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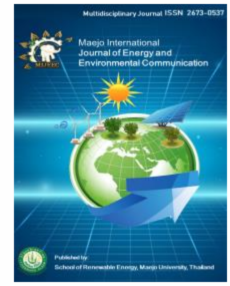

ARTICLE

SHF, SSF, and co-fermentation of alkali and steam delignified agro-residues to bioethanol

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A B S T R A C T

Five agro-residues, including sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, were tested for bioethanol production by different fermentation methods using alkali-pretreated, enzymatically saccharified substrates. Co-fermentation of *S. cerevisiae* and *C. shehatae* was found to be more efficient in the bioethanol production than SHF and SSF. S. cerevisiae and *C. shehatae* complemented one another in the fermentation of hexose and pentose sugars. Together, these organisms had the highest ethanol yields from substrates: sugarcane bagasse (278.40 mg/g), sugarcane tops (262.75 mg/g), sugarcane trash (241.42 mg/g), corn husk (232.36 mg/g) and corn stover (239.82 mg/g). A scaled-up study was also carried out for sugarcane bagasse in optimised conditions. 1) 3.0% NaOH treatment (8 hours at room temperature) followed by autoclaving at 121oC for 1 hour. 2) Saccharification by cellulase enzymes (cellulase 15 FPU/g, b-glucosidase 10 IU/g and xylanase 5 U/g). 3) Fermentation by dual cultures (*S. cerevisiae* and *C. shehatae*). One kilogramme of pre-treated sugarcane bagasse with a 5% substrate concentration yielded 223 grammes of ethanol, 22.30 percent (w/w) of the pre-treated substrate.

1. Introduction

The growing global energy crisis, greenhouse gas emissions, and accelerating climate change have added new urgency to the need for sustainable and low-carbon energy alternatives to fossil fuels (Onyemowo et al., 2024; Reansuwan et al., 2024). Rapid industrialization, growth in urban populations and growing transportation needs continue to put pressure on conventional petroleum resources, which contribute to environmental degradation and energy insecurity (Oteikwu et al., 2024; Pandi et al., 2024). In this scenario, the use of renewable biofuels has become a strategic solution that can contribute to the reduction of carbon emissions and, at the same time, contribute to sustainable development and the circular economy (Ramaraj et al., 2023; Sharma et al., 2023; Tongsiri et al., 2023). Among these, the second-generation bioethanol made from Lignocellulosic agro-residues has received great attention, as it has the potential to use non-food resources and reduce competition with agricultural supply chains (Manmai et al., 2023; Trejo et al., 2023).

However, owing to the inherent recalcitrance of lignocellulosic materials (mainly based on the complex association of cellulose, hemicellulose and lignin), direct conversion by microorganisms is limited and effective pretreatment methodologies such as alkali and steam delignification are needed

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to improve the accessibility of enzymes (Nasution et al., 2024; Taechawatchananont et al., 2024). Following pretreatment and saccharification, various configurations for fermentation, such as Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) and co-fermentation, are utilized to optimize the production of both Hexose and Pentose carbohydrates as ethanol (Kongchan et al., 2022; Sophanodorn et al., 2022). A systematic comparison of such strategies is needed in order to improve the efficiency of processes, maximize the ethanol yield, and achieve economic scalability of second-generation bioethanol production (Vu et al., 2022).

Several categories of raw materials are currently used in the production of bioethanol, which are focused in producing first-, second-, and emerging third-generation feedstocks based on their origin and technological maturity level. First-generation feedstocks include readily fermentable sugar-rich materials such as molasses and sugar juices and starch-based crops, including the cereal grains. These substrates can be converted to the fermentable sugars via relatively simple processing steps thereby making them technologically attractive and commercially established. Nevertheless, the fact that they are directly linked to the human food chain and animal feed industry significantly raises their market price and raises ethical and sustainability concerns of food versus fuel competition. Fluctuating world grain markets, land-use pressures, and debates on policy if addressing food security issues have further the limitations of using edible crops in large-scale bioethanol production. Consequently, research and industrial endeavors have gradually moved towards second generation lignocellulosic feedstock, which are plentiful, cheap and do not compete directly with food resources (Faria et al., 2024).

A large variety of agricultural residues with predominantly lignocellulosic structure have been explored for their potential to produce fermentable sugars for ethanol manufacture. These are cotton stalks (Kerem et al., 1992), wheat straw (Zayed & Meyer, 1996), alfalfa fibre (Sreenath et al., 2001), sunflower hulls (Sharma et al., 2004), rice straw (Karimi et al., 2006) and various crop residues such as bagasse, sugarcane leaves, sugarcane trash, corn husk and corn stover (Shankarappa et al., 2015). The attractiveness of these materials is that they are available widely, have a low economic value and contain high levels of carbohydrates in the form of cellulose and hemicellulose. Globally, millions of tonnes of such residues are produced each year as a by-product of agricultural operations, and handling such residues will often lead to environmental problems such as open-field burning, greenhouse gas emissions and soil nutrient loss if the residues are disposed of improperly. Valorization of such residues into bioethanol does not only contributes to the renewable energy production but is also good for waste management, rural incomes and the circular bioeconomy principles.

