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SODIUM HYDROXIDE PRETREATMENT AND ENZYMATIC HYDROLYSIS METHOD USING XYLANASE ENZYME FOR FUEL ETHANOL PRODUCTION FROM ARECA NUT (*ARECA CATECHU* L.) HUSK WASTE BY YEASTS AND *ZYMOMONAS MOBILIS* NCIM 2915

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ABSTRACT

Pretreatment of lignocellulose has received considerable research globally due to its influence on the technical, economic and environmental sustainability of cellulosic ethanol production. The study involved the NaOH pretreatment and use of xylanase enzyme to degrade the complex lignocellulosic biomass to simple sugars. Sugars so formed in turn are converted to ethanol by employing suitable yeast strains and bacterium Zymomonas mobilis NCIM 2915. Different fermentation process like separate hydrolysis and fermentation process (SHF) and simultaneous saccharification and fermentation process (SSF) have been evaluated for ethanol production. In SHF process, it showed the higher ethanol production in P. stipitis NCIM 3498 (42.60±3.90 g/L) and low ethanol production in S. cerevisiae NCIM 3095 (25.24±2.45 g/L). In SSF process, it showed the higher ethanol production in S. pombe NCIM 3457 (45.76±4.10 g/L) and low ethanol production in S. cerevisiae NCIM 3095 (23.67±2.66 g/L) was monitored after the fermentation process. Hence, NaOH pre-treatment and enzymatic hydrolysis is more effective for ethanol production. The superiority of ethanol yield and productivity was very high in both SSF and SHF methods. Areca nut husk was revealed as a suitable substrate for ethanol production.

KEY WORDS

NaOH pre-treatment, Enzymatic hydrolysis, Areca nut husk, Yeasts, Zymomonas mobilis NCIM 2915, Ethanol.

1. INTRODUCTION

Production of biofuel from lignocellulosic feedstock has attracted significant interests worldwide. Lignocellulosic biomass including various agricultural residues is an abundant and renewable resource on earth [1]. Cellulose is the predominant polymer in lignocellulosic biomass, with a small amount of hemicellulose and lignin [2]. Enzymatic hydrolysis of cellulose is a promising method for the conversion of waste cellulose to glucose. Many factors, such as the content of lignin, crystallinity of cellulose, degree of polymerization (DP), moisture content and particle size, affect the hydrolytic efficiency of the cellulose present in the lignocellulosic biomass. Lignin is one of the most abundant polymers in nature. The complex three-dimensional (3D) polyaromatic matrix of lignin prevents enzymes from accessing some regions of the cellulose polymers [3]. In addition, the amorphous heteropolymer is also water-insoluble and optically inactive, which make it very difficult to be degraded.

Areca nut (*Areca catechu* L.) is one of the most important commercial crops in India. India ranks first in areca nut production in the world. In India the cultivation of areca nut is mostly confined to Karnataka, Kerala and Assam in terms of total area under cultivation and production is around 83 % [4, 5]. Areca nut popularly known as betel nut or supari is one of the most important plantation crop in Shivamogga district

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[6]. The area under areca nut cultivation has increased more rapidly in Shimoga district as compared with Dakshina Kannada and Uttara Kannada districts, Karnataka state, India [4, 5]. The areca nut husk fibers are predominantly composed of cellulose and varying proportions of hemicelluloses, lignin, pectin and protopection. The total hemicellulose content varies with the development and maturity, the mature husk containing less hemicellulose than the immature ones. The lignin content proportionately increases with the development until maturity [7].

Alkali pre-treatment has been observed to decrease cellulose crystallinity and the degree of polymerisation, which is considered to occur due to swelling of the internal cellulose microfibrils increasing the amount of amorphous regions of cellulose micro fibrils [8], thus increasing access for enzymes [9]. Alkali pre-treatment can induce the conversion of cellulose Iβ into cellulose II hydrate, which possesses a larger structure and faster enzymatic hydrolysis rate [10]. It is also known to increase amorphous cellulose regions and decrease degree of polymerisation increasing the susceptibility to enzymatic degradation of the lignocellulose [9].

