjected to ultrasonication. For the steam-assisted acid-hydrolyzed sample, it was transferred to a beaker, and ultrasonic treatment was administered at a frequency of 40 KHz, maintaining a temperature of 60 °C for a duration of 5 min.

2.3. Micro-organism and Culture Conditions

Zymomonas mobilis subcultures were grown at 35 °C in a pH-6.0 agar slant medium with 20 gm of glucose, 10 gm of yeast extract, 2 gm of potassium hydrogen phosphate, 1 L of distilled water, and 15 gm of agar. At pH 6.0 and 35 °C, glucose (4 g/100 mL), yeast extract (2 g/100 mL), and KH_2PO_4 (0.4 g/100 mL) were used to make the inoculum. While maintaining the broth culture, the 0.6–0.8 OD at 610 nm was monitored [31].

Kluyveromyces marxianus was propagated on agar slants made from glucose (10 g/L), yeast extract (3 g/L), malt extract (3 g/L), peptone (5 g/L), agar (20 g/L), and distilled water (1 L). We maintained pH 7.0 and 25 °C. Inoculum was made using yeast extract, peptone, KH_2PO_4, pH 7.0, 25 °C (*w/v*), and glucose. The best broth culture OD was 0.6–0.8 at 680 nm.

2.4. Enzyme and Surfactant

Tween 80, sodium azide, and commercial *Trichoderma reesei* (ATCC 26921) cellulase was purchased from HIMEDIA lab, Mumbai, India. The enzyme activity was estimated using filter paper assay and expressed in filter paper units, FPU [32].

2.5. Simultaneous Saccharification and Fermentation

SSF was conducted in a 250 mL shake flask with a 50 mL working volume under aerobic conditions. The flask contained pre-treated substrate and 0.05 M citrate phosphate buffer (50 mmol/L, pH 5). Subsequently, the mixture underwent autoclaving at 121 °C and 15 psi for a duration of 20 min, followed by a cooling process. Additionally, commercial cellulase at a concentration of 12 FPU/g of substrate and 1 mL of inoculum were added, and the process was carried out in a controlled environment with an agitation speed of 120 rpm. To aid enzymatic action and prevent microbial contamination, Tween 80.0 (1% *v/v*) and sodium azide (0.005%) were included. Samples were periodically withdrawn from the SSF media (at intervals of 12 h up to 96 h), followed by centrifugation at 10,000 rpm for 10 min. The concentrations of reducing sugar and cellulosic ethanol in the supernatant were determined using the DNSA method and gas chromatography (GC), respectively. The pellet was dried, and its dry cell weight (DCW) was measured as the biomass concentration. This experimental approach was employed while varying pH, substrate concentration, temperature, and inoculum volume to determine the optimal conditions for cellulosic ethanol production.

2.6. Analytical Methods

The composition of the components in the woody stem of *M. oleifera* from the sequential pretreatment (3% (*v*/*v*) nitric acid) and combination of pretreatment was analyzed. Cellulose, hemicellulose, lignin, ash, as well as hexose and pentose sugars were analyzed and quantified. The pretreated hydrolysate sample was subjected to HPLC using a refractive index detector and a Thermofisher Dionex CarboPac column, eluted with 0.01 M sodium hydroxide at a flow rate of 0.5 mL/min. The reducing sugars concentration was determined by di-nitro salicylic acid (DNSA) method [33]. The concentration of total carbohydrates present in the pretreated hydrolysate after sequential pretreatment was estimated via the Anthrone method [34].

To determine the biomass concentration, a 5 mL culture sample was taken and centrifuged at 10,000× g for 10 minutes. The resulting supernatant was used for estimating reducing sugar and cellulosic ethanol, while the pellet collected at the bottom of the Ep-

pendorf tube was dried at 70 °C until a constant mass was achieved. The concentration of cellulosic ethanol was assessed using the conventional potassium dichromate method [35] and a gas chromatography instrument. The specifications include Clarus 500 gas chromatograph equipped with an elite-wax column (30.0 m × 0.25 mm) at an oven temperature of 85 °C and a flame ionization detector (FID) set at 200 °C (Perkin Elmer MA, United States). Ethanol standards were created utilizing commercially available ethanol of high quality (Merck, Darmstadt, Germany). As a carrier gas, nitrogen was used at a flow rate of 0.5 mL/min. The second-stage distilled sample obtained (woody stem of *M. oleifera)* from the SSF process under a controlled environment was estimated using GC-MS and GC-FID, which gives the retention time (min) and concentration of cellulosic ethanol (%*v*/*v*), respectively.