Despite their benefit, lignocellulosic materials have a complex and recalcitrant structure of cellulose microfibrils bounded by hemicellulose and lignin. This complex architecture provides mechanical strength and resistance to microbial attack and therefore there is a lack of efficient enzymatic hydrolysis. Therefore, pretreatment is an important step to break the lignin barrier, decrease the crystalline of cellulose, increase the surface area, and enhance the access of enzymes. Numerous pretreatment strategies have been developed that include physical (milling, grinding, steam explosion), chemical (acid, alkali, organosolv), physicochemical (ammonia fiber expansion, liquid hot water), and biological approaches. Among these, alkali pretreatment using sodium hydroxide (NaOH) has attracted a great deal of attention because of its efficacy in solubilizing lignin and swelling the hemicellulose (Shankarappa & Geeta, 2013). Alkali treatment disruptsester linkages between lignin and carbohydrates and leads to the formation of more pores and the improvement of biomass digestibility by cellulolytic enzymes (Schell et al., 2003; Mosier et al., 2005). Consequently, hydrolysis of the carbohydrate fraction to monomeric sugars, can be performed faster and with greater yields compared to the untreated substrates.

Following pretreatment, the process of enzymatic hydrolysis is a major part of the breakdown of structural polysaccharides into sugars that can be fermented. The cellulase enzyme system is a synergistic complex that consists of three major enzymatic activities: (a) endoglucanases (EC 3.2.1.4), (b) exoglucanases including cellobiohydrolases (EC 3.2.1.74 and EC 3.2.1.91), and (c) β-glucosidases (EC 3.2.1.21). Endoglucanases cleave internal (random) beta-1,4 glycosidic bonds in amorphous regions of the cellulose which results in new ends of the chains and soluble oligosaccharides of various chain lengths. Exoglucanases act processively on the reducing and non-reducing ends of cellulose chains to liberate either glucose or cellobiose as the chief products of hydrolysis (Bisaria & Ghose, 1981). These enzymes are especially efficient at the degradation of microcrystalline cellulose by peeling off cellulose chains from the fibrillar structure. Beta-glucosidases then hydrolyze soluble cellodextrins and cellobiose to glucose and thereby prevent product inhibition of upstream enzymes and improve the overall hydrolysis efficiency. Parallel with this, hemicellulases and xylanases break down hemicellulosic polymers into pentose sugars like xylose and arabinose to increase the ranges of fermentable sugars.

The efficient integration between pretreatment and enzymatic saccharification is of critical importance for the maximum sugar recovery and minimised formation of inhibitors. Parameters like enzyme loading, temperature, pH, substrate concentration, and mixing intensity have a great impact on the kinetics and sugars yield. In order to improve process economics and to achieve scaled-up processes, the optimization of these parameters has been widely described. Moreover, improvements in enzyme engineering, strain improvement and recycling strategies of enzymes have helped to overcome the cost of enzymes - one of the major bottlenecks in second-generation bioethanol production.

In our previous studies, low cost and abundantly available agro-residues, such as sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover have been subjected to alkali pretreatment to obtain effective delignification and subsequent enzymatic saccharification using commercial cellulase preparations. The resulted hydrolysates with elevated content of both hexose and pentose sugars were fermented into bioethanol with varying process configurations. These were separate hydrolysis and fermentation (SHF) (Samaksaman et al., 2024), simultaneous saccharification and fermentation (SSF) (Misbah et al., 2022) and also co-fermentation strategies using mixed or engineered microorganisms that could take advantage of both pentose and hexose sugars (Gunasekaran & Chandraraj, 1999, Amutha & Gunasekaran, 2001, Kemita et al., 2024). Each strategy was assessed to see its impact on the ethanol yield, fermentation efficiency, and integration of the whole process. The utilization of pentose and hexose using microbial population was especially significant in maximizing the total sugar conversion and consequently ethanol productivity and supporting the economic feasibility of the lignocellulosic bioethanol production.

2. Materials and Methods

Feedstocks included sugarcane bagasse (sourced from Malaprabha Sahakari Sugar Factory, M. K. Hubli, Belgaum, Karnataka), sugarcane trash and sugarcane tops (Co-8014) from the farms of Mr. Basavaraj, Yettinagudda, Dharwad, Karnataka and corn stover and corn husk (Arjun) harvested from the Main Agricultural Research Station, University of Agricultural Sciences, Dharwad, Karnataka. These materials were transported to the laboratory and were chopped into small sizes, dried at 60 °C in a hot-air oven for 12h and then grinded with a Willey dry mill to reach a particle size of 0.5 mm. The resulting substrates were subjected to a 3.0% NaOH pretreatment for 8h at ambient conditions, followed by a steam pretreatment (Kumar et al., 2010) for 1h at a temperature of 121 °C and 15psi. An aliquot of 50mL NaOH solution was added to 10g of dry substrate in each Erlenmeyer flask. Bagasse needs 10mL additional NaOH solution to ensure enough wetting (Shankarappa & Geeta, 2013). After pretreatment, remaining alkali was eliminated by successive washes with tap water and distilled water rinsed until the pH of the effluent water reached near 7.0, and finally, the material was neutralised using acetic acid. The neutralised residue was then dried in a hot air oven at 60 °C to constant weight. The cellulose content of the delignified agro-residues was 0.633, 0.613, 0.613, 0.620 and 0.613 g g-1 with sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, while the hemicellulose content was 0.180, 0.173, 0.193, 0.180 and 0.193 g g^{-1} for the above-mentioned feedstocks

