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BIOCOMPATIBILITY PROFILING OF MOLTEN INORGANIC ZINC CHLORIDE PRE-TREATMENT MEDIUM USING CORN COB AS CARBON SOURCE

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ABSTRACT: Previous studies on the application of molten hydrate salts, such as zinc chloride tetrahydrate, in biomass refining have proven their efficiency as non-derivatising pre-treatment solvents. The possibility of using this salt media as a platform for simultaneous pretreatment of biomass and bioconversion of resulting sugars into biocommodities could be strengthened by investigating the physiological acclimatization of selected microbial agents capable of biocatalysis in the relatively high salt media. This study sought to investigate the biocompatibility of $ZnCl_2$ salt pretreatment media with a mixed-consortia of microbes using dissolved corncob and glucose as carbon sources. Quantification of DNA extracted from product stream at 24 h intervals revealed the negative influence of the salt concentration on general microbial life, but the microbial activity during the period was immensely reduced, making the application of molecular techniques for identification of the active microbial agents inevitable. 16s rRNA metagenomics analysis of the raw cow dung and product samples revealed a total 1765 operational taxonomic units (OTUs). Overall, species belonging to the *Clostridia* and *Bacilli* were the most represented genera at the analyzed salt concentration (0.9 M L^{-1}). The findings documented herein could be instrumental to developing more advanced and consolidated bio/chemo catalytic process for lignocellulosic biomass conversion to biocommodities.
Keywords: corn cob, conversion systems, pretreatment, fermentation

1 INTRODUCTION

Lignocellulosic biomass (LCB) is an abundant, renewable and proven resource for sustainable production of bio-commodities. Their centrality to modern industrial revolution stems from the carbon neutrality of their processing, especially in comparison to fossil sources [1]. Fossil refining and consumption processes are characterized by threats of high greenhouse gases (GHG) emission which have in turn necessitated the need for more organic and sustainable sources of energy generation [2]. Biomass is an important alternative being proposed globally [3,4], hence, the emphasis on the development of efficient and feasible biomass conversion technologies [5]. Lignocellulosic biomass especially waste agricultural residues are an important feedstock because of their minimized socio-economic effects. LCBs comprise majorly of cellulose, hemicellulose and lignin; all of which are immense repositories of process-able organic carbon [2]. Biomass processing to value-added products requires an activating pretreatment step (Fig. 1) prior to enzymatic saccharification and bioconversion. LCBs naturally have a very ordered and ultra-defensive structure which makes them recalcitrant to bio/chemo catalytic agents [6]. As such, pretreatment being a function of the disorganization and deconstruction of the ordered biomass matrix [7] results in an increase in the susceptibility of biomass to bio/chemo catalysis. Pre-treatment as a stand-alone step is crucial to the techno-economic feasibility of the entire biorefining process [8, 9].

Currently explored pretreatment methods in industry are expensive, usually involving energy-intensive, corrosive, chemical treatments. Yang and Wyman, 2008 in a feasibility study of dilute-acid pretreatment reported that the pretreatment step alone accounts for at least 25 % of the entire processing cost in the production of second-generation bioethanol [8].

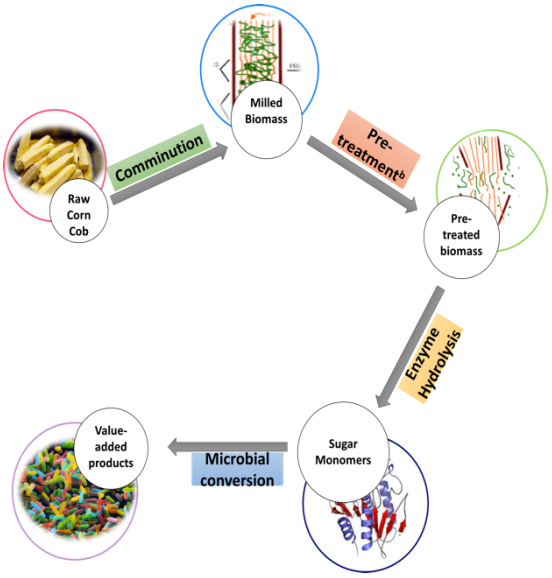

Figure 1: Illustration of the processing steps for biomass conversion to value-added bio-based products ([b]- includes steps such as washing and filtration).