The availability of areca nut is very high in Shivamogga district due its area and high productivity in this region [4, 5]. Areca nut husk is most abundant renewable energy source that may be considered as potential feed stock for ethanol production by microbial fermentation [11]. There is no report available on ethanol production from areca nut husk.

The major objective of the present investigation was to evaluate the effect of alkaline pretreatment and enzymatic hydrolysis method on areca nut husk for improved yield of reducing sugar and bioethanol production by yeasts and bacterium *Zymomonas mobilis* NCIM 2915. The process was carried out in two methods like separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation process (SSF) have been evaluated for the ethanol production.

2. MATERIALS AND METHODS

Collection of areca nut husk:

Areca nut husk waste was collected from the Shivamogga region, Karnataka state, India. The sample was brought to the laboratory and was maintained at room temperature for microbiological study.

Milling of areca nut husk:

The areca nut husk sample was sun dried for 24 hours in order to remove the moisture content present. And later the Areca nut husk was kept in hot air oven 80 °C for 24–48hr. Then, the Areca nut husk was completely air dried and the areca nut husk was poured to the milling machine for hammer milling, where the milling was done in order to cut the areca nut husk into small pieces. And, later areca nut husk were sewed using sewing machine having larger pore size and the bigger particles were obtained after sewing, bigger size particles were again allowed for the milling. So that the particles of small size having 3mm diameter have been obtained, later they obtained areca nut husk was again subjected to flour milling machine. The areca nut husk was finely powdered and the powder was sewed using a sewing machine, in order to obtain the fine sized particles (0.28 ± 0.01 mm) (Figure 1) [6, 12].

Figure 1a

Figure 1b

Figure 1c

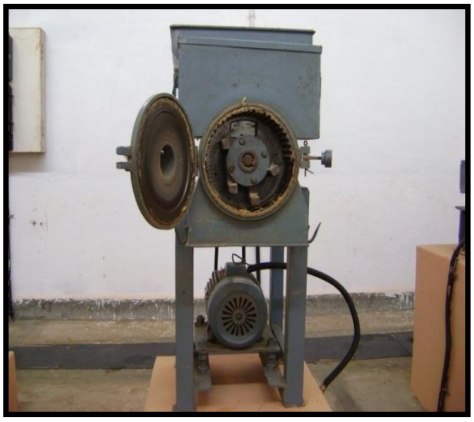


Figure 1d

Figure 1e

Figure 1f

Figure 1: A. Areca nut palm (plant), B. Areca nut husk, C. Dry areca nut husk, D. Hammer milling machine, E. Areca nut husk flour sample–'A' (fine size powder, 0.28±0.01mm), F. Areca nut husk flour sample–'C' (coarse size powder, 0.64 ± 0.01mm)

Procurement of xylanase enzyme:

The xylanase enzyme was obtained from Sigma Aldrich Company with product number X2753–10G, off-white powder derived from culture of *Thermomyces lanuginosus*. Xylanase preparation had >2500 units activity per gram and to maintain the temperature – 4 °C in refrigerator for further study.

Selection of yeast cultures and bacteria:

The standard yeast strains and bacteria used for the fermentation process were *Saccharomyces cerevisiae* NCIM 3095, *Candida shehatae* NCIM 3500, *Saccharomyces uvarum* NCIM 3455, *Pichia stipitis* NCIM 3498, *Schizosaccharomyces pombe* NCIM 3457 maintained on MGYP medium (Composition of MGYP medium: Malt extract 3g, Glucose 10g, Yeast extract 3g, Peptone 5g, Agar 20g, distilled water 1000 mL, Adjust p^H to 6.4-6.8) and bacterium *Zymomonas mobilis* NCIM 2915 was maintained on nutrient agar with 2% glucose (Composition of nutrient agar: Beef extract 10g, Sodium chloride 5g, Peptone 10g, Glucose 20g, Agar 20g, distilled water 1000 ml, Adjust p^H to 7.0–7.5). These yeast cultures and bacteria were procured from the National Chemical Laboratory (NCL), Pune, India [13, 14].

