EFFECT OF SUBSTRATE COMBINATION ON THE YIELDS OF FERMENTABLE SUGARS FROM LIGNOCELLULOSIC BIOMASS HYDROLYZED BY FUNGAL ISOLATES

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Abstract
The sustainability and economic viability of biofuel production depend largely on the abundance and cost effectiveness of lignocellulosic biomass which is subject to seasonal and regional variability. This study investigated the effect of combining some locally available lignocellulosic biomass in Nigeria, on the yields of fermentable sugars, which are intermediates in the conversion of lignocellulose to biofuel, due to fungal hydrolysis. Rice husk (RH), corn cob (CC), sawdust (SD), wheat bran (WB) and guinea corn husk (GCH) were separately milled into powder and pretreated using 0.25N sodium hydroxide (NaOH) solution. The substrates were suspended in mineral salt media individually, and in various combinations. Fungi were isolated and assayed for cellulolytic activities. Aspergillus oryzae produced the highest activities as clearance zone (60.00±0.06 mm) on carboxyl methyl cellulose agar, endoglucanase (0.35 IU mL^{-1}) and exoglucanase (0.66 IU mL^{-1}) among the four fungal isolates, and was used to hydrolyze the substrates both in solid and submerged states. The product of hydrolysis was assayed for reducing sugar, pentose and total sugar. The mixture of CC, WB and GCH yielded the highest reducing sugar (49.50 mg mL^{-1}). The highest pentose (17.40 mg mL^{-1}) and total sugar (18.90 mg mL^{-1}) were from WB as a single substrate. Higher sugar yields were also obtained from combinations involving two and three substrates compared to the single, quadruple and quintet. In most cases, higher amount of each of the sugars were obtained in the solid-state hydrolysis of the substrates than the submerged state. In this study, mixture of CC, WB and GCH yielded higher fermentable sugars on hydrolysis with A. oryzae compared to the individual substrates in solid-state hydrolysis. Substrate combination is therefore recommended to improve the yield of fermentable sugars from lignocellulosic materials and for the sustainability of biofuel production.

Introduction
Economic viability and sustainability of biofuel production is highly dependent on the derivation of fermentable sugars from abundant and renewable feedstock. Lignocellulosic biomass offers a great potential resource because it is largely abundant and inexpensive. It includes agricultural residues, municipal and industrial wastes which have served as major feedstocks for bio-refineries in the production of biofuel and other value-added products such as organic acids (Ahorsu *et al.*, 2018; Xuneng *et al.*, 2018). Plant parts that remain on the field when crops are harvested and those generated during processing are considered as wastes. These are lignocelluloses comprising mainly of cellulose, hemicellulose and lignin which are not used for food and may constitute environmental nuisance. Their use for biofuel production therefore serves a dual purpose of waste management and wealth creation.

Wastes generated from food crops include chaffs, peels, shells, pulp, cobs and pods, which are by-products of processing and are lignocellulosic. Rice husk, a waste product generated during the processing of rice (*Oryza sativa*) comprise of cellulose 35%, hemicellulose 25%, and lignin 20% (Abbass & Ansumali, 2010). Corncob which is between 27 to 30% of maize agro-wastes contain approximately 39.1% cellulose, 42.1% hemicellulose, 9.1% lignin (Barl *et al.,* 1991). Saw dust is a municipal waste generated at wood processing factories. Its lignocellulosic composition depends on the type of wood from which it is derived. Sawdust from hardwood contains 40-55% cellulose, 20-24% hemicelluloses and 18-25% Lignin.

(Kumar *et al.,* 2009) while softwood's components are 55% cellulose, 11% hemicellulose and 26% lignin (Hongzhang, 2015). Wheat bran which is a by-product of wheat grain (*Triticum aestivum*) processing contains about 70% carbohydrates, comprising approximately, 35% cellulose, 43% hemicellulose, 14% starch and 8% sugar. (Fraser and Holmes, 1959). Guinea corn husk is high in cellulose (40.2%) and hemicellulose (41.2%) but low in lignin (14.6%) content (Waghmare *et al*., 2018), making it a good candidate for biofuel production.