In addition to designing pretreatment steps for improved yield of fermentable sugar, reducing cost of pretreatment is essential for process development. The non-derivatizing pretreatment of biomass at relatively mild conditions using biocompatible, non-corrosive green solvents as a strategy have the potential to redefine the biorefining platform. The reduction in energy costs and simultaneous bioconversion in pretreatment solvent system without a post-pretreatment washing/filtering step will contribute to the cheaper production of bio-based products. This and similar strategies [10] for the integration of the pretreatment step with the bioconversion steps in "one-pot" are rare in literature. An understandable

trend, considering the obvious challenges with such hypothesis; such as the physiological limitations of microbial catalysts, protein nature of enzymes and the harsh conditions of pretreatment media; all pointing towards an impossible task. Thus, investigation into the development of cheap pretreatment methods showing potential for biocompatibility and physiological acclimatization of microbial catalysts is very essential.

This study investigated microbial activities in $ZnCl_2.4H_2O$; a molten hydrate salt (MHS) biomass solvating systems. MHS by definition, are inorganic materials with a water to salt molar ratio close to the coordination number of the strongest hydrated cation [11]. Using the mixed consortia of microorganisms in anaerobically digested cow dung inoculum as the spectrum, the study sought to provide insights for biocatalysts engineering for $ZnCl_2$ media by identifying available biotransformation opportunities at selected salt concentration. The main objective of this study was to identify the microbial elements with potential for microbial activity in a conceptual fully-integrated bio-based process at selected pretreatment severity conditions.

2 MATERIALS AND METHODS

2.1 Materials

Locally sourced corncob and standard glucose, dissolved in $ZnCl_2.4H_2O$ was used as fermentation process carbon source. Anhydrous Zinc chloride (purchased from Sigma Aldrich (Pty) South Africa) and deionized water were used in this study as well.

2.2 Raw material and Inoculum preparation

The inoculum used in this study was digested cow dung collected from a dairy farm in Soweto (Johannesburg, South Africa) while the carbon source was from the cobs of local hybrid (Zama star) of *Zea mays* collected from a maize farm in the province of Limpopo (23.89°S, 29.44° E). The characteristics of the cow dung used for the study were as follows: pH: 7.4 ± 0.1, total solid (TS): 35± 1.1 g/L, Volatile solids (VS): 21.5 ± 3.7 g/L, Protein:19.7 ± 2.3 g/L , Carbohydrates: 6.1 ± 0.4 g/L, Lipids: 3.1 ± 0.3 g/L. Details of the bio-compositional analysis of the biomass used are shown in Table I.

2.3 Biomass pretreatment and anaerobic fermentation

Anhydrous zinc chloride was stored in an oven set at 100 ^oC over-night to ensure there is no residual moisture content prior to dissolution of up to 65.4 %w/w in deionized water. The MHS solvents were then transferred to autoclave vials containing 5 g of corn cob biomass at a solid to liquid ratio of 1:12 and autoclaved at 70 ^oC for 1 h. The physicochemical characteristics of the resulting slurry have been described in earlier studies and are shown in Table II. The salt concentrations of the resulting slurries were varied by diluting with sterilized deionized water (slurry to water dilution ratios of 100:0 (4.79 m/L) 80:20 (3.5 m/L), 50:50 (2.39 m/L) and 20:80 (0.9 m/L)) towards the biocompatibility profiling of the pretreatment media. The runs A-D were all prepared in triplicates while control run comprised sterilized deionized water and corn cob.