Inoculum preparation:

For inoculum preparation, yeast cultures was grown in YPD broth (Composition of YPD broth: Yeast extract 10g, Peptone 10g, Glucose 50g and Distilled water 1000mL) and bacterium *Zymomonas mobilis* NCIM 2915 was grown in nutrient broth with 2% glucose (Composition of nutrient broth: Beef extract 10g, Sodium chloride 5g, Peptone 10g, Glucose 20g and Distilled water 1000mL) at 30 °C in a rotary shaker (150 rpm) for 72 hours, harvested by centrifugation, washed three times with sterile distilled water and suspended in sterile water to get $1x10^6$ cells per mL [15].

Sodium hydroxide pretreatment:

In alkaline hydrolysis pretreatment, 100 gram of dried sample was weighed in to 2 liter conical flask and 1000 ml of 0.25 M NaOH solution was added to the conical flask. The flask was left for one hour, after which the mixture was neutralized with 0.1 M HCl to a pH of 4.5. The flask was allowed to cool at room temperature and filtered. The total reducing and non- reducing sugar content of pretreated raw materials was estimated. This filtrate will be used for further enzymatic saccharification [16].

Separate hydrolysis and fermentation (SHF) process

Step-1: Enzymatic pretreatment:

Separate hydrolysis and fermentation (SHF) was performed with media and it was sterilized (SHF media composition: Yeast extract 5g, Ammonium sulfate 7.5g, Dipotassium hydrogen phosphate 3.5g, Magnesium Sulfate heptahydrate 0.75g, Calcium chloride dehydrate 1g, Substrate 1g, distilled water 1000ml). Then, 1 mL of xylanase enzyme was added to each flask aseptically with filtrate (alkaline pretreated) and incubated at 50 °C for 4–6 hours. Then, the media was filtered using Whatman No. 1 filter paper. The filtrate was used for further studies.

Step-2: Fermentation:

Culture filtrate obtained from the above procedure were further inoculated with each of the organisms as mentioned above individually (3%) and allowed for fermentation for 72 hours. After fermentation, the sample was recovered by distillation unit for spectrophotometric analysis was used for determining ethanol concentration [16, 17].

Simultaneous saccharification and fermentation (SSF) process:

Simultaneous saccharification and fermentation (SSF) was performed with media and it was sterilized (SSF media composition: Yeast extract 5g, Ammonium sulfate 7.5g, Dipotassium hydrogen phosphate 3.5g, Magnesium Sulfate heptahydrate 0.75g, Calcium chloride dehydrate 1g, Substrate 1g, distilled water 1000ml) Then, 1 ml of xylanase enzyme was added to each flask aseptically with filtrate. All the Simultaneous Saccharification and Fermentation experiments were performed at 30 °C. The organism's inoculum was added after two days individually and incubated further for three days. Then, the media was filtered using Whatman No. 1 filter paper. After fermentation, the sample was recovered by distillation unit for spectrophotometric analysis was used for determining ethanol concentration [16].

Ethanol recovery by distillation process:

The fermented broth was dispensed into round bottom flask and fixed to a distillation column attached in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78 °C was used to heat the round bottomed flask containing the fermented broth. When the vapours enter the condenser, condenser will cool the vapours and 10 to 20 mL of distillate was collected in a test tube and immediately plugged in order to avoid escaping the alcohol [18].

Analytical methods

Determination of size, surface area, total solids and moisture content of the sample:

The determination of size, surface area, total solids and moisture content by convection oven method was estimated by using the method, as described by Patel, [16].

Determination of reducing sugar:

The glucose concentration was determined by Dinitrosalicylic acid (DNS) method, as described by Miller, [19]. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose.