2.1 Saccharification

Pre - treated substrates were hydrolysed using commercial preparations of cellulase, beta-glucosidase, and xylanase. Oven-dried, alkali-treated and thermally pre-treated samples (5.0g each) were suspended in 250mL Erlenmeyer flasks separately and filled with different amounts (up to 200mL) of 0.05M citrate buffer (pH4.8). The contents were autoclaved and then supplemented with 15 FPU g-1 of cellulase (MAPs India, Ahmedabad), 10 IUI g-1 of beta-glucosidase (SRL Chemicals) and 5 U g-1 of xylanase (M/s Sigma Industry, courtesy of Godavari Sugar Mills, Sameerwadi). An enzyme ratio of 15:10:5 (cellulase : β-glucosidase: xylanase) was adopted to improve the rate of cellulose hydrolysis and also to reduce the inhibitory effects of cellobiose on the formation of glucose (Sattler et al., 1989; Qi et al., 2023). The volume of the buffer/enzyme mixture was then 100 mL. The cellulase available from M/s MAPS India, Ahmedabad, had an activity of 166 FPU/L of cellulase, while the beta-glucosidase from M/s SRL Chemicals showed an activity of 19.6U/gm of beta-glucosidase and xylanase from M/s Sigma Chemical had an activity of 170U/gm of phenolics. The suspensions were incubated for 12h in a shaking incubator at 50 °C and 150 rpm. Hydrolysis was stopped by immersing the flasks in boiling water for 10 min to denature the enzymes. It is pertinent to note that the concentrations of reducing sugars were measured for the hydrolysates at 655.32, 615.23, 576.28, 558.30, and 550.37 mg/g, which vary for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and corn stover, respectively (Shankarappa et al., 2015; 2016).

2.2 Fermentation medium

The cellulase-treated hydrolysate was added with nutrients (urea 0.64%, KH_2PO4 0.2%, and MgSO4.7H2O 0.1%) to form a fermentation medium (Yu and Zhang, 2004). The medium was fermented to bioethanol with five different yeast cultures viz., Saccharomyces cerevisiae (NCIM-3455), Kluyveromyces marxianus (NCIM-3465), *Pachysolen tannophilus* (NCIM-3445), Pichia stipitis (NCIM-3498) and *Candida shihatae* (NCIM-3500) and one bacterium culture, *Zymomonas mobilis* (NCIM-2428), individually, in combinations and simultaneous saccharification and fermentation separately. The cultures were procured from the National Centre for Industrial Microorganisms (NCIM), Pune, India. The 24-hour-old broth cultures (50 ml in 100 ml Erlenmeyer flask), having approximately 30 × 10^6 cells /ml were used for inoculation. After the fermentation, the amount of ethanol formed (Caputi et al., 1968) and the residual reducing sugars (Miller, 1959) in the broth were estimated.

2.3 Separate Hydrolysis and Fermentation (SHF)

The cellulase hydrolyzed medium was fermented to bioethanol with five different yeast cultures, viz., *S. cerevisiae*, *Kluyveromyces marxianus*, *Pachysolen tannophilus*, *Pichia stipitis* and *Candida shihatae* and one bacterium culture (*Zymomonas mobilis*), inoculated individually @ 4%. The inoculated substrates were incubated under aerobic conditions for 24h at 37 ± 2ºC. After 24h, an anaerobic condition was created by plugging flasks with rubber cork, making provision for trapping carbon dioxide. The flasks were incubated for four days, by this time the carbon dioxide ceased to evolve.

2.4 Simultaneous Saccharification and Fermentation (SSF)

The pre-processed substrates, i.e., sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk, and corn stover at 5% solids, were saccharified for a shorter-time period (6 hours) compared with that described in the previous work for saccharification (12 hours). The resultant pre-hydrolysate, including cellulase enzymes, was subsequently incubated at 37 degC during which shaker and water bath systems were combined for 6 h, and then kept rotundely overnight at 37 degC at a temperature which has been chosen in analogy with the conditions used for the yeast and bacteria fermentation (Wyman et al., 1992).

The Hydrolysing Medium was inoculated separately (5 different yeast cultures and one bacterial culture), using a 4 percent inoculum level (v/v-1). Simultaneous saccharification and aerobic fermentation until 24 h at 37 degC and anaerobic fermentation until the stop of carbon dioxide evolution for 4 d at 37±1 °C were performed.

