Establishing an Effective Multi-Function System for Sugarcane Bagasse Bio-degradation

Said El-Sayed Desouky, Mamdouh Salem El-Gamal, Tamer I.M. Ragab, Samy Bayoumy, Abdel-Rahman Mosaad Khattab

Abstract— Sugar cane processing generates large amount of bagasse. Disposal of bagasse is critical for both agricultural profitability and environmental protection. Sugar-cane bagasse (SCB), being renewable and cheap substrates, has the potential to displace fossil fuels for the production of fuels and value-added chemicals. In this study, a complete degradation for SCB using chemical and biological methods was done to maximize its utilization. Chemical treatment degraded SCB into wax, cellulose, hemicellulose and lignin. Further hydrolysis was carried out by biological treatments using different microbial isolates obtained from different localities in Egypt. Out of one-hundred and twelve bacterial isolates, 40 isolates showed better performance for SCB biodegradation, based on the recoverd reducing sugars. Data analysis was carried out with PRIMER 6 (Plymouth Routines In Multivariate Ecological Research) to analyze species diversity against substrate degradation rate. Isolate CS5 was selected as the most promising strains for SCB biodegradation. Finally, various nutritional and environmental parameters affected SCB biodegradation using isolate CS5 were investigated. The results obtained in this study indicate that strain CS5 has potential value for the degradation of SCB to produce high concentration of reducing sugars that would be easily available for various biotechnological applications especially for the bio-production of green chemicals.

Index Terms — Agriculture wastes, Alkaline treatment, Biodegradation, Chemical degradation, Sugar-cane bagasse, Biotechnology.

1 Introduction

Sugar cane bagasse (SCB), a waste in the process of sugar extraction, is an abundant and low-cost lignocelullosic material [1-3]. The most frequent use for SCB is the energy production by its combustion [3-5]. This provokes a problem of pollution, increasing the emissions of CO_2. Other alternative uses of SCB are the production of chemical compounds such as furfural or hydroxymethylfurfural (HMF) [6, 7], the production of paper paste [8-10]. On the other hand, the application of SCB in bioprocesses not only provides alternative substrates but also helps solve their disposal problems. With the advent of biotechnological innovations, it can be utilized for value added products such as acetone, ethanol, butanol, lactic acid, xylitol,…..etc [3, 11-13]. Therefore, a double effect will be obtained, economic and ecologic.

SCB has a complex structure that consists of cellulose 43.6%, hemi- cellulose 33.8%, lignin 18.1%, ash 2.3% and wax 0.8% on a dry weight basis. These polymers cannot be easily converted to monomeric sugars due to their recalcitrant nature. Therefore, the treatment of SCB is necessary to obtain fermentable sugars as cheap alternative substrates for production of valuable chemicals, thus increasing the economy of the process. SCB hemicelluloses (mainly arabinoxylans) contains a high content of xylose and lower of arabinose [5, 14-18]. D-xylose can be used for xylitol production through microbial hydrogenation. Xylitol, a functional sweetener with important technological properties, can be also used for treatment of diabetics [20]. On the other hand, cellulose is mainly composed of glucose units that is available to most microbial fermentations.

To achieve complete hydrolysis of SCB, several chemical or physical pretreatments followed by enzymatic hydrolysis of pretreated lignocellulosic materials are necessary to maximize fermentable sugars recovery. Chemical treatments uses acids or bases at a moderate temperature (100–150 °C). It usually results in the hydrolysis of hemicellulose fraction to xylose but it does not attack the cellulosic fraction in an appreciable extension. The most used acid are H_2SO_4 [5, 7, 17, 22, 26, 27], HCl [28] or HNO3 [15] while sodium hydroxide (NaOH) or potassium hydroxide (KOH) are usually used for alkaline treatment. NaOH is more suitable for non-woody plants such as straw, husk and bagasse to extract hemicellulose, whereas KOH is much preferable in woody plants such as oil palm frond [29]. Although chemical treatment is resulted in various monomeric sugars (xylose, glucose and arabinose), some soluble materials such as lignin, acetic acid, furfural and hydroxyl methyl furfural are also produced, which can inhibit both growth and sugar utilization of microorganisms during the fermentation process. Therefore, the sugar hydrolysates need further processing before fermentation. In general the following operations are needed: sugar concentration, detoxification, and neutralization prior to medium supplementation and fermentation [21, 23, 24]. The sugar concentration of hydrolysates is usually carried out by evaporation to remove excess water. Detoxification process is usually carried out by adsorp-