3. Results

3.1. Pretreatment of Woody Stem Moringa oleifera

The lignocellulosic stem of *M. oleifera* was subjected to different pretreatment processes. As the temperature increased (60–90 °C) during acid pretreatment, mild charring was observed in the case of nitric acid and hydrochloric acid, but this was found to be heavier for the sulfuric acid pretreatment. The total carbohydrate content (63%) was maximized with minimal charring in the nitric acid pretreatment at 70 °C [36].

3.2. Biochemical Composition of Woody Stem Moringa oleifera

The holocellulose, lignin, and ash of the raw substrate were found to be 57 ± 0.16%, 25 ± 0.09%, and 0.29 ± 0.08%, respectively. After steam, nitric acid, and sonication, *M. oleifera*'s woody stem provided more cellulose (43 0.8%), hemicellulose (21 0.4%), and less lignin (19 0.50%) (Table 1).

Table 1. Composition of the woody stem of *M. oleifera* prior to and following pretreatment.

Sample	Weight (gm)	Cellulose	Hemicellulose	Lignin	Glucose	Fructose	Xylose	Arabinose	Ash
1	50	38 ± 0.1	19 ± 0.06	25 ± 0.09	0.8 ± 0.12	2 ± 0.92	9 ± 0.26	1 ± 0.9	0.29 ± 0.08
2	46.5	41 ± 0.6	23 ± 0.1	21 ± 0.18	1 ± 0.13	2 ± 0.10	10 ± 0.26	1 ± 0.00	0.21 ± 0.83
3	38.5	40 ± 0.5	22 ± 0.08	20 ± 0.75	1 ± 0.75	2 ± 0.03	11 ± 0.33	1 ± 0.25	0.22 ± 0.69
4	37	41 ± 0.9	22 ± 0.09	19 ± 0.36	2 ± 0.28	2 ± 0.07	12 ± 0.90	1 ± 0.11	0.22 ± 0.26
5	44	41 ± 0.6	18 ± 0.07	19 ± 0.85	2 ± 0.62	2 ± 0.18	11 ± 0.80	1 ± 0.03	0.22 ± 0.74
6	43	43 ± 0.8	21 ± 0.4	19 ± 0.50	3 ± 0.02	2 ± 0.33	11 ± 0.41	1 ± 0.02	0.03 ± 0.00

1: Raw sample, 2: Auto hydrolyzed, 3: Auto-hydrolyzed sample placed on a heating mantle at a temperature of 70 °C, utilizing a 3% (*v*/*v*) nitric acid solution, 4: Auto-hydrolyzed sample subjected to sonication being maintained on a heating mantle at 70 °C with the use of a 3% (*v*/*v*) nitric acid solution, 5: Combination of autohydrolysis with 3% (*v*/*v*) nitric acid in an autoclave, 6: Combination of autohydrolysis and a 3% (*v*/*v*) nitric acid sample that was sonicated. Wood composition (g/100 g wood in oven-dry basis ± standard deviation).

3.3. SSF Using Symmons mobilis

3.3.1. Effect of Substrate Concentration

The pretreated woody stem of *M. oleifera* was subjected to SSF in the presence of *Z. mobilis* with different substrate concentrations (1.0–7.0% *w*/*v*). The substrate was slowly consumed in the early phases of the process. After 12 h, a consistent utilization of substrate was observed further beyond 24 h of fermentation, propagation of organism steadily increased. For a substrate concentration of 5.0% (*w*/*v*) with an incubation period of 48 h, 60% (11.45 g/L) of the reducing sugar was converted to 8.25 g/L cellulosic ethanol alongwith a biomass concentration of 5.35 g/L. Figure 1 represents the effect of substrate concentration on the production of cellulosic ethanol.

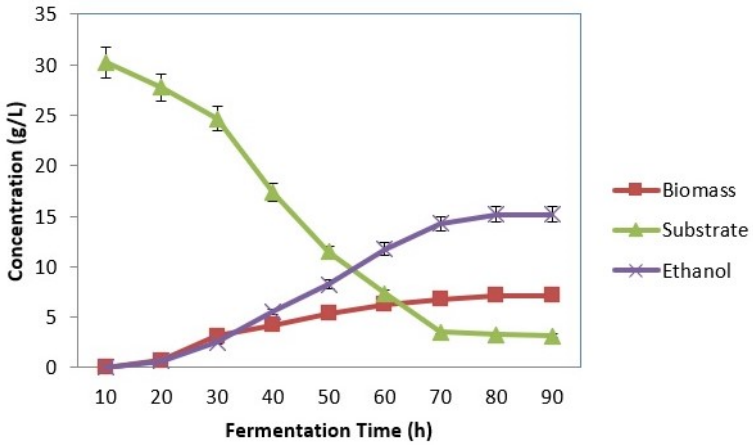

Figure 1. The effect of substrate concentration on the production of cellulosic ethanol.

