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Efficient Bioethanol Production from Lignocellulosic Biomass Using Diverse Microbial Strains

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ABSTRACT

Forestry residues (FR) and medicinal-aromatic plant waste (MAPW) are considered potential resources for energy recovery. In this context, we explore the bioethanol production potential of three microbial strains *Aspergillus niger*, *Zymomonas mobilis*, and *Trichoderma longibrachiatum* using this lignocellulosic hydrolysate as a substrate. The blend of FR and MAPW was pretreated by different methods like acid sulfuric (AS), steam explosion (SE), and enzymatic (E). The ethanol yield was measured by gas chromatography (GC). *Zymomonas mobilis* demonstrated the highest ethanol yield of 5.95% on untreated substrate. Conversely, *Aspergillus niger* exhibited peak performance with an ethanol yield of 10.78% following AS, SE and E combined pretreatment. *Trichoderma longibrachiatum*, yielded ethanol ranging from 1.27% to 2.47%. Furthermore, the use of immobilized *d'Aspergillus niger* strains revealed a small decrease in ethanol yield from 11.34% in the first cycle to only 5.02% in the sixth cycle. In conclusion, *Aspergillus niger* emerges as a promising candidate due to its dual functionality in pretreatment and ethanol fermentation, offering pathways for advancing sustainable biofuel technologies.

Keywords: biomass, bioethanol, lignocellulosic biomass, *Aspergillus niger*, *Zymomonas mobilis*, *Trichoderma longibrachiatum*, pretreatment, fermentation.

INTRODUCTION

Concern for our environment and anxiety related to our dependence on imported oil have rekindled interest in fuels (bioethanol and biogas) and chemicals derived from plants. Fuel production from plant materials supports CO_2-neutral energy production (Antoni et al., 2007; Eloutassi, 2014b). Lignocellulosic biomass, such as forestry residues and waste from aromatic and medicinal plants (Bendaoud 2022; Bendaoud, 2023), are substrates that can be used to produce biofuel. The microbial conversion of sugars derived from plant biomass includes a variety of processes using various microorganisms and enzymes to produce ethanol (Yerizam., 2023).

The yeast *Saccharomyces cerevisiae* is used worldwide as the main ethanol-producing microorganism. Despite its widespread use, it has several disadvantages such as high aeration costs, high biomass production, low tolerance to temperature and ethanol (Saigal, 1993). Consequently, several microorganisms have been tested for ethanol production in recent years. Among these are strains of *Aspergillus niger, Trichoderma longibrachiatum*, and *Zymomonas mobilis*.

Aspergillus niger is a mold widely exploited commercially in various fermentation techniques to produce useful enzymes such as pectinase, lactase, cellulase, and amylase (Gautam et al., 2011; Sheng Xue et al., 2018). *Aspergillus niger* is also capable of consuming and converting other lignocellulose-derived compounds, such as acetic acid (Alhadithy., 2023), furanic aldehydes, and phenolic compounds, which are known as inhibitors of ethanolic fermentation. Similarly, *Trichoderma*

longibrachiatum is a fungus often found on dead wood, construction materials, and sometimes animals. This species can grow over a wide range of temperatures; however, the optimal growth temperature is 35 °C (Druzhinina et al., 2012). T. l is a clonal species that reproduces via smooth-walled unicellular conidia (Bissett et al., 2015). T.l is well-known for producing enzymes that degrade the cell wall. Species of *Trichoderma* are among the most commercially used microbial strains for the production of cellulases.

Zymomonas mobilis has emerged as a potential alternative to the yeast currently used for ethanol production and has been reported by the National Renewable Energy Laboratory (NREL) of Golden, CO, USA, to be capable of efficiently converting C5 sugars, xylose, and arabinose, present in lignocellulosic hydrolysates, into ethanol (Zhang et al., 1995; Deanda et al., 1996). In contrast to the classical ethanologen model, Saccharomyces cerevisiae, which utilizes the Embden-Meyerhof-Parnas (EMP) pathway for glycolysis, Zymomonas mobilis employs the Entner-Doudoroff (ED) pathway. The ED pathway, present in strictly aerobic microorganisms, produces 50% less ATP compared to the EMP pathway, thereby enhancing ethanol yield. Additionally, *Z. mobilis* possesses a higher specific cell surface area and a faster glucose consumption rate than S. cerevisiae, leading to increased ethanol productivity (Conway, 1992).

This study aims to evaluate and compare the ethanol yield of a mixture of lignocellulosic residues by various microbial strains.

MATERIALS AND METHODS

Substrate preparation

The substrates used for ethanol production consist of a mixture of FR residues and waste from aromatic and medicinal plants (MAPW). FR was gathered from Different places in the Fès-Meknès region. The samples were dried at 55 °C for 72 hours until a constant weight was achieved, then they were crushed and sieved to obtain an optimal size of 0.25 mm.