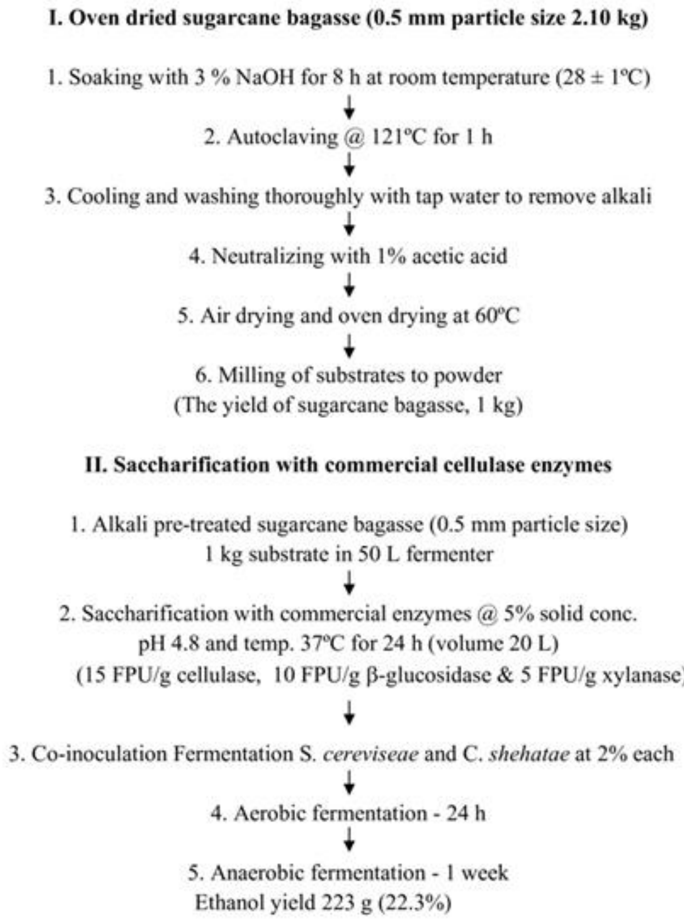

Figure 1. Flow chart of Alkali pretreatment and bioethanol production from sugarcane bagasse (Scale-up study)

2.5 Fermentation by mixed cultures (Co-fermentation)

The medium was fermented to bioethanol with five different yeast cultures and one bacterium culture (*Z. mobilis*), such that the pentose-utilizing *P. stipitis* and *C. shihatae* formed combinations with hexose-fermenting *S. cerevisiae*, *K. marxianus*, *P. tannophilus* as dual inoculums and pentose-utilizing Pichia stipitis (Kumar et al., 2009) and *C. shihatae* forming triple inoculum combinations with *S. cerevisiae* and *Z. mobilis*. Inoculation was done at 2% each for dual inoculation and at 1.33% each for combinations of three cultures. The inoculated substrates were incubated under aerobic conditions for 24 h at 37 ± 1ºC. After 12 h, an anaerobic condition was created by plugging the flasks with a rubber cork, making provision for trapping carbon dioxide. The flasks were incubated till the carbon dioxide ceased to evolve.

2.6 Pilot scale study

The alkali pre-treated (3.0% NaOH for 8 hours followed by autoclaving at 121ºC at 15 lbs pressure for 1h) and enzymatically saccharified bagasse (commercial cellulase enzyme at 15 FPU/g, 10 IU/g β–glucosidase, and 5U/g xylanase at 5% solid concentration) was fermented using a laboratory fermentor of 40 L capacity (Figure 1). Twenty litre fermentation medium consisting of 1 kg pre-treated and saccharified bagasse (5% solids) was added with nutrients (urea 0.64%, KH_2PO_4-0.2% and $MgSO_4.7H_2O$ 0.1%). The medium was fermented to bioethanol using *S.cerevisiae* and *P.stipitis* dual yeast cultures. 24h grown broth cultures were inoculated @ 2% each. The inoculated substrates were incubated under aerobic conditions at 37 ± 1ºC for 24 h. After 24 h, an anaerobic condition was created and incubated for four days.

3. Results and discussion

3.1 Separate Hydrolysis and Fermentation (SHF)

The five different agro-residues viz., sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, were saccharified by using commercial cellulase enzyme and then fermented to bioethanol using six different ethanol fermenting microorganisms. The ethanol content as influenced by the type of microorganism in the fermented broth differed significantly (Table 1a & Figure 2a).

Table 1a. Effect of separate hydrolysis and fermentation (SHF) on ethanol yield (mg/g) from selected pre-treated and saccharified agro-residues

Sl. No	Cultures (A)	Substrates (B)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae*	262.93	246.93	232.00	222.40	220.27	**236.91**
2.	*Zymomonas mobilis*	241.60	228.27	214.40	205.86	219.20	**221.87**
3.	*Kluyveromyces marxianus*	232.00	217.07	202.14	196.80	202.13	**210.03**
4.	*Pachysolen tannophilus*	256.53	240.00	225.07	217.60	222.93	**232.43**
5.	*Pichia stipitis*	146.13	134.40	138.67	114.67	128.00	**132.37**
6.	*Candida shehatae*	155.73	145.07	137.24	123.73	130.67	**138.49**
7.	Control	1.82	1.51	1.42	1.46	1.50	**1.54**
	Mean	**185.25**	**173.32**	**164.42**	**154.65**	**160.67**	
		SE±			CD (1%)		
	Cultures (A)	2.03			7.59		
	Substrates (B)	1.71			6.41		
	Interaction (A x B)	4.53			16.97		

Table 1b. Effect of separate hydrolysis and fermentation (SHF) on residual reducing sugar (mg/g) from selected pre-treated and saccharified agro-residue**s**

Sl. No	Cultures (A)	Substrates (B)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae*	54.96	58.36	53.96	52.53	53.96	**54.76**
2.	*Zymomonas mobilis*	92.90	80.89	73.32	71.21	72.36	**78.13**
3.	*Kluyveromyces marxianus*	94.60	88.00	75.44	70.19	73.20	**80.29**
4.	*Pachysolen tannophilus*	76.38	67.62	59.71	53.25	59.71	**63.34**
5.	*Pichia stipitis*	232.17	195.01	201.27	191.93	198.40	**203.76**
6.	*Candida shehatae*	213.49	189.77	177.56	171.81	176.12	**185.75**
7.	Control	621.64	582.11	572.31	567.64	583.49	**585.44**
	Mean	**198.02**	**180.25**	**173.37**	**168.37**	**173.89**	
		SE±			CD (1%)		
	Cultures (A)	2.03			7.60		
	Substrates (B)	1.72			6.42		
	Interaction (A x B)	4.54			17.00		