Determination of non-reducing sugar:

Non-reducing sugars present in the extracts were hydrolyzed with sulfuric acid to reducing sugars. Then the total reducing sugars were estimated by DNS method. About 100 mg of the sample was taken and the sugars were extracted with 80 % alcohol (hot) twice (5mL each time). The supernatant was collected and evaporated on water bath. Ten ml of distilled water was added to dissolve the sugars. One ml of extract was pipette in to a test tube and 1ml of 1N H_2SO_4 was added. The mixture was hydrolyzed by heating at 49 °C for 30 min. and then 1 or 2 drops of methyl red indicator was added. The contents were neutralized by adding 1N NaoH drop wise from a pipette. Appropriate reagent blanks were maintained. Then total non-reducing sugar was estimated by DNS method [16, 20].

Determination of organic carbon, total nitrogen and protein:

The concentration of organic carbon was estimated by using the method described by Storer, [21]. The total nitrogen content was estimated by using the method described by Sadasivam and Manickam, [20]. The protein concentration was determined by the Lowry's method, as described by Lowry, [22] using bovine serum albumin (BSA) as a standard (0.2 mg/mL).

Determination of cellulose, hemicellulose and lignin:

The concentration of cellulose was estimated by using the method, as described by Updegraff, [23]. Hemicellulose concentration was determined using the method, as described by Deschatelets and Yu, [24]. Lignin concentration was determined using the gravimetric method, as described by Chesson, [25].

Determination of ethanol concentration by spectrophotometric method:

The amount of ethanol content was estimated by spectrophotometric method (JENWAY–6305, UV–VIS Spectrophotometer) as described by Caputi *et al*., [26].

Statistical analysis:

All the results were statistically analyzed using SPSS software to determine the mean of three replicates and its standard error value from independent experiments.

3. RESULTS

Initial composition of areca nut husk:

Areca nut husk flour sample were fractionated in to three graded particles such as sample– 'A' (0.28 ± 0.01 mm), sample–'B' (0.43 ± 0.02 mm) and sample–'C' (0.64 ± 0.01 mm). The initial composition of areca nut husk flour sample–'A' as follows, reducing sugar (3.40 ± 0.12 mg/g), non-reducing sugar (0.91 ± 0.03 mg/g), total sugars (4.79±0.14 mg/g), protein (3.16±0.08 mg/g), carbohydrate (2.14±0.07 mg/g), surface area of the sample (1.93 ± 0.02 m^2/Kg), density of the bed (610.4 ± 4.99), moisture content (3.30 ± 0.20 %), total solids (83.26 ± 0.92 %), organic carbon (31.49 ± 0.59 %), nitrogen (0.52 ± 0.02 %), cellulose (28.4 ± 0.85 %), hemicelluloses (8.30 ± 0.15 %), and lignin (8.0 ± 0.45 %) was estimated, as followed by areca nut husk flour sample–'B' and sample–'C' (Table 1).

Further studies, we have selected areca nut husk flour sample–'A' (fine size), this raw material is suitable and more effective for enzymatic saccharification for production of maximum sugar yield for ethanol production than compared to areca nut husk flour sample–'B' (medium size) and sample–'C' (coarse size).