Pretreatment – hydrolysis and saccharification of lignocellulosic biomass

The mixture of FR residues and MAPW underwent pretreatment using physico-chemical and enzymatic methods. One kilogram of the waste mixture was washed and steam-treated in an autoclave at 200 °C for 30 minutes. Subsequently, an acid pretreatment was conducted by immersing the biomass in a 1% diluted sulfuric acid solution and heating it at 100 °C for 30 minutes, followed by filtration to separate the solids from the acid solution. Enzymatic pretreatment was performed using cellulases extracted from *Aspergillus niger* and *Trichoderma longibrachiatum.*

Microorganisms and inoculum preparation

The strains used to produce cellulase enzymes, *Aspergillus niger* and *Trichoderma longibrachiatum*, were provided by the microbiology laboratory of the Agronomic and Veterinary Institute. Our lignocellulosic substrate serves as the main raw material for inoculum preparation. The strains of *Aspergillus niger* and *Trichoderma longibrachiatum* were inoculated into Petri dishes containing PDA (Potato-Dextrose-Agar) medium prepared with 200g of potatoes, 20g of glucose, and 15 g of agar. For medium preparation, potatoes are boiled in distilled water for 15 to 20 minutes, then the filtrate is collected, and the volume is made up to 1 liter. The mixture is sterilized at 121 °C for 15 minutes. Subsequently, the Petri dishes are incubated at 30 °C until sporulation occurs.

Afterwards, the plates are washed by adding 10 ml of distilled water, and with the help of a sterile platinum loop, the surface of the agar is gently scraped to suspend the fungal spores. The resulting solution is stored in sterile vials and kept at 4 °C until used as inoculum to seed the culture media and produce cellulolytic enzymes. The spore concentration is determined by counting the appropriate dilution using a Thoma cell.

Zymomonas mobilis strains were also provided by the microbiology laboratory of the Agronomic and Veterinary Institute of Rabat. The primary culture medium composed of yeast extract (5 g/L), glucose (20 g/L), and agar (20 g/L) at pH 5, with a culture at 30 °C for 2 days.

Assay of fibers, reducing sugars, and phenolic compounds

Cellulose, hemicellulose, and lignin were determined by the Van Soest method (Van Soest et al., 1991; Godin et al., 2010). Total sugars were measured by the Dubois method (Dubois et al., 1951). Reducing sugars concentration was

determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Phenolic compounds were analyzed using the Folin-Ciocalteu method with gallic acid as the standard, following the procedure described by Singleton and Rossi (1965).

Fermentation

Figure 1 presents the process to follow for producing bioethanol from a hydrolysate of mixed lignocellulosic plant waste:

- culture preparation and inoculation: each strain's suspension culture was introduced into 25 ml of fermentation medium contained in 100 ml Erlenmeyer flasks. This medium included a saccharified solution of lignocellulosic biomass obtained from the previous pretreatment. Additionally, a microorganism extract (2 g/L) was added to the culture medium to promote growth.
- fermentation: the fermentation was carried out at 37 °C with agitation for 48 hours.
- ethanol yield determination: the ethanol yield was determined by gas chromatography (GC) using an HP-5 capillary column equipped with an Agilent series 6890 injector.
- detoxification: to reduce the effect of phenolic compounds on the growth of microorganisms responsible for fermentation, the medium is detoxified. Detoxification is carried out by raising the pH to 9 with calcium hydroxide ($Ca(OH)_2$), then the solution is filtered, and the pH is adjusted to 7 with 2N sulfuric acid (H_2SO_4).

Determination of cellular biomass

Cell growth was determined by measuring the optical density at 625 nm (Spectronic 20 Boch Lamb) and by determining the number of viable cells using the dilution and spread plate technique on solid medium. Biomass was measured by gravimetry, by centrifuging a 10 ml sample at 12.000 g for 15 minutes, followed by washing and drying the cells at 105 °C until a constant weight was obtained. Protein content was determined according to the modified Lowry method, with the standard range made using bovine serum albumin (BSA) at 0.1 mg/ml.