3.3.2. Effect of Inoculum Concentration

The combined auto-hydrolyzed and acid-treated woody stem *M. oleifera* was exposed to SSF using the cellulase enzyme and the Gram-negative bacterium *Z. mobilis* with varying volumes of inoculum (1.0–7.0% *v*/*v*). For an inoculum concentration of 5.0%, the biomass concentration propagated after 12 h and reached a highest of 6.55 g/L after 60 h of incubation, with an ethanol composition of 11.25 g/L. The highest cellulosic ethanol (15.2 g/L) was obtained at 96 h of fermentation with an inoculum concentration (5% *v*/*v*), substrate concentration (3.15 g/L) and biomass concentration (7 g/L). Figure 2 represents the effect of inoculum concentration on the production of cellulosic ethanol.

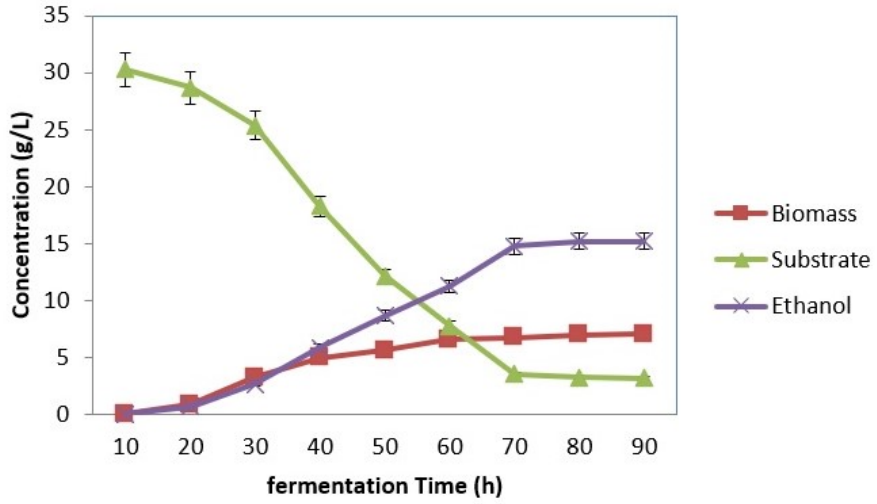

Figure 2. The effect of inoculum concentration on the production of cellulosic ethanol.

3.3.3. Effect of pH

The acid hydrolysate of woody stem *M. oleifera* was subjected to SSF using cellulase and the facultative anaerobe *Z. mobilis*, which was performed with varying pH values (5.0–5.8). Initially, the growth and development were almost the same until after 12 h when propagation started to rise steadily for pH 5.4 and yielded a biomass concentration and cellulosic ethanol concentration of 5.15 g/L and 8.75 g/L, respectively, at the end of 48 h with 55% of the substrate being utilized completely. The maximum cellulosic ethanol concentration was 15.15 g/L for a pH of 5.4, which was obtained after 96 h with a substrate concentration and biomass concentration of 6.45 g/L and 6.75 g/L, respectively. Figure 3 represents the effect of pH on the production of cellulosic ethanol.

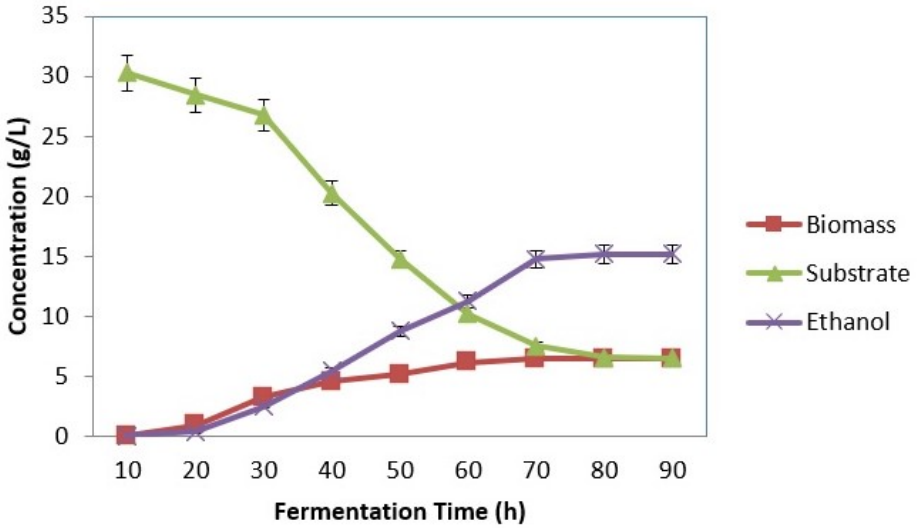

Figure 3. The effect of pH on the production of cellulosic ethanol.