Table 1: Initial chemical composition of areca nut husk

Sl. No.	Parameters	Different sized areca nut husk flour samples		
		Sample– 'A' (Fine size)	Sample– 'B' (Medium size)	Sample– 'C' (Coarse size)
1	Particle size (mm)	0.28 ± 0.01	0.43 ± 0.02	0.64 ± 0.01
2	Reducing sugars (mg/g)	3.40±0.12	3.18±0.09	2.91±0.01
3	Non-reducing sugars (mg/g)	0.91±0.03	0.37±0.001	0.30±0.001
4	Total sugars (mg/g)	4.79±0.14	3.55±0.10	3.21±0.11
5	Protein (mg/g)	3.16±0.08	5.80±0.16	6.21±0.16
6	Carbohydrate (mg/g)	2.14±0.07	2.82±0.04	4.16±0.19
7	Surface area of the sample (m^2/Kg)	1.93 ± 0.02	6.77 ± 0.08	12.60 ± 0.25
8	Density of the bed	610.4 ± 4.99	493.60 ± 2.62	472.47 ± 0.98
9	Moisture content (%)	3.30 ± 0.20	2.90 ± 0.10	2.60 ± 0.15
10	Total solids (%)	83.26 ± 0.92	75.29 ± 0.61	70.86 ± 0.81
11	Organic carbon (%)	31.49 ± 0.59	29.38 ± 0.12	23.64 ± 0.22
12	Nitrogen (%)	0.52 ± 0.02	0.55 ± 0.01	0.59 ± 0.04
13	Cellulose (%)	28.4 ± 0.85	39.5 ± 0.74	46.0 ± 1.53
14	Hemicellulose (%)	8.30 ± 0.15	32.4 ± 0.19	40.3 ± 0.23
15	Lignin (%)	8.0 ± 0.45	11.5 ± 0.35	21.0 ± 0.19

Note: **Results are mean ± S.E. of three replicates (*n*=3).**

Effect of alkaline pretreatment on areca nut husk:

The reducing (95.0±7.0 mg/g) and non-reducing sugar (37.0±3.60 mg/g) yield was maximum than compared with initial composition of areca nut husk in the alkaline treatment of 0.75M NaOH, at room temperature (28 °C) and 1-hour reaction time.

Enzymatic pretreatment:

The NaOH pretreated biomass was fermented with enzymatic saccharification using xylanase enzyme to increases the maximum reducing sugar yield was 105.0±5.49 mg/g and non-reducing sugar was 42.0±4.0 mg/g of the areca nut husk sample.

Separate hydrolysis and fermentation (SHF) process:

The reducing and non-reducing sugars content of the samples, before and after fermentation was estimated. After fermentation, the concentration of residual reducing and non-reducing sugar of the fermented samples treated with different yeast cultures were as follows, *S. cerevisiae* NCIM 3095 (10.2±0.10, 6.0±0.09 mg/g), *C. shehatae* NCIM 3500 (11.6±0.12, 4.2±0.05 mg/g), *S. uvarum* NCIM 3455 (9.31±0.09, 5.2±0.04 mg/g), *P. stipitis* NCIM 3498 (7.0±0.08, 3.6±0.03 mg/g), *S. pombe* NCIM 3457 (11.0±0.10, 3.0±0.03 mg/g) and bacterium *Z. mobilis* NCIM 2915 (10.5±0.14, 4.4±0.06 mg/g). The percentage of sugar used in the fermented samples was estimated (Table 2).

The ethanol production was estimated in the fermented sample by SHF method treated with different yeast cultures were as follows, *S. cerevisiae* NCIM 3095 (25.24±2.45 g/L), *C. shehatae* NCIM 3500 (31.56±3.12 g/L), *S. uvarum* NCIM 3455 (33.13±3.0 g/L), *P. stipitis* NCIM 3498 (42.60±3.90 g/L), *S. pombe* NCIM 3457 (37.87±4.0 g/L) and bacterium *Z. mobilis* NCIM 2915 (41.81±3.85 g/L) (Figure 2).