The fermentation process was initiated by adding 10 g of cow dung in an anaerobic shaking incubator (set at 50 ^OC and 150 rpm) to the biomass slurries. Separate experiments were conducted at the same conditions, using glucose as carbon source. Fermentation lasted for 96 h after which samples were collected at 24 h intervals and stored at -20 ^oC prior to DNA extraction, quantification, fluorometric, metagenomics and chromatographic analysis. All experiments and sampling were conducted in triplicates.

2.4 Analysis of microbial community in process media

Biocompatibility assessment involved the use of next generation sequencing of 16s rRNA gene amplicons, towards a concise profiling and analysis of the bacterial population of samples from the anaerobic processes. Raw cow dung, process media was analyzed for total genomic DNA using the ZR Soil Microbe DNA Kit™ (Zymo research company, USA). The integrity of DNA extracted from samples were verified by agarose gel electrophoresis, while DNA quantification was done using the Qubit 2.0 fluorometer (Invitrogen, California, USA).

For PCR and DGGE profiling, the primer sets 968F (AACGCGAAGAACCTTAC) and 1401R (5´-CGGTGTGTACAAGACCC-3´) [12] targeting the V6-V8 region of the bacterial 16S rRNA gene was used with a 40 bp GC-clamp which was attached to the latter [13]. PCR was carried out in a thermal cycler (SimpliAmp, Applied Biosystems, USA). The reaction mixtures containing 50 ng DNA template, 10 µl of Phusion Flash master mix (Thermo Fisher Scientific, USA), 0.2 µM of each primer and nuclease-free water to make 20 µl in total. The PCR conditions was at 98 °C for 10 s hold, 30 cycles of 98 °C for 5 s, 58 °C for 1 s and 72 °C for 15 s, with a final extension of 72 °C for 1 min. PCR products were verified by agarose gel electrophoresis. Resulting PCR amplicons from each tissue were pooled in equal proportions for each process samples prior to sequencing.

For metagenomics; next-generation sequencing of extracted biomaterials, the first step was the preparation of partial 16S rRNA gene library as described in the Illumina MiSeq 16S library preparation 150 guides. The sequencing method for the V5-V6 region (~336 bp) of the 16S rRNA gene was amplified using forward and reverse primers; 799F (5´-AACMGGATTAGATACCCKG-3´) and 1115R (5´- AGGGTTGCGCTCGTTG-3´) [14]. Both primers used contained Illumina forward and reverse overhangs, respectively (Illumina Inc., California, USA). The components of the PCR included 12.5 µl of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Massachusetts, USA), 5 µM of both forward and reverse primer, 12.5 ng template DNA and nuclease-free water to make a final volume of 25 µl. The PCR was run at 95°C for 3 min, followed by 25 subsequent cycles at 95, 60 and 72 °C for 30 s each and a final extension of 72°C for 5 min held at 4 ^oC. Since the major aim of the study was to obtain a snapshot of the active microbial elements in the respective process media, the respective PCR amplicons were further processed in downstream library preparation procedures; involving purification and indexing steps, using Nextera XT primers (Illumina Inc., CA, USA). After indexing the PCR, the amplicon library was then quantified, normalised and pooled prior to loading on the MiSeq flow cell for a 2x 300 paired-end sequencing.

Upon completion of the sequencing in the MiSeq, reads were de-multiplexed using the on-system MiSeq reporter software. Analysis of the quality of reads was done using the FastQC software (v 0.11.5, Babraham Bioinformatics, UK) before the assembling of both forward and reverse reads using PANDAseq (Masella et al. 2012). The assembled reads were clustered into operational taxonomic units (OTUs) by using the

"pick_open.reference_otus.py" script in QIIME [15] while aligning data against the Silva rRNA database (release 128) [16]. The OTU Table generated from the clustering step data was first ratified, prior to summarizing taxa, and computing alpha and Beta diversity in R software.

3 RESULTS AND DISCUSSIONS

3.1 Feed characterization

Corncob is a major agro-industrial waste upon which the sustainable development of the local bioenergy sector in South Africa could be founded [17]. Corn cob used in study was characterized using NREL standards [18]. The physicochemical properties and organic composition are as detailed in Table I.