3.3.4. Effect of Temperature

The delignified woody stem of *M. oleifera* was exposed to the obligate fermentative bacterium *Z. mobilis* in the presence of cellulase at various temperatures (30–39 °C). At the end of 40 h, the concentration of the substrate became 50% (15.35 g/L) and the corresponding biomass and cellulosic ethanol concentration were found to be 5.05 and 6.75 g/L, respectively. The maximum cellulosic ethanol concentration was determined to be 15.2 g/L for a temperature of 34 °C, with a substrate concentration and biomass concentration of 4.65 g/L and 7.25 g/L, respectively, at 96 h. The production of ethanol increased from 30 °C (12.15 g/L) and reached a maximum of 34 °C. Further increasing the temperature did not show any significant increase in cellulosic ethanol concentration. Figure 4 shows the effect of temperature on the production of cellulosic ethanol.

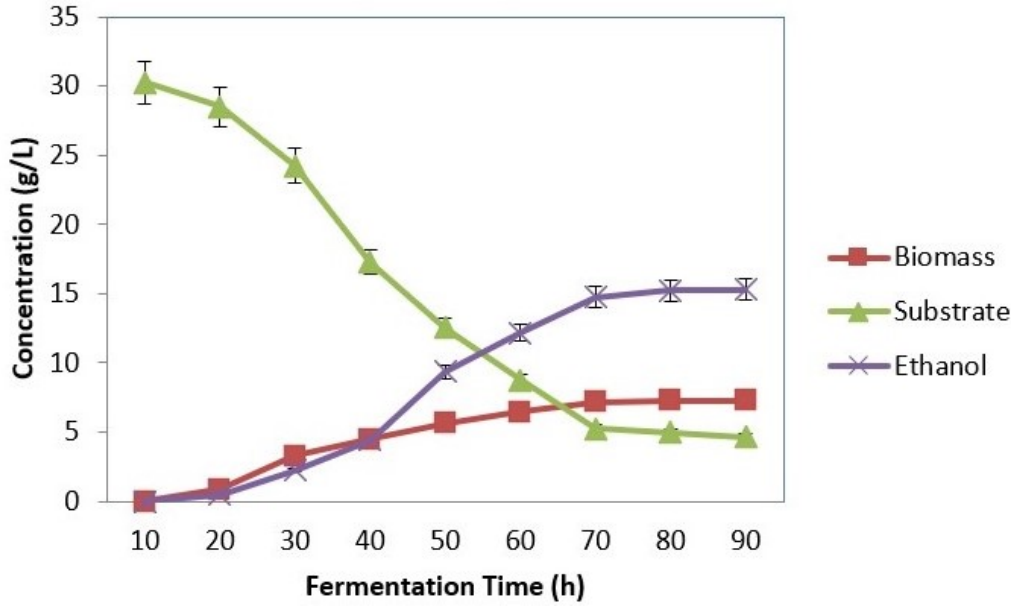

Figure 4. The effect of temperature on the production of cellulosic ethanol.

3.4. SSF Using Kluyveromyces marxianus (MTCC 1389)

3.4.1. Effect of Substrate Concentration

The cellulosic hydrolysate from *M. oleifera*'s woody stem underwent a SSF process in the presence of cellulase and *K. marxianus*, with varying substrate concentrations ranging

from 1% to 7% (*w/v*). Initially, sugar consumption was low, but beyond 12 h, it significantly decreased due to microbial growth. After 20 h, sugar consumption started to rise steadily. For a substrate concentration of 5.0% (*w/v*) and a fermentation time of 48 h, 60% (13.75 g/L) of reducing sugars were converted into 12.25 g/L of cellulosic ethanol, accompanied by a biomass concentration of 6.45 g/L. The highest concentration of cellulosic ethanol (18.15 g/L) was achieved after 96 h of fermentation, with residual substrate and biomass concentrations of 3.75 g/L and 9.15 g/L, respectively. Figure 5 represents the impact of substrate concentration on cellulosic ethanol production.