Bio-refineries will benefit immensely from using materials that are available and easily accessible in their region of operation. The lignin, cellulose, and hemicellulose ratios of lignocelluloses vary among plants and since cellulose is the most energy intensive component, higher value is placed on plants that contains it in high percentage. Such plants are most favoured in bio-refineries. However, seasonal factors may affect sole dependence on particular plants making it pertinent to use a diverse of plants to sustain production. Diverse combinations of lignocellulosic biomass have been used to improve yield from lignocelluloses thereby ensuring economic viability of the process (Oke *et al*., 2016). Some combinations involved lignocellulosic materials within the same category e.g., forest residues, agricultural residues and grasses (Kim *et al.,* 2005; Lim & Lee, 2013), while some cut across different categories e.g., a combination of forest residues, agricultural residue, grass and municipal solid wastes (Mtui & Nakamura, 2005; Shi *et al.,* 2013). Also, parts of different plants such as rice straw and wheat bran, have been combined (Sherieff *et al.,* 2010). The potentials of improving the yield of fermentable sugars from lignocellulosic biomass through fungal hydrolysis of various combinations of some locally available agricultural residues was investigated in this study.

Materials and Methods

Organism

Fungi were isolated from soil samples that were collected from wood processing factories in Ilorin, Nigeria. They were screened for cellulolytic activities using the agar diffusion method of Hankin and Anagnostakis (1977). Isolates that showed clearance zone greater than 5.0 mm on mineral salt agar medium containing carboxyl methyl cellulose were assayed for endoglucanase and exoglucanase activities using the IUPAC (Ghose, 1987; Mandels *et al.,* 1976) methods respectively, and identified using their morphological characteristics. The culture characteristics used for identification were appearance, shape, colour, change in colour and colour of the reverse of the plate. For the microscopic characterization, 7day old fungal isolate was teased in a drop of lactophenol blue and viewed under the x10 and x40 objectives of a light microscope. Characters examined were nature of hyphae, shape and appearance of spores. Identification was based on description in Onions *et al*. (1981a and b).

The isolate that exhibited the highest cellulolytic activities was chosen for the hydrolysis process and was characterized by molecular techniques. The molecular identification was carried out at the International Institute of Tropical Agriculture (IITA), Ibadan Nigeria. Briefly, genomic DNA was extracted from a one-week-old PDA culture of the fungus using QIAGEN DNeasy Plant Mini Kit. The primers ITS 4 (5' TCCTCCGCTTATTGATATGS 3') and ITS 5 (5' GGAAGTAAAAGTCGTAACAAGG 3') were used to amplify ribosomal internal transcribed spacer (ITS). The PCR product was purified using the QIA quick PCR purification kit (Bao *et al*., 2012) and sequenced using Big Dye Terminator sequencing kit. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI) (Javadi *et al*., 2012).

Collection and pretreatment of lignocellulosic biomass samples

The lignocellulosic materials used in the current study were rice husks, corn cobs, guinea corn husks, wheat bran and saw dust. All the samples were collected from their respective processing sites in Ilorin, Kwara State, Nigeria. The lignocellulosic materials were milled separately using electrical milling machine and sieved through a mesh of approximately 100 µm. The resulting powder was pretreated with alkali as described by Vyas *et al.*, (2005). The powder was soaked in 0.25 N sodium hydroxide solution at 20% w/v, boiled for 5 minutes and allowed standing on the bench for 24 hours. The alkali was drained off by sieving the materials through four layers of muslin cloth, and washing with several changes of water until neutral pH was attained. The pretreated materials were air dried by spreading on the work bench for 24 hours and dried in hot air oven at 60ºC to constant weight. The dried samples were stored in airtight containers until required.