Table I: Overview of the physicochemical characteristics and bio-composition of lignocellulosic corn cob biomass feed.

	Corncob
Proximate (wt.%)	
Ash	2.1±1.1
Volatile matter	77.93±2.6
Fixed Carbon	19.97
Moisture	7.37±0.3
Heat of Combustion (MJ/kg)	19.39±1.7
Ultimate	
Carbon	44.70±1.2
Hydrogen	5.69±1.5
Nitrogen	>0.1
Sulfur	-
Oxygen (by diff)	49.62±1.1
Structural components (wt.%)	
Cellulose[a]	40.34±2.6
Hemicellulose[a]	45.35±4.5
Acid insoluble lignin[a]	5.5±1.2
Acid soluble lignin[a]	2.40±0.2
Extractives	9.38±0.7

[a]-estimated on per gram of "dry raffinate biomass" basis
± represents standard deviation for reported values from 5 runs.

With up to 85 % composition of fermentable sugars, corn cob was unarguably ideal (in the pretreated form) for bioconversion. The dissolution of corncob biomass in $ZnCl_2.4H_2O$ has been reported [19, 20]. Table II describes the sugar composition and other physicochemical properties of the resulting slurry from $ZnCl_2.4H_2O$ pretreatment.

Table II: Overview of sugar yield and slurry characteristics of corncob biomass dissolved in $ZnCl_2.4H_2O$

	Pre-treatment slurry
Solid Fraction (g/100g of corn cob)[a]	
Glucan (% w/w)	28.46 ± 1.6
Xylan (% w/w)	30.9 ± 0.5
Lignin (% ,w/w)	7.5 ±1.7
Liquid Fraction (mg/ml)	
Glucan	7.34
Xylan	14.33
Arabinan	1.35
Mannan	2.1
Physicochemical properties	
Viscosity (Pa.S)	7.5
Salt Concentration (m/L)	4.79
pH	0.44
CS[b]	0.45

[b]- pretreatment severity (CS) was calculated using equation 1 (1)

The environmentally benign, cheap and simple biomass solvating media proved to be an efficient platform for the pre-treatment of corncob especially at relatively mild conditions (50-70 ^oC). A promising finding considering the salt system has been proven to be an exceptional media for the heterogeneous chemocatalytic derivatization and conversion of cellulose to valuable products like isosorbide. [21, 22]. The proof of mild, non-derivatizing pre-treatment step is essential for the subsequent microbial conversion of the pre-treated biomass in concentrated salt medium. The conversion of organic carbon by fermentation of pretreated biomass has been optimized for several valuable processes; such as methanogenesis, hydrolysis, acidogenesis and alcohol fermentation [23] and none of them was reported at conditions exceeding 70 ^oC. Hence, investigating sugar yield at mild pretreatment condition and investigating the effect of salt concentration of the pre-treatment medium on microbial activity are important steps in developing "one-pot" pretreatment coupling bioconversion unit in a biomass process. In addition, identification of active microbes in such unit could be instrumental to developing molecular engineering techniques for process-specific enzyme development [24] and conceptual cell-free bioprocess design [25].

3.2 Quantification of microbial DNA.

Fermentation was carried out for pretreated slurry diluted to give four different salt concentrations. Biological activity was measured by quantifying the extracted DNA in product samples. Fig. 2 illustrates the effect of salt concentration on the overall microbial activities *in situ*.