Immobilization of aspergillus spores

The immobilization process involves encapsulating yeast cells in a biopolymeric matrix that provides a protective environment for the cells and allows them to maintain their metabolic activity. Alginate was used as the immobilization matrix due to its biocompatibility and easy gelling properties, while cellulose provides mechanical strength and stability to the beads. The immobilization of *Aspergillus niger* spores was carried

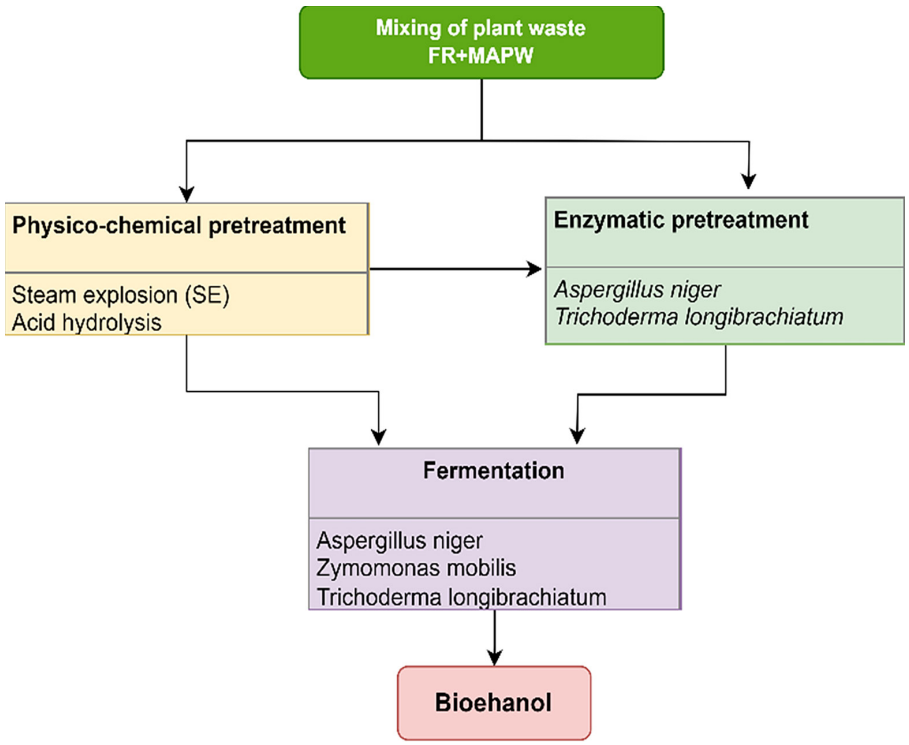

Figure 1. Process for producing bioethanol from lignocellulosic waste

out under sterile conditions in sodium alginate. For 180 ml of alginate solution (30 g/L), 20 ml of mold spores suspended in sterile water were added. Each mixture was then added dropwise to 300 ml of cold, slightly agitated 0.1 mol/L calcium chloride, and allowed to solidify for 1 hour (Ahmad Raus et al., 2021).

Statistical analysis

Data processing and statistical analysis were conducted using RStudio, as documented by the R Core Team in 2022. The comparison of efficiency between the different strains studied in the degradation of polysaccharides and the production of ethanol was executed through a one-factor ANOVA test, with a significance level set at $p < 0.05$.

RESULTS

After physico-chemical pretreatment

Table 1 presents the results of the chemical composition analysis after physico-chemical hydrolysis. Initially, these results revealed variable concentrations of monosaccharides (glucose, xylose, mannose, galactose). After steam pretreatment and acid hydrolysis, these concentrations showed significant changes. Notably, a progressive increase in the concentrations of glucose and other simple sugars was observed, suggesting the release of these compounds from more complex substrates.

Simultaneously, the concentrations of cellulose and hemicellulose decreased significantly after the treatments, highlighting substantial degradation of these polysaccharides. In contrast, lignins showed relative stability after acid hydrolysis, indicating some resistance to these treatments. Total phenolic compounds showed a sharp increase after acid hydrolysis and a slight increase following steam pretreatment.

After enzymatic pretreatment

Table 2 presents the results of the chemical composition analysis after enzymatic hydrolysis. These results revealed variations in the concentration of different components of the culture medium following various treatments. Initially, different concentrations of total phenolics, total sugars, lignins, hemicelluloses, cellulose, galactose, mannose, xylose, and glucose were present. Significant changes in the concentrations of various components were observed after steam pretreatment and acid hydrolysis. Subsequent enzymatic treatments led to additional variations in the concentrations of these components. Notably, total phenolic concentrations significantly increased after acid hydrolysis but decreased after enzymatic treatments. Total sugars also showed variations after the different treatments, with increases observed in some cases and decreases in others. Lignin, hemicellulose, and cellulose were significantly degraded after enzymatic treatments. The concentrations of monosaccharides such as galactose, mannose, xylose, and glucose were also affected by the treatments, showing variations consistent with the degradation and conversion processes of polysaccharides.