- *Abdel-Rahman Mosaad Khattab is currently pursuing master degree program in Faculty of Science (Boys Branch), Al-Azhar University, Cairo, Egypt, PH-01114017513. E-mail: abdelrhmankhattab@gmail.com*
- *Said El-Sayed Desouky and Mamdouh Salem El-Gamal are currently in Faculty of Science(Boys Branch), Al-Azhar University, Cairo, Egypt*
- *Tamer I.M. Ragab is currently in National of Research center(NRC), El-Dokki, Cairo, Egypt*
- *Samy Bayoumy is currently in Egyptian Petroleum Research Institute (EPRI), Nasr City, Cairo, Egypt*

International Journal of Scientific & Engineering Research Volume 5, Issue 10, October-2014
ISSN 2229-5518

tion on charcoal to remove the growth inhibitors. In this operation, phenolic compounds proceeding from lignin can also be removed [7, 19, 25]. The neutralization process uses chemicals (e.g. calcium carbonate) to neutralize the hydrolysates, forming salts [26, 27]. These salts have low solubility and are normally removed by filtration. Finally, the processed hydrolysates are supplemented with several nutrients to be a favorable fermentation medium. These nutrients contribute the nitrogen and micronutrients needed for the growth of the microorganisms.

In contrast to hemicellulosic fraction, enzymatic hydrolysis of the cellulosic fraction by cellulase enzymes is the widely used approach for saccharification processes. However, the high cost of commercial cellulase enzymes is still problematic hindering the effective utilization of lignocellulosic materials. Therefore, use of such materials and low cost degradation process would significantly reduce the cost of fermentation products. Direct microbial degradation as a second step for chemically pretreated SCB as carbon sources would achieve high degradation efficiency as well as enhance the overall treatment process cost.

The aim of this work was to determine the conditions for lignin extraction from sugarcane bagasse, and to investigate the optimal chemical and microbial treatment for hydrolysis of sugarcane bagasse in order to obtain the hydrolysate containing high fermentable sugars for use as a substrate for value added products.

2 Material and Method

2.1. Collection and preparation of SCB sample

SCB was collected from the local market in Egypt, air dried then chipped, ground and sieved to size (0.5–1.0 cm). Ground materials were then stored at room temperature until analysis and treatment.

2.2. Physical and Chemical pretreatments

2.2.1. Physical pretreatment

Milling pretreatment: Ten grams of chipped and ground substrate were put into a 250 mL Erlenmeyer flask. It was then moistened with distilled water; the flasks were incubated for 2 h at room temperature. The solid material was then mixed vigorously with 100 mL distilled water for extraction of soluble reducing sugars, then filtered to separate the contents into liquid and solid parts. The filtrate was centrifuged at 10,000 rpm for 10 min.

2.2.2. Chemical Decomposition

Chemical decomposition was carried out as illustrated in Fig. 1. Bagasse Dewaxing: wax was removed from bagasse by immersing the bagasse cuts in toluene/ethanol (2:1, *v/v*) mixture for overnight. The mixture was then filtrated, the oily residue was accurately weighed after evaporation of the solvent under reduced pressure to determine wax content. Free wax bagasse residue were then dried at 60 °C for 16 h. All weights and calculations were made on an oven-dried (60 °C, 16 h) basis.

