ISOLATION AND CHARACTERISATION OF MICRO-ORGANISMS WITH INDUSTRIAL IMPORTANCE FROM SISAL BOLE ROTS

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ABSTRACT

Investigation of microorganisms naturally acclimatized to *Agave* hybrid H 11648 (sisal bole rot) was conducted, with the aim of isolating and characterizing *Aspergillus niger* strains for industrial use. Microorganism were identified morphologically and then confirmation made by polymerase chain reaction (PCR). Results showed the existence of four major groups, listed in order of abundances as follows; *Aspergilli* (36.0±0.8) %, *Penicillin* (28.0±0.1) %, *Yeast* (15.0±1.6) %and *Fusarium* (10.0±0.12) %. The main groups of *Aspergilli* strains were *A. nidulans*, *A. tamari* and *A. niger* in ratios (3:2:2), respectively. Several endo-spore forming non-enteric gram (-) rods and coccid bacteria identified by API 20 NE identification systemincluded,*Brevundimonas diminuta sp, Shewanella putrefaciens sp, Brevundimonas vesicularis sp* and *Pasteurella sp*. Results showed that sisal bole rot stems hosts a high bio-diversity of microorganism species other than *A. niger*. Exploitation of the individual strains is recommended. This could eventually produce strains for precursors of industrially and therapeutically metabolites.

Key words: *Sisal, filamentous-fungi, Aspergillus niger, yeast, non-enteric gram -ve bacteria*

INTRODUCTION

Microbial communities are ubiquitous in the environment and have been implicated in various processes including opportunistic infections to living organisms of both the animal and plant kingdoms. Microorganisms also play a significant role in the global carbon cycle and provide a wide range of hydrolytic and oxidative enzymes, which are usually involved in digestive systems, breaking down of plant carbohydrates and lignocelluloses. Varieties of these enzymes are also important in chemical and for biotechnology industries. The rapid humification (rot) of carbohydrate rich sisal plants "carbon-cycling" by fermentative microorganisms is not desired in sisal agronomy as it lowers sisal fiber yields. Sisal bole rot (SBR) destruction by fungi has been well documented and strong measures have been emplaced to wade away the chances for manifestation such as proper tillage drainage and moisture control (KATANI Ltd. 1994; Lock, 1969). Since sisal life cycle extends to about 10 to 12 years, this time lapse supports a high microbial biodiversity, for that matter sisal bole rot manifestation suggested the survival of beneficiary microorganisms and that little effort might be needed for the isolation of strains for industrial production purposes. In principle the microbiology of sisal bole rots seems to be limited to essentially some bacteria, fungi and yeast species those which are tolerant to harsh

environmental conditions characterized by comparative low pHs' of sisal saps, hard lignified stem-bark and cuticle layer that covers plant leaves (Taiz and Zeiger, 1998). Presence of carbohydrate in forms of a complex inulin molecule necessitates molecular size reductions by hydrolysis prior the microbial assimilation. This could be another reason for the co-existence of high microbial diversity, where by some produces hydrolytic enzymes while others get access to the readily assimilated hydrolysates sugars. During the extreme dry conditions of Tanzanian coastal summer, sisal plants respiration and osmosis is regulated by the stored complex inulin-carbohydrate (Edelman and Jefford, 1968). Either at this point essentially most of the microorganisms enter the dominancy, forms spores or form a symbiotic alliance that is; waiting for others to break huge molecules for them to assimilate and survive.

This research therefore was aimed to isolate and characterize microorganisms, with industrial importance from sisal bole rots (SBR) and apply them in selective fermentation production of bio chemicals. Research findings is presumed to be applied in promotion of utilization of the whole sisal plant, which could lead to the expansion of production and marketing of non-traditional sisal products for example, bio ethanol enzymes and carboxylic acids (CFC/UNIDO 2003;KATANI Ltd. 1994).

MATERIALS AND METHODS

Materials

Sisal boles rot chunks from *Agave* H 11648, were randomly collected from Highland estate, and were handled aseptically. Samples were analyzed the same day; otherwise, stated sisal boles were frozen below -20ºC in a deep freezer. Microbial culture media used were from Sigma Aldrich and Fluka that is Sabouraud Dextrose Agar (SBD) CM0041, Oxytetracycline Yeast Extract Agar (OGYE) 66481 HiCrome, Czapek yeast autolysate agar (CYA) potato dextrose agar (PDA)were purchased from Sigma Aldrich. Standard methods and protocols by the respective suppliers were used throughout (AOAC, 1995; Collins *et al*., 1998; Domsch *et al*., 1980; Tran *et al*., 1998).Isolation recovery screening and confirmation of indigenous *A. niger* from sisal bole rot was done at the UDSM-College of Engineering and Technology (CPE) and KTH Environmental Biotechnology Department Sweden (KTH-Bio). Representative samples were sent to Nadicoma sequencing company in Germany (Nadicom, 2006).