Ethanol yield

Preliminary tests showed that the strains are capable of multiplying in the medium containing our substrate as a source of carbon and energy without

Table 1. Measurement of sugars and phenolic compounds in the lignocellulosic substrate after physicochemical treatments

Component	Initial concentration (g/l)	Steam pretreatment (g/l)	Acid hydrolysis (g/l)
Glucose	5.29 ± 0.04	6.57 ± 0.02	11.68 ± 0.05
Xylose	3.41 ± 0.12	4.27 ± 0.02	4.75 ± 0.02
Mannose	1.02 ± 0.08	1.12 ± 0.02	2.39 ± 0.02
Galactose	0.65 ± 0.07	1.29 ± 0.04	1.93 ± 0.04
Cellulose	11.37 ± 0.14	5.14 ± 0.12	1.24 ± 0.03
Hemicellulose	5.73 ± 0.11	4.36 ± 0.13	0.41 ± 0.02
Lignine	4.71 ± 0.2	4.7 ± 0.03	1.09 ± 0.07
Total sugar	25.19 ± 0.13	NA	NA
Phenolic compounds	3.68 ± 0.03	5.14 ± 0.03	14.25 ± 0.03

Note: Data reported as mean standard deviation from three replicate determinations. NA: not available.

Table 2. Measurement of sugars and phenolic compounds in the lignocellulosic substrate after enzymatic treatment in the three studied microbial strains

Components (g/l)	Initial concentration	Steam treatment and acid hydrolysis	Enzymatic treatment			Enzymatic treatment without physicochemical hydrolysis		
			A n	Z m	Tl	A n	Z m	Tl
Phenolic compounds	3.68± 0.03	14.25 ± 0.03	NA	NA	NA	4.5 ± 0.17	5.1 ± 0.2	5.68 ± 0.15
Lignine	4.71 ± 0.2	1.09 ± 0.06	NA	NA	NA	2.91 ± 1.02	2.22 ± 0.02	2.07 ± 0.03
Hemicellulose	5.73 ± 0.11	0	0	0	0	3.51 ± 1.05	4.02 ± 0.08	3.56 ± 0.12
Cellulose	11.37 ± 0.14	0	0	0	0	4.12 ± 0.03	1.79 ± 0.02	1.47 ± 0.02
Galactose	0.65 ± 0.07	1.29 ± 0.04	1.93 ± 0.04	1.75 ± 0.02	1.68 ± 0.03	1.11 ± 0.02	0.85 ± 0.02	0.26 ± 0.03
Mannose	1.02 ± 0.08	2.32 ± 0.02	3.46 ± 0.1	2.94 ± 0.05	2.35 ± 0.06	1.25 ± 0.02	1.03 ± 0.03	0.93 ± 0.05
Xylose	3.41 ± 0.12	4.25 ± 0.02	5.57 ± 0.04	5.15 ± 0.03	5.05 ± 0.04	3.37 ± 0.06	2.78 ± 0.02	2.15 ± 0.03
Glucose	5.29 ± 0.04	11.68 ± 0.04	13.97 ± 0.05	9.87 ± 0.14	9.52 ± 0.03	9.18 ± 0.08	8.96 ± 0.03	8.56 ± 0.1

Note: data reported as mean standard deviation from three replicate determinations. NA: not available.

any treatment. Thus, they were tested for ethanol production. A comparison was also made. The results are presented in Figure 2. After 48 hours of fermentation, Z.m shows the highest ethanol yield with a rate of 5.95%, followed by A.n with 1.48%, and T.l with 1.27%. These results suggest that Z.m is the most efficient in converting glucose into ethanol, while A.n and T.l have lower yields.

By applying a preliminary treatment with steam explosion (Figure 3), A.n has the highest ethanol yield with a rate of 6.97% (g/g), followed by Z.m with 3% (g/g) and T.l with 2.47% (g/g). When applying a combined physicochemical treatment with an enzymatic treatment (Figure 4), the highest ethanol yield was obtained in A.n with a content of 10.78%, followed by Z.m and T.l with respective rates of 4.27% and 3.49%.

The results of the ANOVA test conducted to evaluate the differences between experimental conditions for three variables: glucose concentration, xylose concentration, and ethanol yield, are presented in Table 3. The results show that the differences in glucose concentration between the different strains tested are significant ($F = 45.09$, $p = 0.00024$). In contrast, the results for xylose concentration ($F = 0.025$, $p = 0.976$) and ethanol yield ($F = 0.849$, $p = 0.474$) did not reveal significant differences. This suggests that while glucose concentration is significantly influenced by the experimental conditions, xylose concentrations and ethanol yields remain relatively constant regardless of the condition. These results highlight the influence of the microbial strain on the production of glucose from lignocellulosic biomass, while indicating relative stability for the other measured parameters. Based on these statistical results, the Aspergillus niger strain appears to be the most effective in hydrolyzing polysaccharides into glucose.