Hydrolysis of pretreated lignocellulosic samples

Spores of the fungal isolate that showed the highest cellulolytic activity were harvested from a seven days old slant culture using normal saline. The spores were enumerated using hemocytometer and standardized by diluting with normal saline. Aliquots, 5 ml of the spore suspension at approximately 4.5 x 10^3 spores/ml were used to inoculate 100 ml of mineral salt medium containing 1% and 2% w/v of the pretreated lignocellulosic materials for submerged and solid-state hydrolysis respectively, in 250 ml Erlenmeyer flasks. The lignocelluloses were used singly and in various combinations as shown on Table 1. All the flasks were incubated for seven days. Flasks for the submerged process were placed on gyratory shaker at 150 rpm while those used for solid state hydrolysis were left on the work bench. After the incubation period, the product of the hydrolysis was obtained by filtering through Whatman number 1 filter paper. For the submerged process, the culture was filtered directly while the flasks used for the solid-state fermentation were first placed on gyratory shaker at 150 rpm for 30 minutes after adding 100 ml sterile distilled water, before filtering (Hong *et al.*, 2011). The filtrates were assayed immediately for reducing, pentose and total sugars.

Assay for reducing, pentose and total sugars in the products of hydrolysis of lignocellulosic samples

The amount of reducing sugar in the product of hydrolysis was determined using the dinitro salicylic acid (DNSA) method of Miller (1959) and absorbance was taken at 540 nm using Genesys-20 spectrophotometer. The reducing sugar content was read off glucose standard curve and expressed as mg/ml. For pentose sugar, absorbance was read at 480 nm using the phenol-sulphuric acid method described by Nielsen (2010) and xylose was used for the standard curve. The phenol-sulphuric acid method (Wood and Bha*,* 1988) was also used to determine the total sugar content of the hydrolysates, the absorbance was taken at 490 nm and glucose was used for the standard curve.

Results and Discussion

Cellulolytic organisms

Four fungal isolates showed clearance zones greater than 5 mm (Table 2) and were characterized using their morphologies (Table 3). There were two *Aspergillus niger*, one *A. oryzae* and one *Penicillium* sp. The endoglucanase and exoglucanase activities of the fungi further confirmed their cellulolytic properties (Figures 1 and 2). The highest enzymatic activity of 0.35 IU ml^{-1} endoglucanase and 0.66 IU ml^{-1} exoglucanase was produced by *A. oryzae*. This result compares favorably with our earlier reported activities of 0.1494 IU ml^{-1} endoglucanase, and 0.0820 IU ml^{-1} exoglucanase from *A. niger* (Saliu & Sani, 2013). A higher endoglucanase activity (11.42 IU ml^{-1}) was however reported from *A. oryzae* (Begum & Alimon, 2011). In the

current study the isolate identified as *A. oryzae* FG 20 with Ascension no EU030335.1 and identity of 99.3% by molecular techniques was used for the downstream hydrolysis step. The endoglucanase (0.19 IU ml^{-1}) and exoglucanase (0.49 IU ml^{-1}) activities of *Penicillium* sp., obtained in this study compare favourably with that of *P. decumbens* which were 0.1111 IU ml^{-1} and 0.0216 IU ml^{-1} respectively from an earlier study by Saliu and Sani (2012).