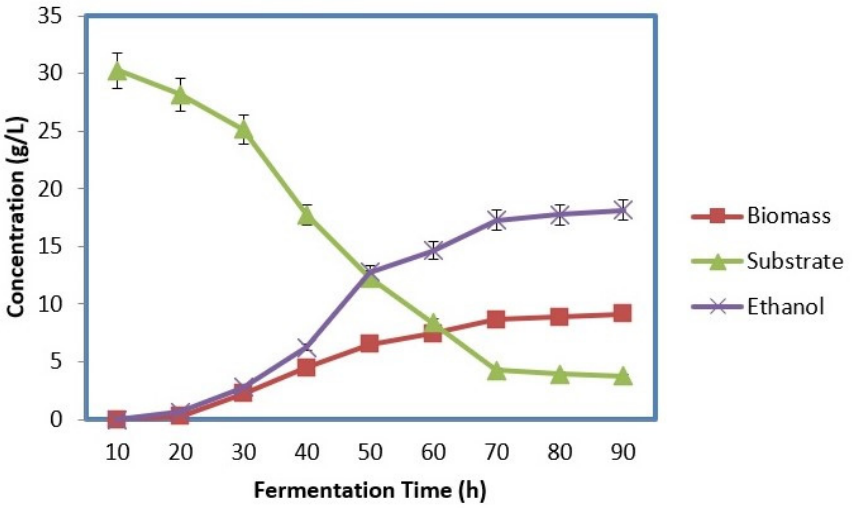

Figure 5. The impact of substrate concentration on cellulosic ethanol production.

3.4.2. Effect of Inoculum Concentration

Various inoculum concentrations ranging from 1% to 5% (*v/v*) were tested to assess their impact on the pretreated lignocellulosic woody stem of M. oleifera in the presence of *K. marxianus* and cellulase. The utilization of fermentable sugars played a crucial role in enhancing biomass production. Beyond the 36 h mark, the organism demonstrated rapid consumption of the medium, leading to increased biomass and ethanol production as it metabolized the resources efficiently. Optimal results were observed with an inoculum concentration of 3% (*v/v*) after 48 h of fermentation, where cellulosic ethanol and microbial concentrations reached 70% of the total concentration, amounting to 12.75 g/L and 6.35 g/L, respectively. Figure 6 illustrates the impact of inoculum concentration on cellulosic ethanol production.

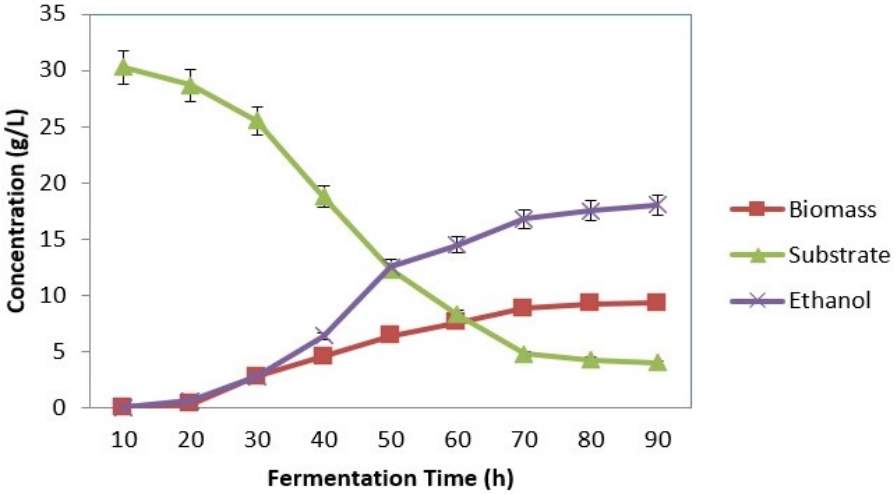

Figure 6. The impact of inoculum concentration on cellulosic ethanol production.

3.4.3. Effect of pH

The pH level was adjusted within the range of 4.5 to 5.5 while keeping the substrate concentration fixed at 5% (*w*/*v*) and the inoculum concentration at 3% (*v*/*v*). Cellulosic ethanol production showed steady improvement beyond 24 h, ultimately reaching a concentration of 70% after 60 h of fermentation at an optimal pH of 5.1. Under these conditions, when the substrate concentration was approximately 30% (10.25 g/L) of the initial value, the product and biomass yields were 14.25 g/L and 7.65 g/L, respectively. Figure 7 illustrates the impact of pH on the production of cellulosic ethanol.