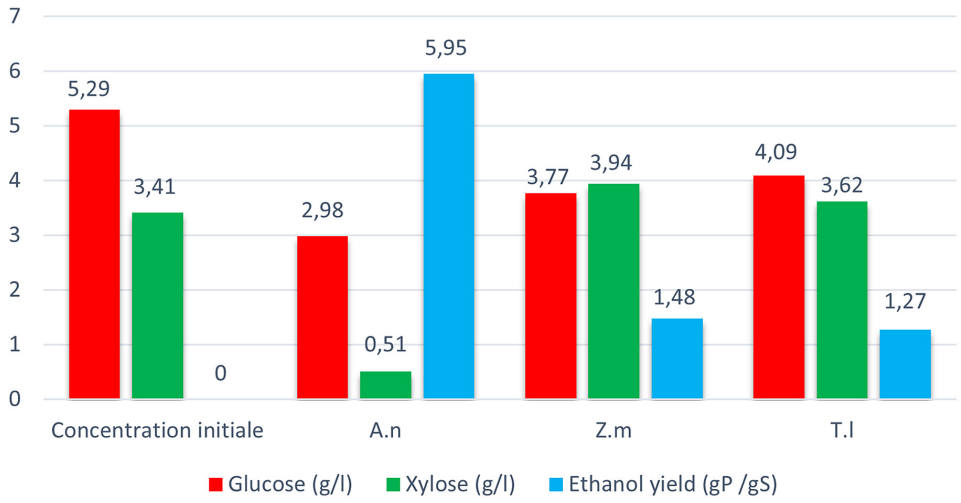

Figure 2. Evaluation of ethanol fermentation on untreated substrates by various studied strains

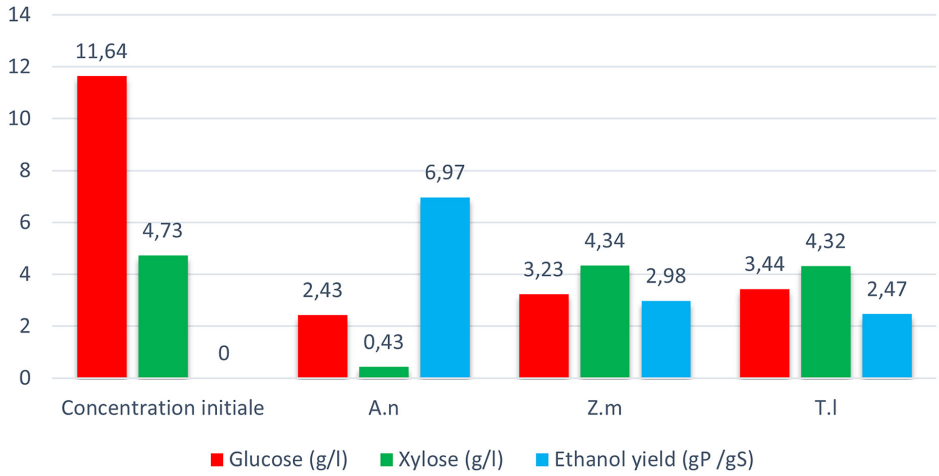

Figure 3. Evaluation of ethanol fermentation on substrates pretreated by steam explosion by various studied strains

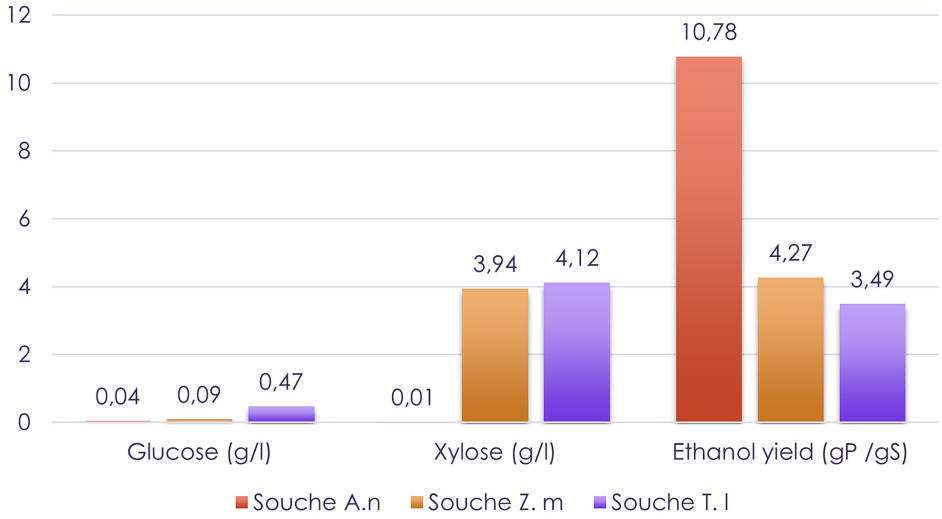

Figure 4. Evaluation of ethanol fermentation on pretreated substrates (physicochemical and enzymatic) by various strains