The mean maximum ethanol content of 236.91 mg per g substrate was produced by the yeast Saccharomyces cerevisiae and it was at par with Pachysolen tannophilus 232.43 mg per g ethanol. These two yeast cultures were significantly superior over other ethanol fermenting yeasts viz., K. marxianus (210.03 mg/g), P. stipitis (132.37 mg/g), C. shehatae (138.49 mg/g), over bacterial culture, Z. mobilis (221.87 mg/g), and control treatment (1.54 mg/g). The ethanol content due to agro-residues also differed significantly, with a mean maximum ethanol content of 185.25 mg per g in sugarcane bagasse. It was found to be significantly superior to other substrates.

The interaction between ethanol-fermenting microorganisms and different substrates indicated significant differences in ethanol yield (Table 1a). The yeast S. cerevisiae produced the significantly highest ethanol yield of 262.93 mg per g in bagasse, which was on par with *P. tannophilus* (256.53 mg/g) also in bagasse and significantly superior to the rest of the ethanol-fermenting microorganisms. *S. cerevisiae* recorded the highest ethanol yield in all the agro-residues except in corn stover, where *P. tannophilus* produced the highest ethanol yield (222.93 mg/g). The *S. cerevisiae* and *P. tannophilus* were found to be at par with respect to ethanol yield in respective agro-residues and they were significantly superior to the rest of the ethanol-fermenting microorganisms. The higher ethanol production by *S. cerevisiae* and *P. tannophilus* organisms indicates their efficiency in converting hexose sugars into ethanol (Sharma et al., 2004).

The influence of alcohol fermenting microorganisms and different substrates showed variations in residual reducing sugar content. Among the alcohol fermenting microorganisms (Table 1b), *P. stipitis* fermented substrates showed the highest residual reducing sugar content in all the substrates, sugarcane bagasse (232.17 mg/g), sugarcane tops (195.01 mg/g), sugarcane trash (201.27 mg/g), corn husk (191.93 mg/g) and in corn stover (198.40 mg/g), whereas *S. cerevisiae* showed the lowest residual reducing sugar content in all the substrates, it reflects about the relative ability of the organism to utilize maximum reducing sugars for higher ethanol production (Samaksaman et al., 2024).

3.2 Simultaneous Saccharification and Fermentation (SSF)

The simultaneous saccharification and fermentation of five agro-residues viz., sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, was tested by using six different ethanol fermenting microorganisms. All six ethanol-fermenting microorganisms showed significant differences in ethanol production (Table 2a & Fig. 2b). The mean ethanol production ranged from 126.97 to 243.67 mg per g across the different microorganisms. The mean maximum ethanol production (243.67 mg/g) was observed with the yeast *S. cerevisiae* and it was significantly superior to other ethanol-fermenting microorganisms. The lowest mean ethanol production of 126.97 mg per g was noticed with P. stipitis. The different agro-residues that were subjected to fermentation also showed significant variation in ethanol yield. The mean maximum ethanol yield of 184.07 mg per g was recorded in sugarcane bagasse, followed by sugarcane tops (175.59 mg/g), corn stover (164.25 mg/g), sugarcane trash (162.73 mg/g) and corn husk (162.37 mg/g). The substrate bagasse was found to be significantly superior over other substrates in ethanol production. The next best substrate was sugarcane tops. The sugarcane trash, corn husk and corn stover were observed to be at par with each other and significantly low in yielding ethanol.

Significant differences were also observed in the combined interaction between ethanol-fermenting microorganisms and substrates regarding ethanol yield. The highest ethanol (269.84 mg/g) yield was observed in sugarcane bagasse when saccharified with cellulase enzymes and fermented simultaneously with S. Cerevisiae over other SSF microorganisms (Table 2a). S. cerevisiae also produced the highest ethanol in three different substrates, bagasse (269.84 mg/g), sugarcane tops (252.41 mg/g) and sugarcane trash (237.16 mg/g), whereas Z. mobilis produced the highest ethanol yield in two substrates, corn husk (231.29 mg/g) and corn stover (232.00 mg/g). However, the two microorganisms were on par with each other in ethanol yield in all the substrates, except in the case of bagasse, where *S. cerevisiae* (269.84 mg/g) was significantly superior to *Z. mobilis* (251.78 mg/g).

Table 2a. Effect of simultaneous saccharification and fermentation (SSF) on ethanol yield (mg/g) from selected pre-treated and saccharified agro-residues

Sl. No	Cultures (A)	Substrates (B)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae*	269.84	252.41	237.16	227.50	231.41	**243.67**
2.	*Zymomonas mobilis*	251.78	244.78	235.75	231.29	232.00	**239.12**
3.	*Kluyveromyces marxianus*	219.63	214.17	213.69	212.70	209.49	**213.94**
4.	*Pachysolen tannophilus*	247.12	239.80	228.17	223.82	224.70	**232.72**
5.	*Pichia stipitis*	143.82	135.29	120.18	117.33	118.22	**126.97**
6.	*Candida shehatae*	154.49	141.15	102.75	122.49	132.45	**130.67**
7.	Control	1.82	1.51	1.42	1.46	1.50	**1.54**
	Mean	**184.07**	**175.59**	**162.73**	**162.37**	**164.25**	