Alkaline treatment: bagasse residue was mixed with dilute sodium hydroxide solution (1%) at 1:20 *w/v* solid to liquid ratio and then incubated at 55°C for 1h. The reaction mixture then was filtered to get solid bulk (cellulose). This step was repeated for three times. The filtrate (hemicellulose+ lignin) was then neutralized with HCl and then filtered to separate lignin and hemicellulose fractions. Analyses of the main fractions (cellulose, hemicelluloses and lignin) were carried out using alkaline hydrolysis under standard conditions by the modified method of [40].

2.3. Biological treatment

2.3.1 Media

Nutrient agar (NA) medium was used for isolation, purification and maintenance of microbial isolates [30, 31, 32]. Minimal salt medium (MSM) supplemented with different saccharides separately was used for screening of microbial utilization of different saccharides according to [33] .

2.3.2. Isolation and purification of different microorganisms

Ten grams of the substrate was added under aseptic conditions to 90 mL of sterile saline solution (8.5 g/L, NaCl) in 250 mL conical flasks. The flasks were shaken at 200 rpm for 60 min. After that, the suspensions were serially diluted and inoculated onto NA agar plates. The plates were then incubated at 30 °C for 24 h. The well-grown colonies were picked up, streaked on a MSM medium for purification. Pure separated single colonies were maintained on sterile slants at 4 °C for further investigation. The culture slants were sub-cultured monthly. The selected isolates were preserved in micro-tubes containing 1:1 (*v/v*, glycerol [30%]: pure isolate in appropriate

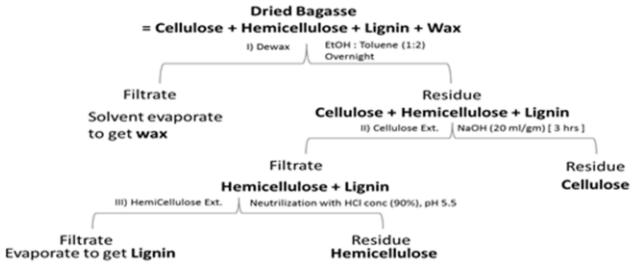

Fig. 1. Schematic diagram showing the dewaxing and alkaline treatment processes of sugar cane bagasse.

International Journal of Scientific & Engineering Research Volume 5, Issue 10, October-2014
ISSN 2229-5518

medium) at minus 40°C.

2.3.3. Screening for microbial utilization of different saccharides as sole carbon and energy sources

All the isolates strains were streaked on minimal salt medium supplemented with 0.6% (w/v) of substrate [bagasse, dewaxed bagasse, cellulose and hemi cellulose].

2.3.4. Microbial biodegradation of SCB

112 isolates were evaluated for their ability to degrade the different treatments of bagasse separately as the sole carbon source. Sampling: samples were collected from different localities: (1) Bagasse Soil, Banha governorate, (8) Textile Co. Soil, (9) Gas Station (Badrashin), (10) El-Kok Co. Soil, (11) Sludge soil (12) Swage soil, (13) Pesticided soil, (14) Paints Co. soil, (15) South Sinai soil, (16) Marine (On shore) soil. All samples were collected in clean and sterile bottles and plastic bags.
Saccharification: The saccharification percentage was also calculated as described by Uma et al. [21] by the following formula:

Saccharification %=(Reducing sugar×0.9×100)/(Cellulose of pretreated substrate)