Kinetics of ethanol production by *Aspergillus niger*

Figure 5 presents the results of the kinetics of ethanol production by Aspergillus niger. Initially, a high glucose concentration of 13.86 g/L gradually decreases, suggesting a preferential use of this sugar as a substrate for ethanol production. This decrease is accompanied by a slight reduction in the concentrations of xylose, mannose, and galactose, indicating their utilization for ethanol production as well, although their contribution is less significant compared to glucose.

Ethanol production begins after approximately 6 hours and increases significantly thereafter, reaching a peak production of 10.78% after 40 hours. However, it is important to note that ethanol production from substrates other than glucose, such as xylose, mannose, and galactose, while present, remains less significant, suggesting a preference of the microorganisms for glucose as the primary substrate. In addition to its role in ethanol production, glucose consumption also supports cell growth (Figure 6). Analysis of this graph shows a significant evolution of single-cell organism protein concentrations and glucose over time. As time progresses, protein concentration progressively increases, reaching a peak of 15.94 g/L at the 26th hour before stabilizing. This continued cell growth suggests an efficient use of the culture medium's resources. Simultaneously, glucose concentration decreases rapidly, indicating active consumption by the cells for their growth and metabolism. This efficient use of resources could be crucial in biotechnological contexts for the production of biomass or other products of interest.

Table 3. Statistical analysis by ANOVA test of sugars concentration, and ethanol yield by the different strains tested

Variable	F-statistic	P-value	Significant ($p < 0.05$)
Glucose (g/l)	45.09	0.00024	Yes
Xylose (g/l)	0.025	0.976	No
Ethanol yield (gP/gS)	0.849	0.474	No

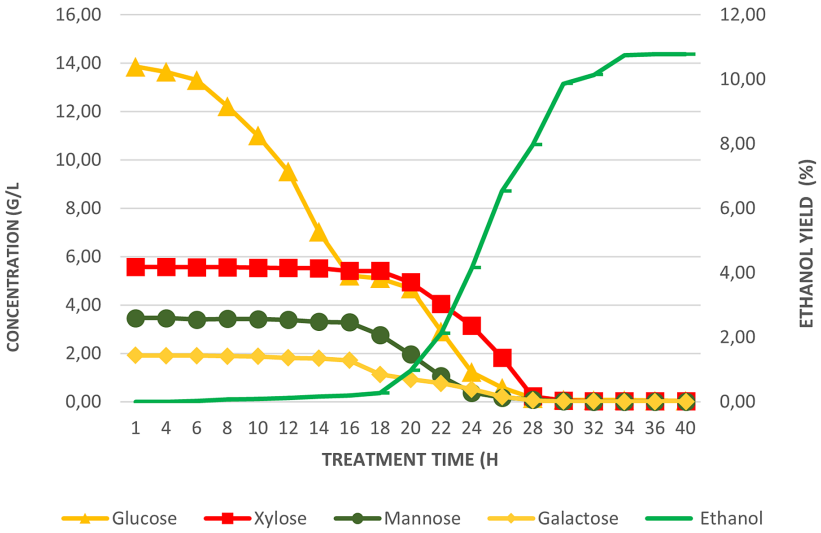

Figure 5. Kinetics of ethanol production by *Aspergillus niger*

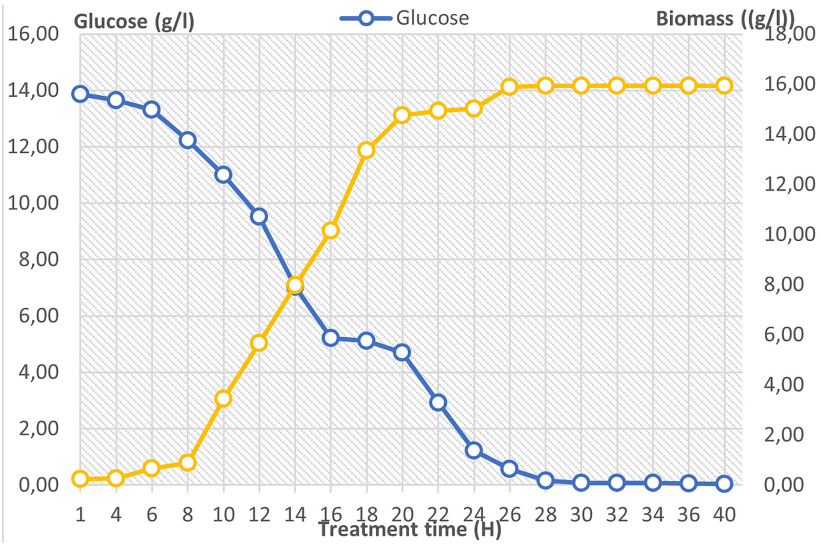

Figure 6. Evolution of cell growth during ethanol fermentation in Aspergillus niger