	SE±	CD (1%)
Cultures (A)	0.94	3.53
Substrates (B)	0.80	2.98
Interaction (A x B)	2.11	7.89

Table 2b. Effect of simultaneous saccharification and fermentation (SSF) on residual reducing sugar (mg/g) from selected pre-treated and saccharified agro-residues

Sl. No	Cultures (A)	Substrates (B)					**Mean**
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae*	45.85	41.03	39.59	40.31	41.03	**41.56**
2.	*Zymomonas mobilis*	64.98	59.94	60.58	54.47	59.81	**59.96**
3.	*Kluyveromyces marxianus*	87.02	74.83	61.22	55.02	60.50	**67.72**
4.	*Pachysolen tannophilus*	58.28	52.53	45.34	41.75	46.06	**48.79**
5.	*Pichia stipitis*	232.89	224.98	207.02	204.86	207.74	**215.50**
6.	*Candida shehatae*	217.80	194.09	180.19	179.71	181.87	**190.73**
7.	Control	495.31	475.21	472.99	476.87	481.62	**480.40**
	Mean	**171.73**	**160.37**	**152.42**	**150.43**	**154.09**	

	SE±	CD (1%)
Cultures (A)	1.39	5.21
Substrates (B)	1.18	4.41
Interaction (A x B)	3.11	11.66

The higher ethanol yield observed in SSF with *S. cerevisiae* and *Z. mobilis* could be due to efficient conversion of sugars released by cellulase enzymes into ethanol by ethanol-fermenting microorganisms (Misbah et al., 2022), and the supplementation with beta-glucosidase would have enhanced the availability of reducing sugars at an optimum level for ethanol conversion (Harikrishna et al., 2001). The residual reducing sugar content due to microbial inoculations and different substrates differed. The pentose-fermenting yeast *P. stipitis* recorded the significantly highest residual reducing sugar content for respective substrates (Table 2b). It was found to be significantly inferior compared to other alcohol fermenting microorganisms with regard to the conversion of reducing sugar. The significantly lowest residual reducing sugar content was observed with the yeast *S. cerevisiae* for all the agro-residues subjected to alcoholic fermentation.

3.3 Co-fermentation

The combined inoculation of hexose and pentose-fermenting microorganisms showed significant variation in ethanol yield. The mean maximum ethanol yield of 250.95 mg per g ethanol was obtained with the coinoculation of S. cerevisiae and C. shehatae and it was statistically superior over the next best combined inoculation of *P. tannophilus* and *P. stipitis* (236.45 mg/g) and over all other dual and triple inoculations of microorganisms (Table 3a & Fig. 2c). The lowest mean ethanol production of 147.66 mg per g was observed with the combined inoculation of *S. cerevisiae*, *Z. mobilis* and *P. stipitis*. Different saccharified substrates indicated significant variations in ethanol yield. The mean maximum ethanol yield of 213.50 mg per g was observed in the substrate sugarcane bagasse, which wasstatistically high over the rest of the substrates. The significantly lowest ethanol yield (180.45 mg/g) was observed with the corn husk substrate. Variation in ethanol yield was also observed due to the combined interaction of ethanol-fermenting microorganisms and substrate. The highest ethanol production was recorded in sugarcane bagasse inoculated with S. cerevisiae and C. shehatae together.

Table 3a. Effect of co-fermentation on ethanol yield (mg/g) from selected pre-treated and saccharified agro-residues

Sl. No	Cultures (A)	Substrates (B)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae* + *Pichia stipitis*	186.13	171.73	165.15	161.25	166.04	**170.06**
2.	*Zymomonas mobilis* + *Pichia stipitis*	251.38	234.49	219.55	211.55	218.67	**227.13**
3.	*Kluyveromyces marxianus* + *Pichia stipitis*	237.33	220.27	207.82	201.78	205.16	**214.47**
4.	*Pachysolen tannophilus* + *Pichia stipitis*	260.80	243.38	230.04	222.05	225.96	**236.45**
5.	*Sacchoromyces cerevisiae* + *Zymomonas mobilis* + *Pichia stipitis*	165.87	145.78	142.76	141.15	142.75	**147.66**
6.	*Sacchoromyces cerevisiae* + *Candida shehatae*	278.40	262.75	241.42	232.36	239.82	**250.95**
7.	*Zymomonas mobilis* + *Candida shehatae*	251.20	235.91	220.45	209.42	216.18	**226.63**
8.	*Kluyveromyces marxianus* + *Candida shehatae*	243.73	227.02	208.18	205.15	262.75	**229.37**
9.	*Pachysolen tannophilus* + *Candida shehatae*	256.53	240.89	223.82	216.00	222.93	**232.04**
10.	*Sacchoromyces cerevisiae* + *Zymomonas mobilis* + *Candida shehatae*	215.46	205.51	189.16	182.75	188.62	**196.30**
11.	Control	1.68	1.52	1.50	1.47	1.50	**1.54**
	Mean	**213.50**	**199.02**	**186.35**	**180.45**	**190.04**	
			SE±			CD (1%)	
	Cultures (A)		0.97			3.61	
	Substrates (B)		0.66			2.44	
	Interaction (A x B)		2.18			8.08	

Again, co-inoculation of these organisms resulted in the highest ethanol yield in all the substrates, sugarcane bagasse (278.40 mg/g), sugarcane tops (262.75 mg/g), sugarcane trash (241.42 mg/g), corn husk (232.36 mg/g) and in corn stover (239.82 mg/g). The above co-inoculation of hexose and pentose fermenting microorganisms statistically highest ethanol yield over other combinations (Table 3a). The significantly lowest ethanol yield was recorded with the combined inoculation of *S. cerevisiae*, *Z. mobilis*, and *P. stipitis* in all the saccharified agro-residues.