Yield of fermentable sugars from lignocelluloses

The highest yield of reducing sugar (49.50 mg ml^{-1}) obtained after hydrolysis using *Aspergillus oryzae* FG 20, was from the combination of corn cob, wheat bran and Guinea corn husks (Figure 3). This yield compares favorably with the 2.65 mg ml^{-1} reported from corncob (Saliu & Sani, 2013), 0.8 mg ml^{-1} from wheat straw and 1.5 mg ml^{-1} from guinea corn husks (Kshirsagar *et al*., 2017) but lower compared to 58.8 g/100g of wheat bran reported by Farkas *et al*. (2019). The lowest reducing sugar yield of 1.95 mg ml^{-1} was from sawdust as a single substrate. Begum and Alimon (2011) also obtained low reducing sugar yield of 1.12 mg ml^{-1} from sawdust. Similarly, Olanipekun *et al*. (2016) reported a lower reducing sugar yield from sawdust compared to corncob. Guinea corn husks produced the highest yield of 8.30 mg ml^{-1}in the single substrate category although the difference was insignificant ($p \leq 0.05$) when compared with corn cob and wheat bran. Higher yields (66.0 mg ml^{-1}) of reducing sugar have previously been reported from guinea corn husks although a combination of enzymatic and acid hydrolysis was employed in that study (Ahmed-El-Imam *et al.*, 2019). In the double substrate category, highest yield (3.20 mg ml^{-}) was obtained from a combination of CC and GCH. Both are high in cellulose and hemicellulose, but low in lignin contents as mentioned previously in this report and therefore have reduced recalcitrance which make them more available for enzymatic saccharification.

In this study, the combined substrates generally yielded higher amounts of reducing sugar compared to the singles. High cellulase activity was obtained from a mixture of rice straw and wheat bran hydrolyzed using *Aspergillus fumigatus* (Sherieff *et al*., 2010). In like manner, 98% of theoretical maximum of mannose and galactose, were produced from 100 g of mixed bark-rich sawmill residues (Kim *et al*., 2005). Also, ethanol yields up to 50 g L^{-1} was achieved with feedstock mixtures of wheat straw and corn stover although using steam treatment before enzymatic hydrolysis (Nielsen, *et al*., 2020) to produce the fermentable sugars which was far higher compared to the 2.165 mg ml^{-1}obtained in this study. Yields from combining four and five substrates in the current study were significantly ($p \leq 0.05$) lower than the triple and double substrates. This could be due to the presence of substrates that have low cellulose and high lignin contents such as sawdust in the mixture. In all cases, solid-state hydrolysis yielded higher reducing sugar than the submerged process.

Unlike reducing sugar, the highest pentose was from WB (17.3 mg ml^{-1}) as a single substrate in solid-state hydrolysis and from the mixture of CC, SD, WB and GCH (17.55 mg ml^{-1}) in submerged culture. Pentose yields up to 40 g L^{-1} was previously reported from wheat bran (Sanjust *et al*., 2004); 14.22 g/L from sorghum husk (Camargo *et al*., 2019); and 32.7 g L^{-1} from corncob (Jin *et al.,* 2018). Yield from sawdust (0.16 mg ml^{-1}) in the current study, was quite low compared to the 17.9 g L^{-1} reported by Rafiqul and Sakinah (2011). The other single substrates also yielded lower amount of pentose sugars individually, compared to the mixed substrates. Among the combined substrates the pentose sugar yields were higher in the solid-state hydrolysates than the submerged although the differences were insignificant in many cases (Figure 4).

The total sugar yields were generally high and did not differ significantly among most of the substrates (Figure 5). In similarity to the pentose sugar, the highest yield (18.89 mg mL^{-1}) was from WB and the mixture of CC, SD, WB and GCH while the lowest (3.63 mg mL^{-1}) was

from sawdust. Rice husks produced a relatively high yield of 15.10 mg mL^{-1} compared to the 0.0751 g L^{-1} reported by Salimi *et al*., (2017). In contrast, the total sugar yield of 16 .80 mg mL^{-1} from corncob in this study was not comparable with the 46.29 g L^{-1} obtained by Boonsombuti *et al*. (2016). Except for a few of the mixed substrates, solid-state hydrolysis was most favored in the current study.

Conclusion and Recommendation

This study shows that combined substrate hydrolysis particularly involving corn cob, wheat bran and guinea corn husks, using *Aspergillus oryzae,* yielded higher amounts of fermentable sugars compared to the single substrates. Combination of these substrates is therefore recommended for the production of fermentable sugars from lignocellulosic materials by fungal hydrolysis.