Evolution of ethanol production in immobilized *Aspergillus niger strain*

The results of ethanol production by the immobilized Aspergillus niger strain (Figure 7) over multiple cycles (Figure 8) revealed an increase in sugar levels, from 0.09 g/L in the first cycle to 12.14 g/L in the sixth cycle. This increase suggests a continuous accumulation of sugars due to the reduced consumption by the fungal strain. Similarly, the ethanol yield also decreases significantly, from 11.34% in the first cycle to only 5.02% in the sixth cycle. This decrease indicates a decline in the efficiency of sugar conversion into ethanol as the cycles progress.

Additionally, the concentration of single-cell organism proteins in the culture medium also decreases over the cycles, from 17.56 g dry weight/L in the first cycle to only 3.86 g/L in the sixth cycle, indicating a reduction in cell biomass.

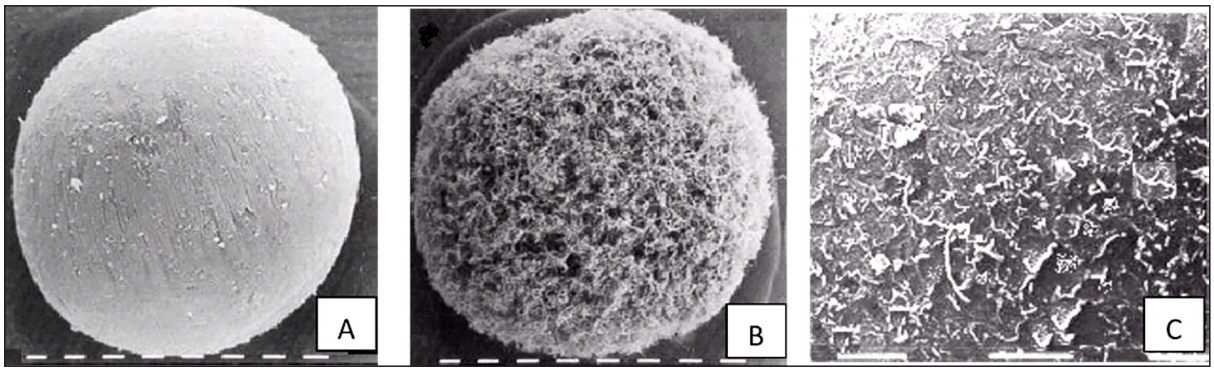

Figure 7. Electronic photograph of a calcium alginate bead containing immobilized A.n concentrate. (a) 1 cycle, (b) after 6 cycles, (c) surface of bead

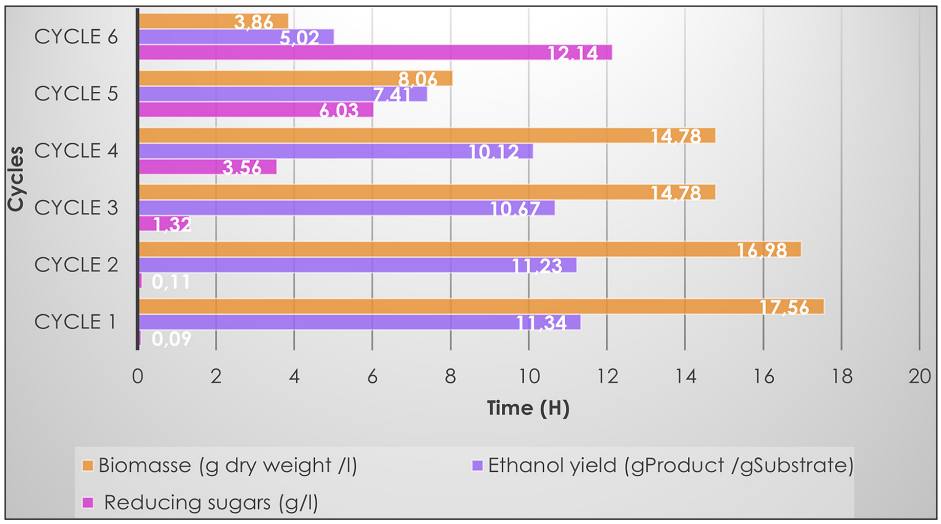

Figure 8. Ethanol and biomass yield from immobilized Aspergillus niger strain over multiple cycles