Simultaneous saccharification and fermentation (SSF) process:

The concentration of reducing and non-reducing sugars of the samples, before and after fermentation was estimated. Before fermentation, the concentration of reducing sugar was 105.0±5.49 mg/g and non-reducing sugar was 42.0±4.0 mg/g in the sample using xylanase enzyme for enzymatic saccharification. After fermentation, the concentration of residual reducing and non-reducing sugar of the fermented samples treated with different yeast cultures were as follows, *S. cerevisiae* NCIM 3095 (11.0±0.10, 3.2±0.04 mg/g), *C. shehatae* NCIM 3500 (4.0±0.09, 4.0±0.06 mg/g), *S. uvarum* NCIM 3455 (12.3±0.14, 6.6±0.09 mg/g), *P. stipitis* NCIM 3498 (6.4±0.08, 3.0±0.02 mg/g), *S. pombe* NCIM 3457 (5.0±0.06, 5.0±0.08 mg/g) and bacterium *Z. mobilis* NCIM 2915 (10.0±0.20, 3.8±0.09 mg/g). The percentage of sugar used in the fermented samples was estimated (Table 3).

The ethanol production was estimated in the fermented samples by SSF method treated with different yeast cultures were as follows, *S. cerevisiae* NCIM 3095 (23.67±2.66 g/L), *C. shehatae* NCIM 3500 (28.40±2.99 g/L), *S. uvarum* NCIM 3455 (41.02±3.45 g/L), *P. stipitis* NCIM 3498 (39.45±3.40 g/L), *S. pombe* NCIM 3457 (45.76±4.10 g/L) and bacterium *Z. mobilis* NCIM 2915 (42.60±4.0 g/L) (Figure 2).

Table 2: Production of reducing and non-reducing sugar (mg/g) from areca nut husk treated by NaOH pretreatment and enzymatic hydrolysis (xylanase enzyme) during separate hydrolysis and fermentation (SHF) process.

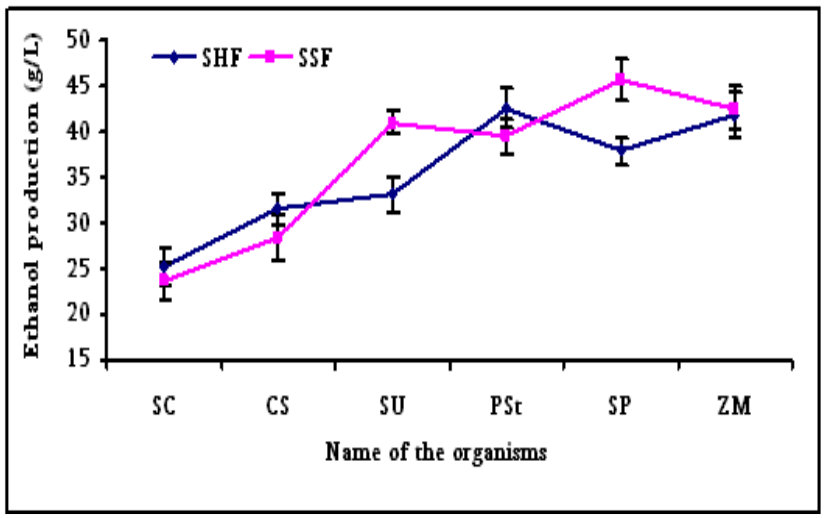
Sl. No.	Name of the organisms	Reducing sugar				Non-reducing sugar			
		Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)	Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)
1	*S. cerevisiae* NCIM 3095	105.0±5.49	10.2±0.10	94.8±3.52	90.28±4.0	42.0±4.0	6.0±0.09	36.0±2.70	85.71±4.30
2	*C. shehatae* NCIM 3500	105.0±5.49	11.6±0.12	93.4±3.85	88.95±4.56	42.0±4.0	4.2±0.05	37.8±2.90	90.0±4.0
3	*S. uvarum* NCIM 3455	105.0±5.49	9.31±0.09	95.69±2.89	91.13±3.65	42.0±4.0	5.2±0.04	36.8±3.10	87.61±3.85
4	*P. stipitis* NCIM 3498	105.0±5.49	7.0±0.08	98.0±2.80	93.33±3.89	42.0±4.0	3.6±0.03	38.4±3.50	91.42±4.0
5	*S. pombe* NCIM 3457	105.0±5.49	11.0±0.10	94.0±3.80	89.52±4.10	42.0±4.0	3.0±0.03	39.0±2.40	92.42±3.98
6	*Z. mobilis* NCIM 2915	105.0±5.49	10.5±0.14	94.5±4.25	90.0±4.20	42.0±4.0	4.4±0.06	37.6±3.0	89.52±4.22

Note: Results are mean ± S.E. of three replicates (*n*=3).