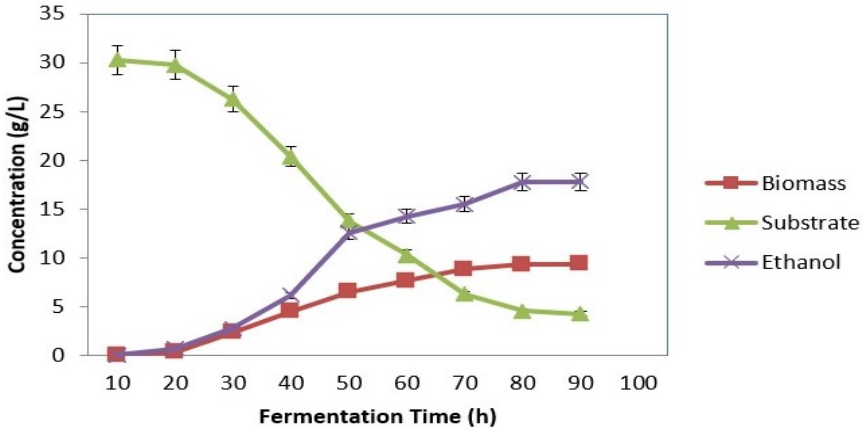

Figure 7. The impact of pH on the production of cellulosic ethanol.

3.4.4. Effect of Temperature

The delignified biomass obtained from M. oleifera was subjected to SSF in the presence of cellulase and K. marxianus. While keeping the other variables constant, the temperature was varied within the range of 33 to 43 °C. It was observed that both cellulosic ethanol and biomass concentrations increased significantly after the 24 h fermentation period. After 48 h, it was determined that the optimal temperature for maximum production was 41 °C, resulting in 65% (12.85 g/L and 6.85 g/L) of the total concentration. Figure 8 illustrates the impact of temperature on the production of cellulosic ethanol.

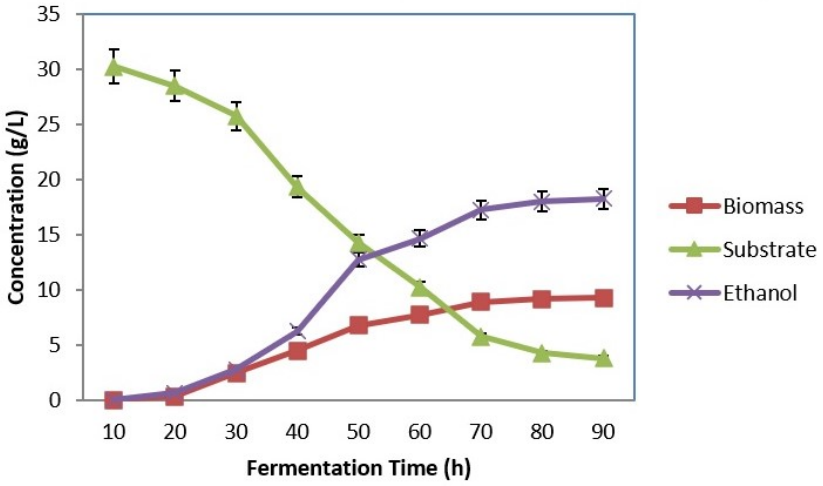

Figure 8. The impact of temperature on the production of cellulosic ethanol.

3.5. GC-MS & GC-FID Studies of Cellulosic Ethanol Production

3.5.1. Woody Stem of *Moringa oleifera* and *Zymomonas mobilis*

When subjected to SSF in the presence of *Z. mobilis* and cellulase at optimized values of substrate conc. 5% (*w*/*v*), inoculum conc. 5% (*v*/*v*), pH (5.4), and a temperature of 34 °C,

the woody stem of *M. oleifera* yielded an ethanol conc. Of 15.2 g/L. The product cellulosic ethanol after second stage distillation was analyzed and validated. The percentage area obtained for a retention time of 7.028 min (peak 5) was found to be 100%. The concentration of cellulosic ethanol from GC-FID was found to be 20.43%.

3.5.2. Woody Stem *Moringa oleifera* and *Kluyveromyces marxianus*

When subjected to SSF in the presence of *K. marxianus* and cellulase at optimized values of substrate conc. 5% (*w*/*v*), inoculum conc. 3% (*v*/*v*), pH (5.1), and a temperature of 40 °C, the woody stem of *M. oleifera* yielded an ethanol conc. 18.05 g/L. The product cellulosic ethanol after second-stage distillation was analyzed and validated. The percentage area obtained for a retention time of 7.044 min (peak 5) was found to be 100%. The concentration of cellulosic ethanol from GC-FID was found to be 23.77%.