2. 4. Analytical methods

Hemi-cellulose, cellulose, and lignin percent in bagasse samples were determined before treatment [as total carbohydrate (TC) and low molecular weight carbohydrate (LMWC) according to [34]] and after hydrolysis. Moisture content was determined by direct subtracting the substrate dry weight, after drying in an oven at 105 °C to a constant weight. Ash content obtained in a muffle furnace by heating bagasse of known moisture content, at 350°C till a constant weight [34]. Total lipids and fats determined using SCB was extracted separately with petroleum ether in a soxhelt apparatus for 48h, the oily residue was accurately weighed after evaporation of the solvent under reduced pressure. After soxhlet extraction LMWC was determined by dissolving in 85% ethanol for 24 h. The resulting alcoholic extract was decolorized by boiling with charcoal, concentrated under reduced pressure at 45°C and then examined by paper chromatography using *n*-butanol-Acetone-Water (4:5:1, v/v) as a solvent mixture [35]. Detection of spots was achieved by spraying the papers with Aniline-phthalate reagent [36] and analine-xylose. Glucose, Arabinose and Xylose in appropriate ratios were determined in the extract using phenol-sulfuric acid method [37]. The details of this method were as follows: After suitable dilution, 1 ml of 5% phenol solution was added to 1 ml of the resulted diluted solution. After mixing, 5 ml conc. H_2SO_4 was added rapidly to the mixture, shaked and set aside for 10 min at room temperature, then at 20-30 °C (in a water bath) for 20 min. Thereafter, the color density was measured at 480 nm for pentoses and 490 nm for hexoses and total carbohydrate by phenol-sulfuric acid hydrolysis according to [38]. Total reducing sugars ware determined by the 3,5-dinitro salicylic acid (DNS) method [39] and glucose was used as a standard. The samples were stored in a fridge at -18°C until analysis to prevent spoilage by microbes.

2.5. Statistical analysis

All experimental data were carried out in triplicates and averages are given. All experimental data were carried out in triplicates and averages are given. To compare the isolates population with SCB substrates use the PRIMER (Plymouth Routines in Multivariate Ecological Research) program to calculate and plot the similarity of each pair of samples. Construct an Excel table that contains the data from all the samples.

3 Results and discussion

3.1. Chemical composition of sugar-cane bagasse

The chemical composition of SCB was investigated as described in 'Material and method' section. As shown in Table 1, SCB consists of various components including wax, lipid, protein and carbohydrate. Of these, the carbohydrate content is the major component at 54.6 %. To be accessible for microbial degradation, several treatment methods are further required.

TABLE 1
CHEMICAL COMPOSITION OF SUGAR-CANE BAGGAGE (%, W/W)

Content	% (w/w)
Moisture	21.0 ±1.0
Ash	4.00 ±0.17
Lipids	2.80 ±0.03
LMWC	26.4 ±0.9
Wax	4.50 ±0.15
Protein	0.670 ±0.040
Total Carbohydrates	54.6 ±0.8

LMWC, low molecular weight carbohydrates; ±SD, ± standard deviation

3.1. Chemical hydrolysis of sugar-cane bagasse

The chemical hydrolysis of SCB was carried out using two steps to separate its components. Ethanol/toluene mixture was used in the first step to remove waxes. Alkaline treatment using sodium hydroxide was used to separate SCB into major components. Our results showed that SCB contains cellulose, hemicellulose and lignin at 64.3%, 14.8 % and 6.8 %, respectively. Lignin and wax separation can be used in many applications. Alkaline treatment of lignocellulosic substances disrupts the cell wall by dissolving hemicelluloses and lignin and by swelling cellulose, and decreasing the crystallinity of cellulose.