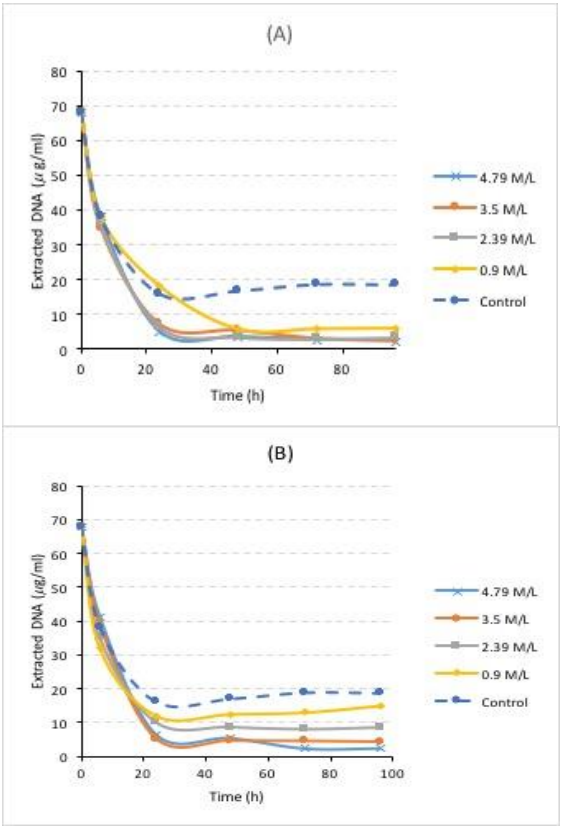

Figure 2: Quantification of DNA extracted from fermentation product (anaerobic set-up, 50 ºC) at varying $ZnCl_2$ salt concentrations using different carbon sources (A) – pre-treatment corn cob slurry (B) Glucose.

Figure 2 shows that extracted DNA reduced by over 70 % in the first 24 h in all runs. This could be attributed to the process conditions, mostly, temperature. From literature, non-spore forming microbes such as those belonging to the genus *Enterobacter and Klebsiella* can display activity after treatment at temperature as high as 105 ºC [26]. While mesophilic bacteria such as *Escherichia coli* are more sensitive to temperatures over 40 ºC [26]. The detection of biology activity at highly saturated media was negligible (<2.5 μg/ml). At more dilute and lesser salt concentrations, DNA extracted increased relatively after 24 h reaching the highest value of 18.6 μg/ml for the control run.

3.3 Microbial Analysis

Sequence reads spanning the V5-V6 region of bacterial 16S rRNA gene were recovered from the batch fermentation process fed with the most diluted corn cob slurry and glucose ($ZnCl_2$, 0.9 M/L). Upon trimming for higher-quality reads, the sequences were processed by rarefactioning at a depth of 1500 reads per sample, corresponding to 1765 operational taxonomic units (OTUs) in the process samples (Table II).

Table III: Diversity indices of OTUs in process samples.

		Inoculum	$ZnCl_2$ (0.9 M/L)	
Diversity Indices			Glucose	Corn cob
OTUs		676	535	554
Shannon (H')	index	7.88	7.13	7.38
Simpson (D')	index	86.86	34.69	59.96
Chao1		1939.71	784.03	1740.06

The inoculum was found to have the highest amount of OTUs while the process sample with a single source of organic carbon had the least. Likewise, the alpha diversity indices as presented in Table III shows that at the same salt concentration, the process with the corn cob slurry had higher Shannon and Simpson indexes than glucose feeds. The chao1 index used in estimating the total number of species present in the reactor microbiota showed that the process with the pretreated corn cob biomass feed had a higher true OTU richness than for the glucose feed.

The bacterial diversity assessment done by OTU clustering analysis, taxonomically assigned the observed OTUs in all samples into 15 bacterial phyla (Fig. 3a), 43 classes, 80 orders and 266 genera. At the phylum taxa, *Firmicutes* was dominant in the inoculum and even more in both process samples. Other well represented phyla include *Actinobacteria, Bacteriodetes, Proteobacteria and Plantomycetes.* With the exception of *Firmicutes,* majority of the featured phyla in the inoculum either reduced in abundance with time and at the select salt concentration (e.g *Bacteriodetes, Proteobacteria Therm1,* and *Plantomycetes*) or completely removed (e.g *Verrucomicroia).*