4. Discussion

4.1. SSF Using Zymomonas Mobilis (MTCC 2427)

4.1.1. Effect of Substrate Concentration

When 30.25 g/L of cellulosic hydrolysate (obtained from woody stem *Moringa oleifera*) was subjected to SSF in the presence of *Z. mobilis*, the maximum cellulosic ethanol obtained was 15.25 g/L after 96 h of combined treatment of auto-hydrolysis and 3% (*v*/*v*) nitric acid. This maximum concentration of cellulosic ethanol was obtained for a substrate concentration of 5% (*w*/*v*), with a substrate concentration and biomass concentration of 3.15 g/L and 7.15 g/L, respectively, after 96 h. When comparing to sulfuric acid, the application of nitric acid for pretreatment is better due to the presence of nitrogen that acts as a medium source during the fermentation process. When the substrate concentration was raised to 7% (*w*/*v*), no significant increase in cellulosic ethanol was observed. In ethanol production by *Z. mobilis* using head waste from sunflower for various substrate concentrations, it was found that at 5.0% (*w*/*v*), the maximum concentration of ethanol was found to be 18.56 g/L, with a reducing sugar concentration of 41.63 g/L, as obtained by the combined treatment of auto-hydrolysis and 0.2 N sulfuric acid [37].

4.1.2. Effect of Inoculum Concentration

The utilization of pretreated agave leaves with an inoculum concentration of 5% of *Zymomonas mobilis* yielded a maximum cellulosic ethanol concentration of 5% [38]. *Z. mobilis* with rice straw produced 10.02 ± 1.18 g/L of ethanol, whereas, with wheat straw, it yielded 9.70 ± 2.32 g/L, with an inoculum volume of 5% [39]. Above 5% (*v*/*v*) of inoculum concentration, there was no increase in concentration of cellulosic ethanol.

4.1.3. Effect of pH

After 96 hours, a substrate concentration of 6.45 g/L and a biomass concentration of 6.75 g/L produced 15.15 g/L cellulosic ethanol at pH 5.4. The presence of components, such as acetic acid (0.9 ± 0.10 g/100 g wood), furfural (0.05 ± 0.4 g/100 g wood), and hydroxyethyl furfural (0.07 ± 0.5 g/100 g wood) may have an inhibitory effect on the production of ethanol [40]. At a pH value of 5.5, the maximum cellulosic ethanol concentration obtained was found to be 14.36 g/100 mL when using waste potatoes as a substrate [41]. When the pH of the fermentation medium was increased above 5.4, the productivity of the micro-organism, with respect to this substrate, decreased. On the other hand, if the pH is reduced below 5.4, the micro-organism cannot sustain and grow, and, hence, the productivity is affected.

4.1.4. Effect of Temperature

Adding steam explosion pretreatment to SSF with *T. reesei* cellulase and *Zymomonas mobilis* generated 1.10 g/L reducing sugars, 0.94 g/L ethanol, and ethanol yield [42]. Since temperature is an important factor in cellulosic ethanol, beyond 35 °C bacteria cannot grow at a higher temperature, and the production is affected. At lower temperatures, due to the

characteristics feature of the substrate, it cannot grow, thereby reducing the concentration of cellulosic ethanol.

4.2. SSF Using Kluyveromyces marxianus (MTCC 1389)

4.2.1. Effect of Substrate Concentration

The increase in substrate concentration above 7.0% (*w*/*v*), decreases the interaction between cellulase and the substrate; hence, it leads to substrate inhibition. If the enzyme-to-substrate ratio is altered, then a substantial increase in cellulosic ethanol could be observed. The higher production of cellulosic ethanol at a reduced substrate concentration can be attributed to the decreased accumulation of glucose at the conclusion of the fermentation process [43]. When substrate concentration is present in excessive amounts, it leads to detrimental outcomes in the manufacture of cellulosic ethanol. At a high concentration of substrate in the mixing vessel during SSF process, the process becomes a difficult task, which leads to poor aeration [44].

4.2.2. Effect of Inoculum Concentration

At an ideal inoculum concentration (3% *v*/*v*), cellulosic ethanol reached 18 g/L after 96 h of fermentation, with biomass and substrate concentrations of 9.3 and 4 g/L, respectively. Cellulosic ethanol from carob fruit extracts and waste potato mash utilizing *S. cerevisiae* as fermenting microbe yielded 42 g/L and 27 g/L respectively for an inoculum concentration of 3% (*v*/*v*) [45,46].

4.2.3. Effect of pH

The highest concentration of cellulosic ethanol achieved was 17.8 g/L after approximately 96 h of incubation, accompanied by substrate and biomass concentrations of 4.25 g/L and 9.35 g/L, respectively. Modifying the pH level, whether by increasing it to 5.5 or decreasing it to 4.6, disrupts the enzymatic hydrolysis process, resulting in a decline in cellulosic ethanol production. In a different scenario, when utilizing ricotta whey for cellulosic ethanol production in the presence of *Kluyveromyces marxianus* CCT 7735 (UFV-3), a pH level of 5.0 during the SSF process yielded an impressive 49.65 g/L of cellulosic ethanol. However, altering the pH to either 5.5 or 4.5 led to a substantial reduction in cellulosic ethanol production [47].