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ISSN 2229-5518

TABLE 2
THE COMMON ISOLATE DEGRADATION RATE OF SCB SUBSTRATE WITH AVERAGE DISSIMILARITY FOR EACH ISOLATE

Core isolates	B*	D*	C*	H*	Av. Diss.					
					B\D	B\C	D\C	B\H	D\H	C\H
CS1	0.80	1.60	2.10	2.10	0.00	0.17	0.00	0.16	0.00	0.00
Cs2	2.00	1.25	2.40	2.25	0.00	0.00	0.00	0.00	0.10	0.00
CS3	1.60	1.15	2.95	2.05	0.00	0.12	0.18	0.00	0.10	0.00
CS4	0.50	1.10	2.55	2.75	0.00	0.84	0.83	0.79	0.78	0.00
CS5	0.40	1.00	1.85	1.85	0.00	0.78	0.77	0.72	0.71	0.00
HS1	1.05	0.55	1.45	0.75	0.00	0.00	0.16	0.00	0.00	0.10
HS2	1.35	0.53	1.85	2.55	0.15	0.00	0.21	0.11	0.25	0.00
HS3	0.85	1.05	2.60	3.20	0.00	0.21	0.17	0.23	0.20	0.00
HS4	1.45	0.65	2.00	1.65	0.00	0.00	0.19	0.00	0.14	0.00
LS2	0.75	1.45	2.65	1.15	0.00	0.23	0.00	0.00	0.00	0.15
BD	1.17	0.75	1.60	1.47	0.00	0.00	0.00	0.00	0.10	0.00
BC	1.05	0.85	1.65	0.58	0.00	0.00	0.00	0.00	0.00	0.16
BL	1.50	1.50	1.52	1.09	0.00	0.00	0.00	0.00	0.00	0.00
CIIB	1.10	1.70	1.05	1.44	0.00	0.00	0.00	0.00	0.00	0.00
CIIH	1.14	2.37	1.95	2.00	0.00	0.00	0.00	0.00	0.00	0.00
FB	1.34	2.40	1.60	1.80	0.00	0.00	0.00	0.00	0.00	0.00
FD	0.90	1.55	0.70	1.80	0.00	0.00	0.00	0.11	0.00	0.15
FC	1.00	2.50	2.05	1.15	0.17	0.13	0.00	0.00	0.14	0.10
FH	1.40	2.20	1.95	1.90	0.00	0.00	0.00	0.00	0.00	0.00
GB	0.85	1.15	1.70	1.55	0.00	0.00	0.00	0.00	0.00	0.00
GH	1.10	1.90	1.80	1.80	0.00	0.00	0.00	0.00	0.00	0.00
HB	1.50	1.85	1.85	2.00	0.00	0.00	0.00	0.00	0.00	0.00
HD	1.05	1.50	1.30	1.75	0.00	0.00	0.00	0.00	0.00	0.00
ID	1.15	2.15	1.00	1.40	0.00	0.00	0.00	0.00	0.00	0.00
IH	1.25	1.15	1.00	2.20	0.00	0.00	0.00	0.00	0.11	0.14
LL	1.20	2.45	2.25	2.30	0.00	0.00	0.00	0.11	0.00	0.00
MB	0.80	0.80	0.90	0.75	0.00	0.00	0.00	0.00	0.00	0.00
Sh4	1.75	0.50	1.65	1.04	0.20	0.00	0.19	0.00	0.10	0.00
Sh70	1.40	0.40	1.37	0.37	0.19	0.00	0.19	0.19	0.00	0.19
S2I	2.10	1.40	1.45	1.15	0.00	0.00	0.00	0.00	0.00	0.00
S2IV	2.15	1.75	2.00	1.55	0.00	0.00	0.00	0.00	0.00	0.00
S1 V	1.75	0.65	2.00	1.15	0.16	0.00	0.19	0.00	0.00	0.10
S1III'	1.00	1.00	1.15	0.65	0.00	0.00	0.00	0.00	0.00	0.00
S3II	2.55	2.00	1.65	1.55	0.00	0.00	0.00	0.00	0.00	0.00
S3III	2.00	1.75	1.60	1.55	0.00	0.00	0.00	0.00	0.00	0.00
S3IV	2.00	1.15	1.00	1.00	0.00	0.13	0.00	0.12	0.00	0.00
S3VII	2.45	2.00	1.85	1.55	0.00	0.00	0.00	0.00	0.00	0.00
S3VIII	3.00	2.00	1.75	1.50	0.00	0.00	0.00	0.13	0.00	0.00
S4II	2.00	1.45	1.00	1.25	0.00	0.13	0.00	0.00	0.00	0.00
S5I	2.00	1.45	1.15	1.55	0.00	0.00	0.00	0.00	0.00	0.00
MGI	1.45	1.00	1.55	0.88	0.00	0.00	0.00	0.00	0.00	0.09
MGIII	3.00	1.45	1.85	1.15	0.14	0.00	0.00	0.17	0.00	0.08
MGV	1.45	1.65	1.85	1.33	0.00	0.00	0.00	0.00	0.00	0.00
C1	1.65	0.55	1.00	0.55	0.18	0.00	0.00	0.17	0.00	0.09