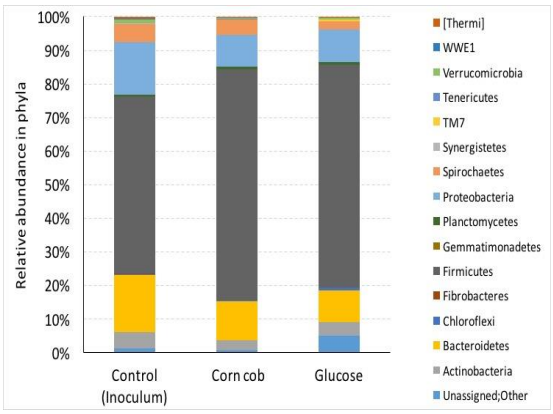

Figure 3: Relative taxonomic abundance of phyla taxa in process stream at $ZnCl_2$ concentration of 0.9 m/L (unassigned refers to OTUs at phylum taxa level which was unclassifiable.)

Microbial diversity, in terms of the effect of carbon source was less extensive. Bacteria assigned to the phylum *Firmicutes* were found to be the most diverse and abundant group in the inoculum and product samples. The *Firmicutes* phylum increased in the presence of the dissolved biomass and glucose, even though the phyla seem to slightly prefer the corn cob feed. The reason for this could be the presence of sugar components other than glucose such as xylose, mannose, arabinose (Table II).

The class taxa abundance of OTUs is presented in Fig. 4. The various classes with abundance more than 1 % in the process samples are as shown in Table IV. The taxa class of *Firmicutes*; *Clostridia* (OTU 17) and *Bacilli* (OTU 16) comprises 52 %, 66 % and 70 % of the microbiome in the control, glucose-feed and dissolved corn cob-feed sample process.

Figure 4: Pie chart diagrams showing the OTU profiles of bacterial populations in A) Inoculum B) Process sample (Corn cob feed) C) Process sample (Glucose) The taxonomic assignments for specific OTUs (1-36) are shown in Table IV

As aforementioned, up to 265 genera was detected, but only 18 genera constituted over 1 % of abundance; in at least one of the process samples (Fig. 5). The behavior of the respective microbial diversity especially at the process conditions and salt concentration with varying carbon sources is important in defining the suitability of the salt media for fermentation. *Clostridium* as a genus of the *Fermicutes* was prominent in both the control and process samples suggesting their tolerance to $ZnCl_2$ up to the concentration of 0.9 m/L in the metagenomic study.
Effect on carbon sources was apparent as the relative abundance in the presence of glucose showed their dominance at the system conditions. *Clostridium* is an essential genus in biobased production systems for biohydrogen generation and Acetate-Butanol-Ethanol (ABE) fermentation. They are proven biocatalysts which have been employed in productive processes using organic carbon sources such as starch and glycerol.

Table IV: Taxonomic assignment for selected OTUs identified in anaerobic

OTU	Taxa (phylum-class)	OTU	Phylum-class
1	*Unassigned; Other*	19	*Planctomycetes-Phycisphaerae*
2	*Actinobacteria-Acidimicrobiia*	20	*Planctomycetes-Planctomycetia*
3	*Actinobacteria-Actinobacteria*	21	*Proteobacteria-Alphaproteobacteria*
4	*Actinobacteria-Coriobacteriia*	22	*Proteobacteria-Betaproteobacteria*
5	*Actinobacteria-Nitriliruptoria*	23	*Proteobacteria-Deltaproteobacteria*
6	*Actinobacteria-Thermoleophilia*	24	*Proteobacteria-Epsilonproteobacteria*
7	*Bacteroidetes-Bacteroidia*	25	*Proteobacteria-Gammaproteobacteria*
8	*Bacteroidetes-Cytophagia*	26	*Spirochaetes-Spirochaetes*
9	*Bacteroidetes-Flavobacteriia*	27	*Spirochaetes-Leptospirae]*
10	*Bacteroidetes-Sphingobacteriia*	28	*Synergistetes-Synergistia*
11	*Bacteroidetes-Saprospirae]*	29	*Planctomycetes-Phycisphaerae*
12	*Chloroflexi-Anaerolineae*	30	*Planctomycetes-Planctomycetia*
13	*Chloroflexi-Thermomicrobia*	31	*TM7-TM7-3*
14	*Fibrobacteres-Fibrobacteria*	32	*Tenericutes-RF3*
15	*Firmicutes-AHT28*	33	*Verrucomicrobia-Opitutae*
16	*Firmicutes-Bacilli*	34	*Verrucomicrobia-Verrucomicrobiae*
17	*Firmicutes-Clostridia*	35	*Verrucomicrobia-Spartobacteria]*
18	*Firmicutes-Erysipelotrichi*	36	*WWE1-Cloacamonae]*