Table 1: The various combinations of lignocellulosic materials used in this study

Single	Double	Triple	Quadruple	Quintus
A, B, C, D, E	AB, AC, AD, AE, BC, BD, BE, CD, CE, DE	ABC, ABD, ABE ACD, ACE, ADE BCD, BCE, BDE CDE	ABCD, ABCE ABDE, ACDE, BCDE	ABCDE

Key: A - Rice husk (RH), B - corn cob (CC), C - sawdust (SD), D - wheat bran (WB) and E - guinea corn husk (GCH)

Legend: All combinations comprised of equal amount (w/w) of the individual substrates.

Table 2: The cellulolytic activities of the isolated fungi as zone of clearance around growth on agar plates

Organisms	Diameter of Growth (mm)	Diameter of clearance (mm)	Clearance zone (mm)	Cellulolytic index
Aspergillus oryzae	15.02±0.07	74.98±0.09	60.00±0.06	4.00
Aspergillus niger 1	16.00±0.20	37.00±0.20	21.00±0.23	1.31
Aspergillus niger 2	6.00±0.09	14.00±0.08	8.00±0.15	1.33
Penicillum sp	10.23±0.15	22.24±0.15	12.00±0.13	1.20

Legend:

Four of the isolated fungi showed cellulolytic activities as clearance zones around the colony that grew on mineral salt agar medium supplemented with 0.5% w/v carboxyl methyl cellulose. After the growth of the fungi, clearance zones were detected by flooding the culture with 1% Congo red solution and washing with 1N sodium chloride solution. The difference between the diameter of clearance and that of growth was taken as the zone of clearance. The cellulolytic index is the clearance zone divided by the diameter of growth. Two *Aspergillus niger* isolates from different sources showed cellulolytic activities and were therefore differentiated as *Aspergillus niger* 1 and *Aspergillus niger* 2.

Table 3: Colonial and microscopic characteristics of the fungal isolates that showed cellulolytic activities

Colonial Characteristics					Microscopic Characteristics	
Isolate	Appearance	Shape	Clr	Clr Change	Clr of Reverse	Hyphae	Spores

							Shape	
Texture								
CD7	Velvety	R	Gr	Y-Gr	Y	Sept	Glo	Rgh
AB4	Floc/comp	R	Bl	W-Bl	Crm	Sept	Glo	Rgh
CD4	Velv/comp	R	Br	W-Br	Y	Sept	Glo	Smt
P4	Velv/comp	R	Gr	W-Gr	Y	Sept	Ellip	Smt

Key: – Clr = colour; floc = floccose; comp = compact; powd = powdery; velv = velvety; R = round; W = white; Bl = black; Br = brown; Gr = green; Y = yellow; Crm = cream; Sept = septate, Ellip = elliptical; Glo = globose; Rgh = rough; Smt = smooth