**B, baggase; D, Dewaxed baggase; C, prepared cellulose; H, prepared hemi-cellulose*

3.2. Isolation of different microbial isolates

One hundred and twelve microbial isolates were isolated from different soil and water samples that have been collected from different localities in Egypt. Many isolates were able to degrade SCB and showed different hydrolysis ratio. Furthermore, the degradability of different forms of treated SCB (bagasse, de-waxed bagasse, prepared cellulose and hemicellulose) were also evaluated using the obtained isolates. These data are presented using PRIMER 6 program and focus on the isolates which contribute in all substrates degradation as are shown in Fig.2. The statistical analysis showed that 40 microbial isolates (out of 112 isolates) have the ability to degrade all of the tested substrates. The core isolates or the most common microorganisms focused on similarity and dissimilarity properties with degradation rate for each substrate using reducing sugar method (Table 2; Fig. 2 and Fig. 3). Table 3 show the most promising 15 isolates that achieved higher hydrolysis percentage and

International Journal of Scientific & Engineering Research Volume 5, Issue 10, October-2014
ISSN 2229-5518

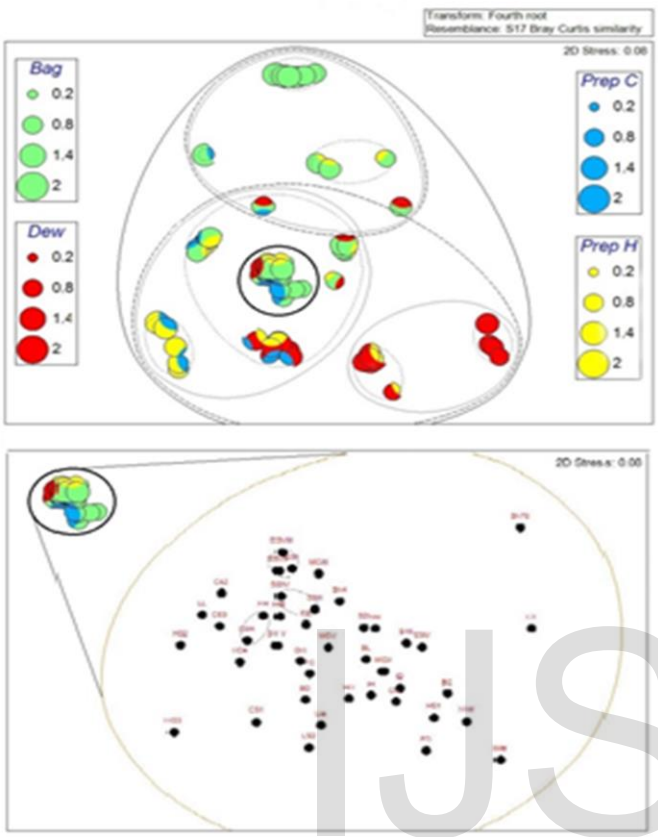
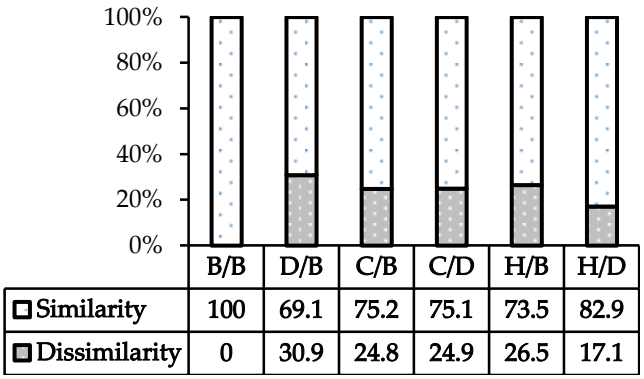


Fig. 2. The similarity and dissimilarity between the 112 isolates using PRIMER 6 with focusing on the common microorganisms.