Table 3b. Residual reducing sugars after co-fermentation of ethanol (mg/g) from selected pre-treated and saccharified agro-residues

Sl. No.	Cultures (A)	Substrates (B)					**Mean**
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cerevisiae* + *Pichia stipitis*	123.01	114.32	92.77	81.27	87.02	**99.68**
2.	*Zymomonas mobilis* + *Pichia stipitis*	27.38	24.50	22.35	21.39	23.07	**23.74**
3.	*Kluyveromyces marxianus* + *Pichia stipitis*	42.61	34.56	31.69	33.13	39.59	**36.32**
4.	*Pachysolen tannophilus* + *Pichia stipitis*	19.47	18.75	16.60	26.66	29.53	**22.20**
5.	*Sacchoromyces cerevisiae* + *Zymomonas mobilis* + *Pichia stipitis*	197.20	191.93	164.62	131.33	156.00	**168.22**
6.	*Sacchoromyces cerevisiae* + *Candida shehatae*	23.78	22.35	17.32	16.60	18.75	**19.76**
7.	*Zymomonas mobilis* + *Candida shehatae*	67.62	55.40	43.19	43.19	44.62	**50.80**
8.	*Kluyveromyces marxianus* + *Candida shehatae*	77.05	68.43	58.28	51.09	56.84	**62.34**
9.	*Pachysolen tannophilus* + *Candida shehatae*	60.12	45.34	33.84	30.97	32.41	**40.54**

10.	*Sacchoromyces cerevisiae* + *Zymomonas mobilis* + *Candida shehatae*	102.11	84.86	74.16	64.74	71.93	**79.56**
11.	Control	622.23	582.70	571.00	565.56	589.16	**586.13**
	Mean	**123.87**	**113.01**	**102.35**	**96.90**	**104.45**	
			SE±			CD (1%)	
	Cultures (A)		2.14			7.95	
	Substrates (B)		1.45			5.36	
	Interaction (A x B)		4.79			17.77	