4.2.4. Effect of Temperature

Ethanol production continued to improve, reaching 40% (17.25 g/L) at 72 h, with 80% (5.75 g/L) of the substrate being utilized. The highest concentration of cellulosic ethanol achieved was 18.25 g/L, occurring at approximately 96 h of fermentation time. This was accomplished at the optimized temperature of 41 °C, resulting in final substrate and biomass concentrations of 4.15 g/L and 9.2 g/L, respectively. It's essential to note that the temperature plays a critical role in enzymatic hydrolysis and fermentation, and it can become a limiting factor in the SSF process. For instance, when utilizing taro waste with *K. marxianus* K21, the highest cellulosic ethanol production was 43.46 g/L at 40 °C. However, as the temperature increased to 50 °C, cellulosic ethanol production decreased to 19.15 g/L. This observation indicates that while saccharification can occur around 50 °C, the effective production of cellulosic ethanol diminishes due to the accumulation of glucose during the SSF process. Ultimately, this high temperature leads to yeast mortality [48].

This optimised substrate concentration (5% *w/v*), inoculum concentration (5% *v/v*), pH (5.4), and temperature (34 °C) produced 18.25 g/L of cellulosic ethanol after 96 h of fermentation, which is better than the Bermuda grass, reed, and rap ethanol (16.1, 16.4, and 15.8 g/L, respectively) [49]. The maximum ethanol produced is due to the presence of minimal inhibitors in the pretreated hydrolysate during the combination of pretreatment methods. Moreover, as the SSF process continued, the micro-organism was capable of uptaking the reducing sugars for the production of cellulosic ethanol. The cellulosic ethanol produced by isolated strain *K. marxianus* K213 using pretreated hydrolysate water hyacinth

at 42 °C progressed smoothly and yielded a maximum ethanol concentration of 7.34 g/L at 24 h, beyond which the production declined, and at the end of 72 h, the concentration was around 6 g/L [50]. For a substrate loading of 5% *w*/*v* and an enzyme loading of 15 FPU/g substrate, the maximum cellulosic ethanol was 17.9 g/L unlike for 5% (*w*/*v*) pretreated hydrolysate *M. oleifera*, with 12 FPU/g of substrate yielding 18.25 g/L [51].

With initial glucose of 91 g/L and temperature of 40 °C, ethanol concentration reached 12 g/L. Cellulosic ethanol generated from a substrate at 5.0% (*w*/*v*) and 41 °C is 18.15 g/L [52]. Bioethanol production rose to 29.4 g/L when 30 FPU/g cellulose of the cellulase complex and 60 CBU/gcellulose of cellobiase were added to CAB-OH at 7.5% solid loading. For an enzyme concentration of 12 FPU/g of substrate, the batch SSF process gave a cellulosic ethanol concentration of 18.25 g/L during the SSF process. Although substrate loading and cellulosic ethanol production are high, the utilization of enzymes for saccharification is also high, which was found to be extravagant [53]. In the case of the 5% sulfuric acid hydrolysis process, the highest ethanol production recorded was 4.02 g/L for whole biomass fermentation and 6.14 g/L for residual biomass fermentation at 72 h. However, these values are notably lower when compared to the cellulosic ethanol concentration of 17 g/L achieved through the 3% nitric acid treatment of *Moringa oleifera*, followed by the SSF process [54].

5. Conclusions

The cellulosic ethanol obtained from agro biomass or lignocellulosic materials is considered a second-generation biofuel, which is recognized globally. In the present study, the woody stem of *M. oleifera* was used for cellulosic ethanol production. The presence of lignin content makes it difficult to perform any type of size reduction process, as it requires a good amount of energy. During the production of cellulosic ethanol with pretreated *M. oleifera* stem, *K. marxianus* (MTCC 1389) was considered to be a better, as well as a promising strain when compared to the Gram-negative bacterium *Z. mobilis* (MTCC 2427). At a high temperature (>42 °C) and a reduced pH (5.0), *K. marxianus* has the tendency to yield better cellulosic ethanol (18.25 g/L) when compared to *Z. mobilis* (15.2 g/L) since under these conditions, the performance of cellulase is found to be at a maximum, and, hence, SSF is considered for cellulosic ethanol production. However, it is assumed that the yield obtained was found to be relatively low for industrial ethanol production processes. Further improvements in terms of genetic manipulations need to be carried out for enhanced cellulosic ethanol production.

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