	B/B	D/B	C/B	C/D	H/B	H/D
Similarity	100	69.1	75.2	75.1	73.5	82.9
Dissimilarity	0	30.9	24.8	24.9	26.5	17.1

Fig. 3. Show the similarity and dissimilarity of dewaxed bagasse (D) and cellulose (C) with bagasse (B) and hemicellulose (H).

TABLE 3
BAGASSE HYDROLYSIS AND REDUCING SUGAR LIBERATION BY THE MOST POTENT 15 ISOLATES IN RELATION TO THE INCUBATION TIME.

Time	24 h		48h		72 h	
Isolate code	RS (g/L)	Hyd. (%)	RS (g/L)	Hyd. (%)	RS (g/L)	Hyd. (%)
CS2	1.82	2.07	2.56	2.91	0.55	0.63
CS3	2.53	2.88	1.11	1.26	0.46	0.52
CS4	2.83	3.22	1.08	1.23	0.62	0.70
CS5	**3.31**	**3.76**	**2.14**	**2.43**	**0.54**	**0.61**
LS5	2.00	2.27	2.59	2.95	1.49	1.69
CIIH	2.48	2.82	2.21	2.51	1.21	1.38
GB	2.00	2.27	2.55	2.91	0.77	0.87
I H	1.44	1.63	2.43	2.77	0.51	0.58
LD	2.09	2.38	2.50	2.84	0.83	0.94
LL	2.02	2.30	2.72	3.09	0.57	0.65
20	1.85	2.11	2.48	2.82	1.14	1.29
S2I	2.28	2.60	2.48	2.82	0.76	0.87
S3IV	1.61	1.83	2.47	2.80	0.72	0.82
MGII	2.34	2.67	2.60	2.96	0.94	1.07
MGIII	2.76	3.14	2.41	2.74	0.86	0.98

RS, reducing sugar g/L; Hyd. %, percent of hydrolysis

liberated high concentration of reducing sugars. Isolate CS5 achieved the highest degradation efficiency after 24 h with liberation of 3.31 g/L reducing sugar at 3.76 % hydrolysis ration. These results indicate that strain CS5 is the most promising strain for saccarification of SCB.

3.3. Optimization of bio-degradation conditions by isolate CS5

Various nutritional and environmental parameters were reported to affect the microbial growth, enzymatic activities and consequently the degradation of polymers. In the present investigation various parameters affecting the biodegradability of SCB using CS5 isolate were investigated. The effect of different nitrogen sources, different carbon concentration, different pH values and different temperatures on SCB were shown in Fig. 4. Sodium nitrate was optimal nitrogen sources that resulted in liberation of 3.31 g/l reducing sugar (Fig. 4 A). The optimal carbon source concentration is 6 g/L that achieved liberation of 4.66 g/l reducing sugar (Fig. 4 B). Optimization of initial pH at 5 have resulted in liberation of 9.4 g/l reducing sugars (Fig. 4 C) that is about 2.83 fold higher than that achieved at pH of 6.0. In addition, high temperature also resulted in better degradation that lower one with the optimal degradation efficiency at 45 °C (Fig. 4D). Therefore, we can conclude the environmental parameters (pH and temperature) were found as more important than nutritional parameters for biodegradation of SCB by isolate CS5 because a significant increase in the liberated reducing sugars was obtained. . Finally, a liberation of 12.1 g/l was achieved under the optimized condition. These fermentable sugars could be easily available for further production of valuable products.