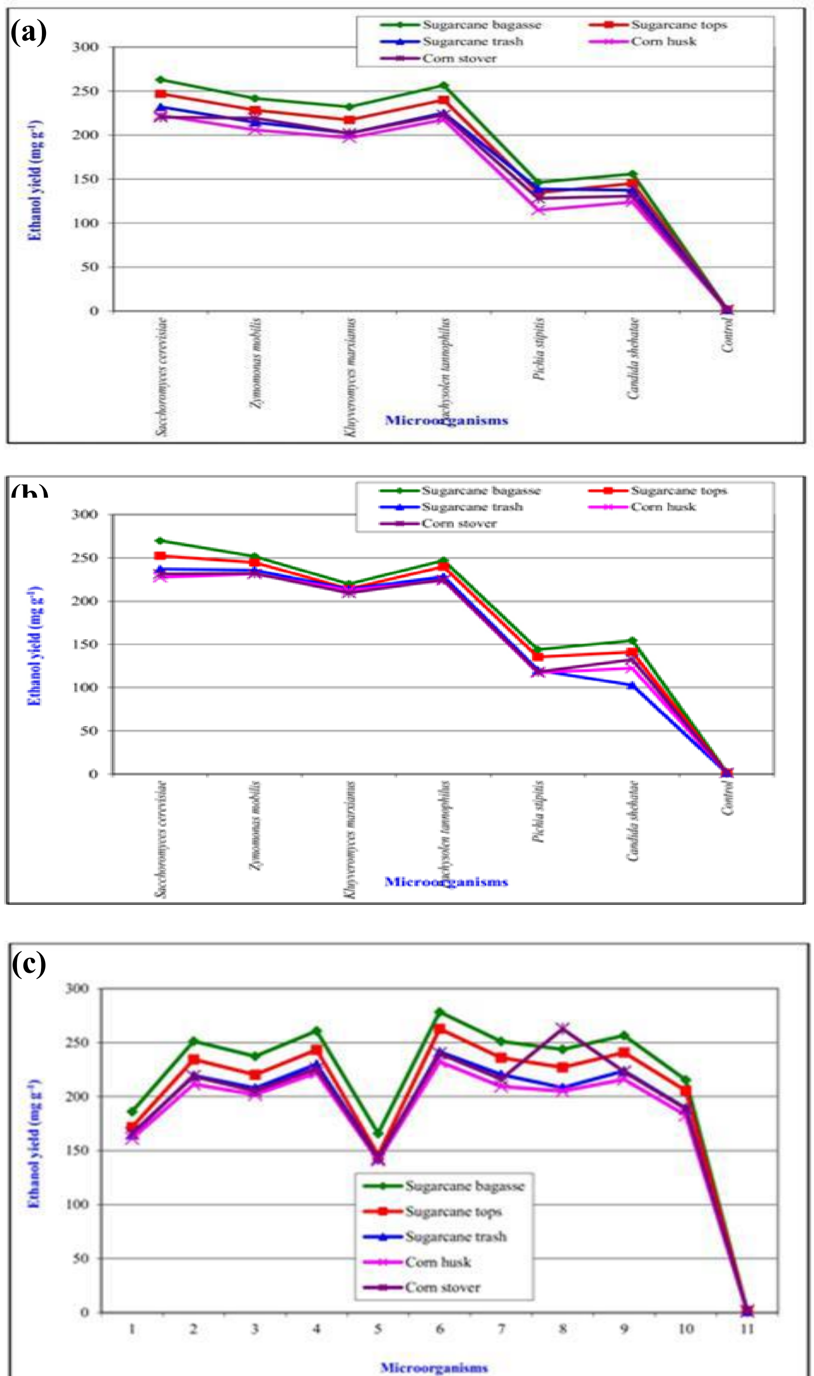

Figure 2 (a,b,c). Bioethanol production by different fermentation systems

The interaction effect of coinoculation of alcohol fermenting microorganisms and different substrates on residual reducing sugar content showed that the control treatment recorded the significantly highest residual reducing sugar content in all the agro-residues tried for bioethanol production. The significantly lowest residual reducing sugar content of 23.78, 22.35, 17.32, 16.60 and 18.75 mg per g was observed in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover respectively with dual inoculation of *S. cerevisiae* and *C. shehatae* (glucose and xylose fermenting yeasts), it indicates that these organisms are compatible for efficient conversion of both hexose and pentose sugars to ethanol (Sreenath et al., 2001). The combined inoculation of *S. cerevisiae*, *Z. mobilis* and *P. stipitis* recorded significantly the higher residual reducing sugar contents in all the substrates (Kemita et al., 2024). This could be due to incompatibility among the microorganisms to grow together and to cause fermentation of ethanol (Table 3b).

3.4 Pilot scale study

The pilot-scale fermentation over 20 L of volume (with 5% of solids loading of 20 L working volume) using a 300 L bioreactor yielded 223 g/kg (223 mg/g) bioethanol from pretreated sugarcane bagasse in the optimized conditions. Although this result proves the technical feasibility of scaling up the process, the ethanol yield was less than that obtained at the laboratory scale (278.40 mg/g in a 5 g substrate/100 mL system). The difference reflects typical scale-up issues related to the production of lignocellulosic bioethanol. One of the main reasons for the decreased yield was probably the lack of a buffering system in the pilot reactor. In laboratory experiments, saccharification and fermentation were carried out in 0.5 M buffer with a pH of 4.8, which resulted in the optimal cellulase activity and stable fermentation conditions. In contrast, pH variations in the pilot system may have diminished the efficiency of the enzymes and may have thus limited cellulose hydrolysis, and hence the availability of fermentable sugars.

Another important factor was the temperature of saccharification. In the pilot research work, the enzymatic hydrolysis was done at 37 °C, while commercial cellulase enzymes tend to have a temperature optimum at 50 °C. Operating at a temperature lower than the optimum temperature may have slowed the kinetics of hydrolysis and decreased the release of glucose, and thus limited the ethanol production (Malinee et al., 2022; Qi et al., 2023). In addition, larger volumes of reactors create mass and heat transfer issues, such as decreased efficiency of mixing and temperature variations, as well as possible accumulation of inhibitory compounds resulting from the degradation of lignin. Such factors can adversely affect the performance of the enzyme as well as the metabolism of the microbe. Similar scale-dependent decreases in ethanol production have been reported for lignocellulosic substrates, such as poplar wood and sunflower hulls, where process control parameters had a significant impact on the overall productivity (Ballerini et al., 1994; Sharma et al., 2004).

Despite these limitations, the outcome of the pilot-scale operation shows the strength and suitability of the integrated pretreatment, saccharification and fermentation (PSS) process. With better pH control, optimised saccharification temperature and better reactor design for mixing and heat transfer, ethanol yields at pilot and industrial scale could come closer to that at the laboratory-scale. These findings highlight the need for stringent control of operations in the scale-up process and are a good guideline to the commercialisation of the second-generation bioethanol from sugarcane bagasse.

4. Conclusions

This study examined second-generation bioethanol production from lignocellulosic agro-residues with the use of alkali-steam pretreatment, enzymatic saccharification and 3 different fermentation strategies (SHF, SSF and co-fermentation). Pretreatment contributed successfully to the improvement of the accessibility of the cellulose, and the hydrolysis of the sugars by enzymes resulted in a high yield of reducing sugars, with the sugarcane bagasse exhibiting the highest yield of sugar. Among the several methods of fermentation, *S. cerevisiae* was able to consistently give superior ethanol production, both in SHF and SSF. However, co-fermentation had the highest efficiency. The dual culture of *S. cerevisiae* and *C. shehatae* yielded the highest total ethanol production, especially from sugarcane bagasse and low residual sugars, attributed to efficient utilisation of hexose and pentose. Triple cultures exhibited decreased performance, most likely as a result of microbial incompatibility. Pilot scale validation proved process feasibility, but yields were modestly lower than the results of the laboratory work because of limitations in the operating conditions, such as suboptimal temperature and pH control. Overall, the combination of alkali-steam pretreatment, enzymatic saccharification, and dual co-fermentation (*S. cerevisiae* + *C. shehatae*) is found to be the most efficient approach for bioethanol production from lignocellulosic agro-residues, particularly sugarcane bagasse, with a good potential of becoming a more industrial process.

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