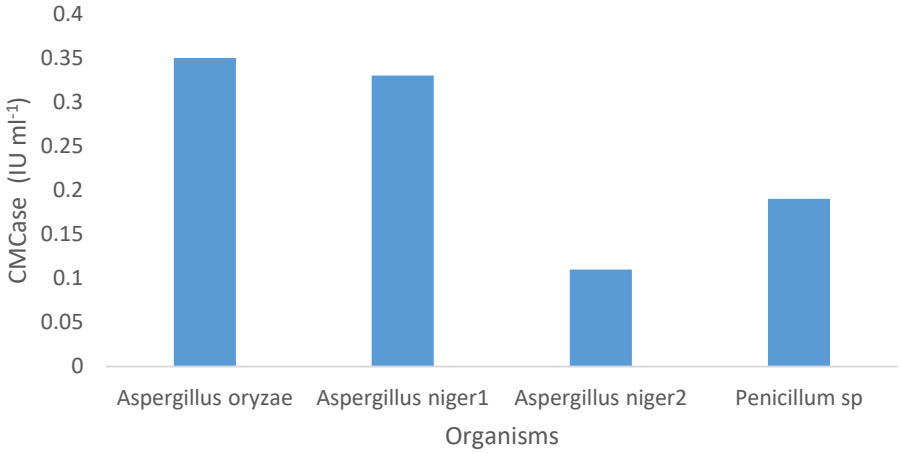

Figure 1: Endoglucanase activity of the isolated fungi

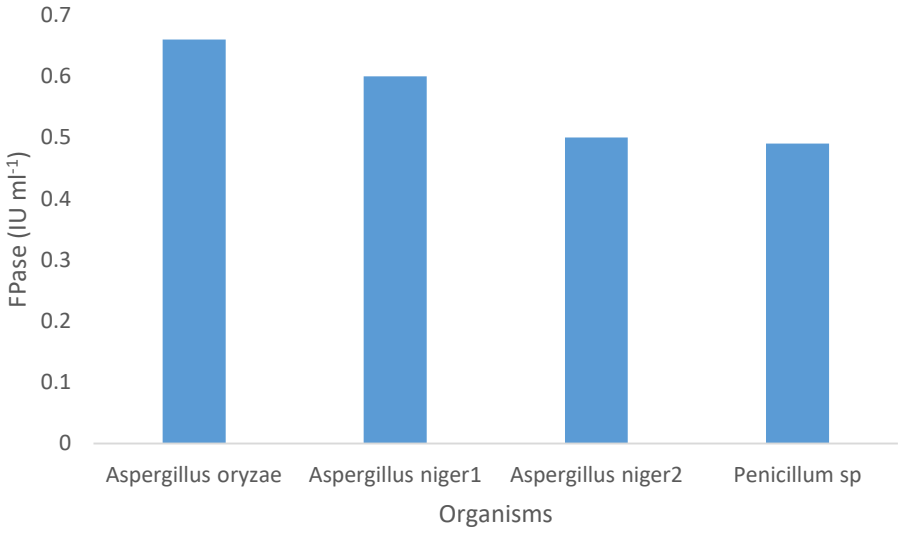

Figure 2: Exoglucanase activity of the isolated fungi

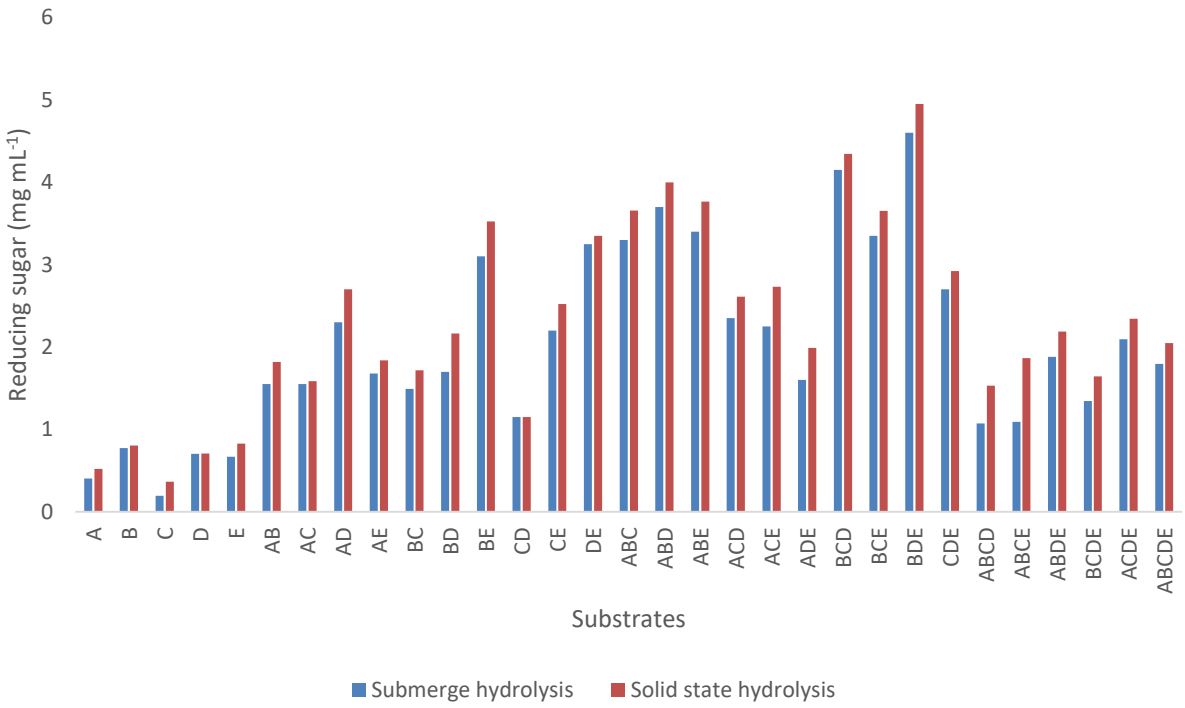

Figure 3: Reducing sugar yield after substrate hydrolysis using fungal isolate identified as *Aspergillus oryzae* FG 20

Key: A - Rice husk (RH), B - corn cob (CC), C - sawdust (SD), D - wheat bran (WB) and E - guinea corn husk (GCH)