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ISSN 2229-5518

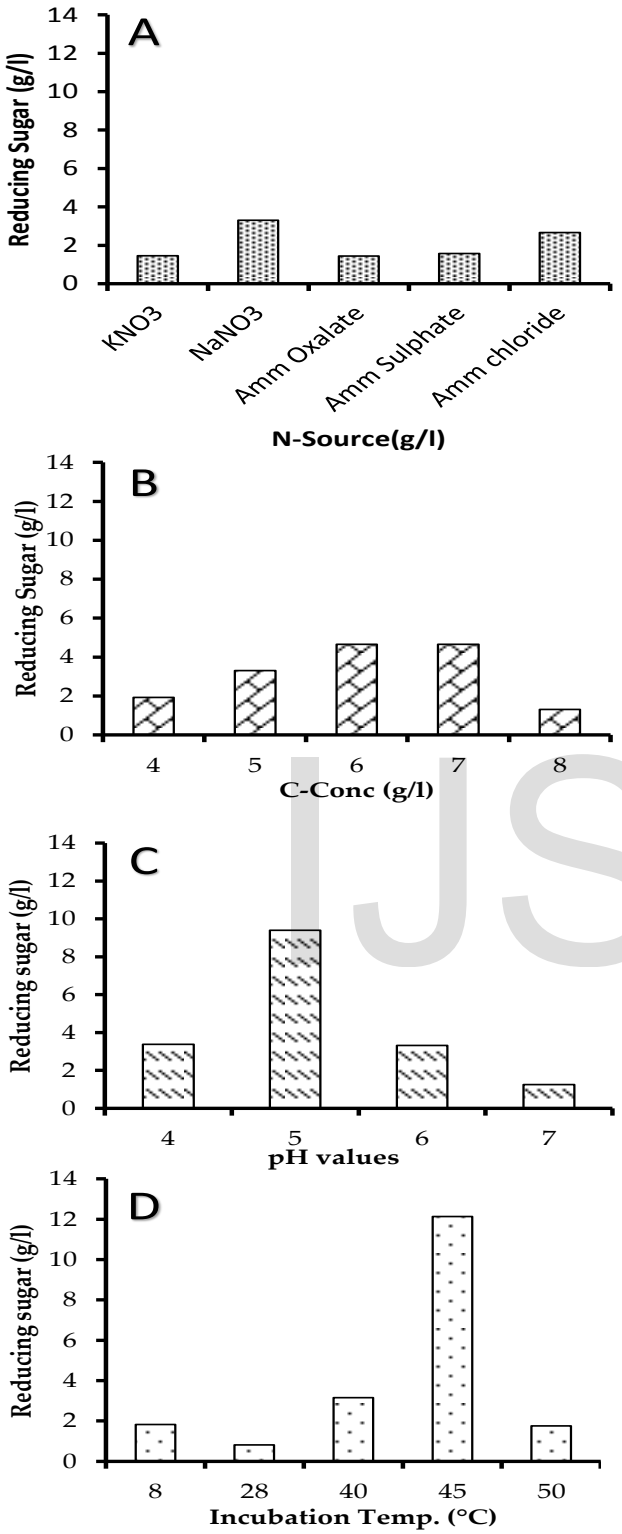

Fig. 4. Optimization of the conditions for SCB degradation using isolate SC5 (a) effect of nitrogen sources, (b) effect of carbon concentration, (c) effect of pH values, and (d) effect of different incubation temperature.

4 Conclusions

Sugarcane bagasse (SCB) is a lignocellulosic material containing polymerized sugars (e.g. cellulose and hemicellulose) that can be liberated by hydrolysis and subsequently fermented by microorganisms to form different valuable chemical products. In this study, dewaxing and chemical decomposition of SCB was carried out using chemical treatments. Dilute base hydrolysis using sodium hydroxide catalyzed the hydrolysis of hemicellulose to its constituents. The cellulose fraction was hydrolysed to fermentable simple sugars using microbes. 112 bacterial isolates were tested for the biodegradability of different forms of treated SCB (bagasse, de-waxed bagasse, prepared cellulose and hemi-cellulose). Species diversity against substrate degradation rate was analyzed using PRIMER 6 (Plymouth Routines in Multivariate Ecological Research). Amongst those isolates, forty microbial isolates were considered as a promising isolates for efficient SCB biodegradation. Out of them, strain CS5 achieved the best degradation of SCB. The amount of sugar recovered from the SCB is more dependent on the temperature and pH values rather than nutritional parameters with the recovery of 12.1 g/l reducing sugars at the optimized conditions. Therefore, we could successfully establish an effective treatment method for SCB using microbial sources rather than expensive cellulase enzymes.

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