Table 3: Production of reducing and non-reducing sugar (mg/g) from areca nut husk treated by NaOH pretreatment and enzymatic hydrolysis (xylanase enzyme) during in simultaneous saccharification and fermentation (SSF) process.

Sl. No.	Name of the organisms	Reducing sugar				Non-reducing sugar			
		Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)	Initial sugar (mg/g)	Final sugar (mg/g)	Sugar used (mg/g)	Sugar used (%)
1	*S. cerevisiae* NCIM 3095	105.0±5.49	11.0±0.10	94.0±3.86	89.52±4.65	42.0±4.0	3.2±0.04	38.8±4.0	92.38±5.20
2	*C. shehatae* NCIM 3500	105.0±5.49	4.0±0.09	101.0±4.0	96.19±5.10	42.0±4.0	4.0±0.06	38.0±3.85	90.47±4.95
3	*S. uvarum* NCIM 3455	105.0±5.49	12.3±0.14	92.7±3.94	88.28±4.80	42.0±4.0	6.6±0.09	35.4±3.66	84.28±4.60
4	*P. stipitis* NCIM 3498	105.0±5.49	6.4±0.08	98.6±4.20	93.90±4.63	42.0±4.0	3.0±0.02	39.0±4.10	92.85±3.89
5	*S. pombe* NCIM 3457	105.0±5.49	5.0±0.06	100±4.51	95.23±3.98	42.0±4.0	5.0±0.08	37.0±3.90	88.09±3.90
6	*Z. mobilis* NCIM 2915	105.0±5.49	10.0±0.20	95.0±5.0	90.47±5.0	42.0±4.0	3.8±0.09	38.2±3.80	90.95±4.0

Note: Results are mean ± S.E. of three replicates (*n*=3).



Note: **SC**–*Saccharomyces cerevisiae* NCIM 3095, **CS**–*Candida shehatae*, NCIM 3500, **SU**–*Saccharomyces uvarum* NCIM 3455, **PSt**–*Pichia stipitis* NCIM 3498, **SP**–*Schizosaccharomyces pombe* NCIM 3457 and **ZM**-*Zymomonas mobilis* NCIM 2915, **SHF-** Separate Hydrolysis and Fermentation, **SSF**- Simultaneous Saccharification and Fermentation process.
Vertical bar indicates the standard error value of three replicates (*n*=3).

Figure 2. Production of ethanol (g/L) from areca nut husk treated by NaOH pretreatment and enzymatic hydrolysis (xylanase enzyme) by SHF and SSF methods.

4. DISCUSSION

Energy dependence on fossil fuels can lead to depletion, environmental degradation, and economic instability. The effect of fossil fuel dependency has increased the number of research-oriented generations of alternative energy sources that are sustainable and renewable. These alternative sources include biodiesel, bioethanol, and biogas. Bioethanol, just like other biofuels, contributes to the reduction of pollutant emissions and prevents the greenhouse effect due to the traditional process of its production from lignocellulosic materials [27].

Lignocellulosic materials mainly contain lignin and a carbohydrate matrix (holocellulose) which, consists of cellulose and hemicelluloses. These materials are connected by chains of ether or ester bonds (lignin and hemicellulose), hydrogen bridges (cellulose and hemicellulose), and other removable and inorganic materials in minor proportions [27, 28].

In the current study, sodium hydroxide pre-treatment was chosen as the best pre-treatment to release cellulose and hemicellulose from lignin in the biomass of areca nut husk. The concentration of 0.75M NaOH was used for the pre-treatment of areca nut husk (sample-A) to yield maximum reducing sugar (95.0±7.0 mg/g) and non-reducing sugar (37.0±3.60 mg/g). The sodium hydroxide pre-treated biomass was fermented with enzymatic saccharification using xylanase to increase the maximum reducing (105.0±5.49 mg/g) and non-reducing sugar (42.0±4.0 mg/g) yield in SHF and SSF methods.