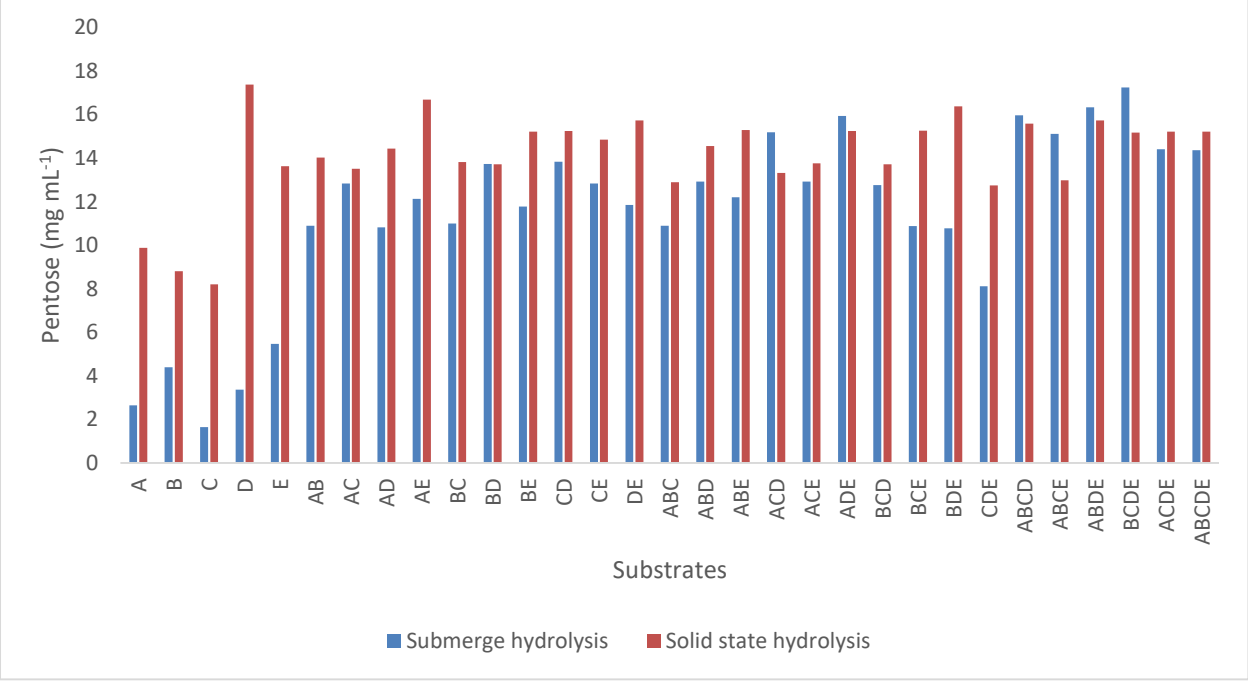

Figure 4: Yield of pentose from hydrolysis of substrate using fungal isolate identified as *Aspergillus oryzae* FG 20

Key: A - Rice husk (RH), B - corn cob (CC), C - sawdust (SD), D - wheat bran (WB) and E - guinea corn husk (GCH)

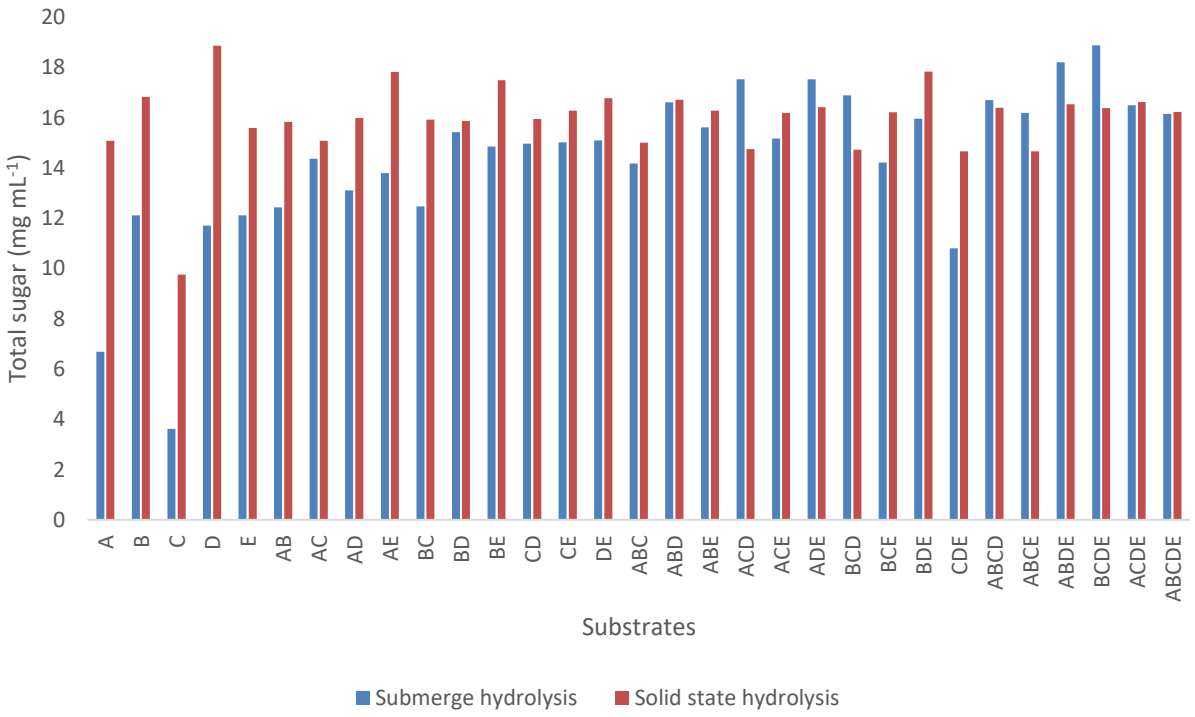

Figure 5: Total sugar yield from hydrolysis of substrate using fungal isolate identified as *Aspergillus oryzae* FG 20

Key: A - Rice husk (RH), B - corn cob (CC), C - sawdust (SD), D - wheat bran (WB) and E - guinea corn husk (GCH)

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