Other genera such as *Corynebacterium, Sulphurimonas, Candidatus poteiras, and Devosia* had reduced activity in both processes. Several *Corynebacterium* species are prominent in the production of organic acids with optimized activities in the presence of glucose. The stunted growth in diluted $ZnCl_2$ salt infers a high sensitivity to the salt concentration hence the observed decline in their relative abundance in the media. *Caloramotor*, and observed genus such as *Coprococcus* and *Sphaerochaeta* in the inoculum, showed a positive response in the salt media but only in the dissolved corn cob and not glucose. Species of this genera have been described as moderately thermophilic anaerobic fermenters of glycerol, xylose and cellobiose [27] Their preference for dissolved biomass at the process conditions and observed tolerance to salt concentration was as such understandable. Choice of organic carbon played an important role in their sustained bioactivity, much like the *Proteinclasticum*, albeit in an inversed role. *Proteinclasticum* was observed to thrive better in the presence of glucose in comparison to dissolved biomass.

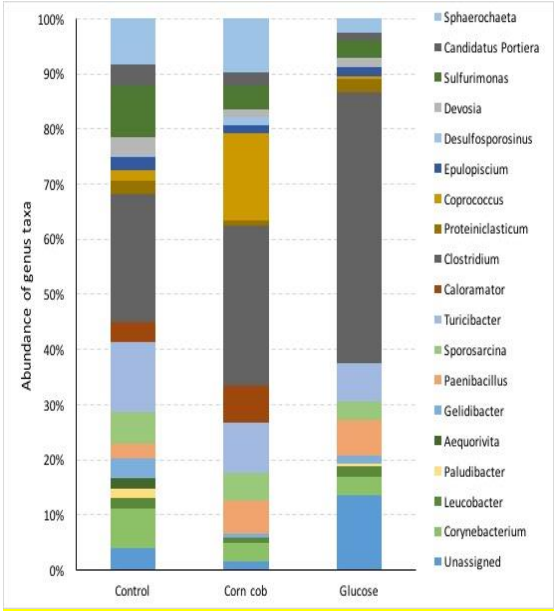

Figure 5: Relative abundance of the genera taxa in process media.

Paenibacillus strains is another observed genus with potential applications in industrial process manufacturing especially in the production of saccharification enzymes such as cellulase, amylases and hemicellulases. Hence the observed increase in activity in the process media. Biocatalysis using *Paenibacillus* strains is not very commercialized currently, but their potential for use due to their tolerance and high-activity and stability at industrially relevant process conditions makes them prospective alternatives to current physiologically ultrasensitive biocatalysts.

4 CONCLUSIONS

From this study, it became clear that:

1) Species of the *Clostridia* and *Bacilli* genera could find applicability in the processing of $ZnCl_2$ pretreated biomass.
2) Cow dung was not a very good source of halophilic anaerobes such as those of the genus *Haloanaerobium.*

5 NOTES

(1) Pretreatment severity factor (CS) was calculated with equation (1):

$$CS = \log\left[t \times \exp\left\{\frac{(T_H - T_R)}{14.75}\right\}\right] - pH \qquad (1)$$

where: CS (unitless) is a function of the severity factor,
T_H,- reaction temperature (°C),
T_R- reference temperature (°C).
t - pre-treatment time (min).

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