DISCUSSION

The results obtained showed, on the one hand, that a high ethanol yield was achieved when fermentation was preceded by pretreatment compared to an untreated substrate. This confirms that pretreatment of lignocellulosic biomass is an essential preliminary step before starting ethanol production. On the other hand, the ethanol yield is higher after combined pretreatment (physico-chemical and enzymatic) compared to steam explosion pretreatment (Bendaoud, 2023; Eloutassi, 2014). It is also observed that after combined pretreatment, the ethanol yield (10.78%) and the concentration of hydrolyzed monosaccharides (glucose and xylose) are higher in *A. niger* compared to the other strains tested.

However, despite the relatively high ethanol yield (5.95%) observed in Z.m compared to the other strains tested, it remains relatively low. Z.m is known as an efficient ethanol producer from substrates such as glucose, fructose, and galactose (Rogers et al., 2007; Panesar et al., 2006), but it is unable to ferment C5 sugars such as xylose and arabinose, which are also components of lignocellulose (Doran-Peterson et al., 2008). Thus, on the one hand, the choice of substrate used in this study and, on the other hand, the lack of pretreatment of this substrate to break down the lignocellulosic complex could influence the ethanol yield produced by the microbial strains tested (Bendaoud, 2022). Moreover, this low yield can be explained by Z.m's inability to tolerate toxic inhibitors present in lignocellulose hydrolysates, such as phenolic compounds, during fermentation.

The kinetic analysis of ethanol production by *Aspergillus niger* revealed a preferential use of glucose as the primary substrate, although other sugars were also metabolized for ethanol production. Additionally, the amount of ethanol increased with the fermentation time. *Aspergillus niger* is well known for its enzymatic power,

secreting cellulases necessary for saccharification of polysaccharides into fermentable monosaccharides (Boondaeng, 2024). However, its role in bioethanol production is a novel finding in this study. Some studies have also examined the role of *A. niger* in ethanol production. For instance, Salih et al. (2019) reported a yield of 0.94 g/g on a honeydew melon substrate. Another study showed a maximum ethanol yield of 12.124% from waste potatoes. The ethanol content can also be influenced by the inoculum concentration and the fermentation duration (Uljanah., 2024).

Working with a microbial strain that possesses both lignocellulolytic enzymatic activity and the ability to ferment sugars from lignocellulosic biomass into bioethanol could significantly enhance the efficiency of the bioethanol production process at a pilot scale. In this study, the three tested microbial strains demonstrated similar bioethanol production capacities. However, regarding polysaccharide hydrolysis and the release of simple sugars, the *Aspergillus niger* strain performed better. Therefore, it could be used for both pretreatment and bioethanol production, offering a more integrated and efficient solution for converting lignocellulosic biomass into biofuel.

To further elaborate, it would be pertinent to mention the specific advantages of such integration. Using a single strain for both critical stages of the process can reduce production costs, simplify logistics, and decrease the time needed to achieve optimal bioethanol yields. Additionally, this approach could potentially increase the sustainability of the process by limiting the use of chemicals and exogenous enzymes. Further studies should be conducted to optimize the culture conditions and maximize the efficiency of the Aspergillus niger strain, considering variables such as temperature, pH, and substrate concentration.

CONCLUSIONS

The results of this study highlight the importance of selecting microbial strains for producing bioethanol from lignocellulosic substrates. The strain *Zymomonas mobilis* exhibited the highest ethanol yield among the strains studied when fermenting untreated substrates after 48 hours. In contrast, the *Aspergillus niger* strain proved to be the most efficient after steam explosion pretreatment, with an ethanol yield of 10.78%. The application of a combined physicochemical and enzymatic treatment also showed similar results, with *Aspergillus niger* presenting the highest yield.

The kinetic analysis of ethanol production by *Aspergillus niger* revealed a preferential use of glucose as the primary substrate, although other sugars were also metabolized for ethanol production. Additionally, the observation of cell growth during fermentation highlighted an efficient use of the culture medium's resources, with significant glucose consumption for cell biomass.

Furthermore, ethanol production by immobilized *Aspergillus niger* strains showed promising results in terms of ethanol yield. Although a decrease in ethanol yield was observed over successive cycles, this could be economically advantageous on an industrial scale.

The observed efficiency of the *Aspergillus niger* strain in terms of pretreatment, saccharification of lignocellulosic biomass, and fermentation for bioethanol production suggests that it could be chosen for the energetic valorization of our lignocellulosic waste substrate for bioethanol production.

This study underscored the importance of microbial strain selection and pretreatment methods in ethanol production from lignocellulosic substrates.

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