It has been shown that alkali pre-treatment increases access to the cellulose by saponification of the intermolecular ester bonds between hemicellulose and lignin. The cleaving of these links can cause solubilisation of lignin and hemicelluloses [9]. Alkali pre-treatment can also result in the removal of acetyl and various uronic acid substitutions on hemicelluloses [2, 29]. Xylans have been shown to be more susceptible to lower concentrations of alkali than glucomannan, suggesting that hardwoods would be more susceptible to lower levels of alkali pre-treatment [30], which is consistent with the results reported by Mirahmadi et al., [31], observed larger yields from hardwoods pre-treated at low temperature with NaOH

In SSF process, higher sugar utilization (100±4.51 mg/g) was observed and percentage of 95.23±3.98 sugar in *S. pombe* NCIM 3457. In SHF process, higher sugar utilization (98.0±2.80 mg/g) was observed and percentage of 93.33±3.89 sugar in *P. stipitis* NCIM 3498 was observed after fermentation period. The NaOH pre-treatment and enzymatic hydrolysis and effective microbial strains were used to enhance the maximum sugar and ethanol production from areca nut husk. We observed that the sugars utilization efficiency was high in the SSF compared to SHF process. Maria *et al*., [32] reported that NaOH pre-treatment causes swelling of the lignocellulosic materials, leading to an increase of the internal surface area, a decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure.

In the present study, we observed that maximum ethanol production was shown by *P. stipitis* NCIM 3498 (42.60±3.90 g/L), and low ethanol production was shown by *S. cerevisiae* NCIM 3095 (25.24±2.45 g/L) in SHF method. In SSF method, we observed that maximum ethanol production was shown by *S. pombe* NCIM 3457 (45.76±4.10 g/L), and lowest ethanol production was shown by *S. cerevisiae* NCIM 3095 (23.67±2.66 g/L).

Simultaneous Saccharification and fermentation (SSF) process was considered as preferably process because of reduced operation all costs, lower enzyme requirement and increased productivity [33, 34]. SSF process can use single reactor and the same temperature for saccharification and fermentation process, so the reduction of operation cost can be achieved. Glucose produced from hydrolysis is simultaneously metabolized by microorganism, thereby alleviating problems caused by product inhibition in SSF process [34, 35].

Moreover, NaOH pre-treatment, enzymatic hydrolysis, different types of yeast strains and bacterium *Z. mobilis* NCIM 2915 used in fermentation would play a very important role in the conversion of the waste biomass into fuel ethanol by fermentation. Only few yeast strains and bacterium *Z. mobilis* NCIM 2915 were used to evaluate the ethanol production and still needs an effective method for improvement of strains to produce ethanol production. The energy sector has played a crucial role in the context of the global economy as well as the socio-economic development. The present study was undertaken to utilize the areca nut husk waste for bioethanol production by microbial fermentation.

CONCLUSION

Saccharification of NaOH pre-treatment and enzymatic hydrolysis (xylanase enzyme) pre-treated sample resulted in higher reducing (105.0±5.49 mg/g) and non-reducing sugar yield (42.0±4.0 mg/g) in both SHF and SSF methods. Xylanase enzyme as potential for the hydrolysis and saccharification of lignocellulosic material in order to obtain fermentable sugars that can be converted into second generation ethanol by fermenting effective yeast strains and bacterium *Zymomonas mobilis* NCIM 2915. Hence, NaOH pre-treatment and enzymatic hydrolysis is more effective for ethanol production. The superiority of ethanol yield and productivity was very high in both the methods such as simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Hence areca nut husk was revealed as a suitable substrate for ethanol production.

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