Isolation of Microorganisms

Isolation and enumeration of microorganisms was done by sub sampling of 10 small chunks of sisal bole rot stems measuring 1-2 mm diameter and weighing 0.2 -0.3 gm. These were gently crushed, and soaked in 5 ml deionized sterile water for 5 minutes. A serial tube dilution for viable cells counting was performed at the dilution range between 10^{-1} and 10^{-7}. Total microbial population numbers was determined by a pour plate count technique on agar plates (PCA) after inoculation and culturing at temperatures 20ºC, 30ºC and 45ºC for 24, 48 and 72 hours (AOAC, 1995; Collins *et al*., 1998; Guarro *et al*., 1999;Domsch *et al*., 1980; Johnson and Case 2004; Tran *et al*., 1998). The growth media was made up by incorporating hundred millilitres of sisal Inulin so as to ensure that media used are suitable for the survival of indigenous species. Pure cultures of Aspergilli were differentiated by using special media Tryptone Agar (Tryptone) Yeast Extract Agar (YEA) Sabourad Dextrose (SB), Oxytetracycline Yeast

Extract Agar (OGYE), and incubation done at 30 º C for 7 days. Filamentous fungi and yeasts were also enumerated from acidified YEA, along with some endo spore forming bacterial that survives the acidic conditions. Isolated strains were purified, and maintained on NA and PDA slants (Johnson and Case, 2004).

Systematic and molecular-biological analyses

Macromorphologic identification were done by the powerful light microscope and Olympus BX-51 fluorescence research microscope that incorporates the advanced phase contrasting (Nomarski DIC system) and the Sony DXC960MD incorporating a 3 chip CCD video camera system. These were available at the KTH-Bio Laboratory. Cultures expressions on agar plates differed in terms of micro- and macro morphological features according to differential media used and microorganisms in question (Collins *et al.,* 1998; Domsch *et al.,* 1980). While systematic identification was done using taxonomical keys by (Guarro *et al.,* 1999; Pitt, 1993), confirmation was done by subjecting microorganisms to (PCR) polymerase chain reaction identification after method by (Gardes and Bruns, 1993; Kure *et al.,* 2003). While polymerase chain reaction identification was done using Sequences of Oligonucleotides Primers "SOP" AD-02, standard primers used had the following Internal Transcribed Spacer (ITS) sequences:

ITS 1 = 5`-TCCGTAGGTGAACCTGCGG-3` and
ITS 4 = 5`-TCCTCCGCTTATTGATATGC-3`;

ITS region was preferred because it is the most widely sequenced DNA region in fungi and it is typically most useful for molecular systematic across species level and even within species, as it allows selective amplification of fungal sequences (Gardes and Bruns, 1993). Genomic DNA samples for the *Aspergilla* was prepared according to Applichem manufacturer's instructions by taking the grown colonies from the respective plates, with an inoculating loop and directly inserting into the DNA Applichem extraction kit. The sequences of the oligonucleotides primers (SOP) used had the following sequence:

M13P = 5'-GAG GGT GGC GGT TCT-3'.

Evaluation of the PCR products of the M13P-PCR were applied on 2% TAE gel and separated at 100 volts, using the agarose gel which was stained with a 0.1% ethidium bromide solution and photographed (Gardes and Bruns, 1993 Kure *et al.,* 2003). After culturing in NA, non-enteric Gram (-) strainsbacteria colony forming units (c.f.u) were carefully observed under powerful light microscope and confirmed by the API 20 NE identification system by "bioMérieux Bv, Boxtel, Netherlands" (Johnson and Case, 2004).

RESULTS AND DISCUSSION

Microorganisms isolation screening and identification

As experiments were carried out in three replicates, results were presented as mean values in tabular form (Table 1 to 6). Four major groups of fungi and yeasts resulted. Individual group isolated were named and averaged in order of abundances; Aspergilli (36.0±0.8) %, Penicillin (28.0±0.1) %, Yeast (15.0±1.6) %and Fusarium (10.0±1.2) %. Remaining fraction included various spore forming bacteria species, of which the dominant were bacilli and coccid. Also seen were coma shaped but were not considered in this study (Table **1** and **2**). Dominant *Aspergilli* species observed were *Aspergillus nidulans*, *Aspergillus tamari* and *Aspergillus niger* in ratios (3:2:2) consecutively.

Table **1**: Total microorganisms counts in (SBR) chunks grab samples (n = 10).

Dilution range	c.f.u -count range	Average counts (x10^5 c.f.u)					
		Mould	Bacteria	Fungi	Yeast	Others	Total
10^{-2}	80 and above	ND	ND	ND	ND	ND	ND
10^{-3}	36-42	2.00	4.00	5.50	1.98	0.67	13.48
10^{-4}	30-40	2.08	3.31	6.60	2.43	0.58	14.42
10^{-5}	28-32	1.80	1.00	7.40	1.62	0.86	11.82
10^{-6}	25-30	3.60	4.30	6.80	5.09	0.78	19.79
10^{-7}	15 and less	ND	ND	ND	ND	ND	ND
Total counts		9.48	12.61	26.3	11.12	2.89	62.40
	Min	1.80	1.00	5.50	1.62	0.58	10.50
	Max	3.60	4.30	7.40	5.09	0.86	21.25
	Average	2.37	3.15	6.58	2.78	0.72	15.60
	Stdev	0.83	1.49	0.79	1.58	0.12	4.81
	Average (%)	15.19	20.21	42.15	17.82	4.63	100

ND=not determined above 80 and below 15 c.f.urange so as to avoid uncertainty errors

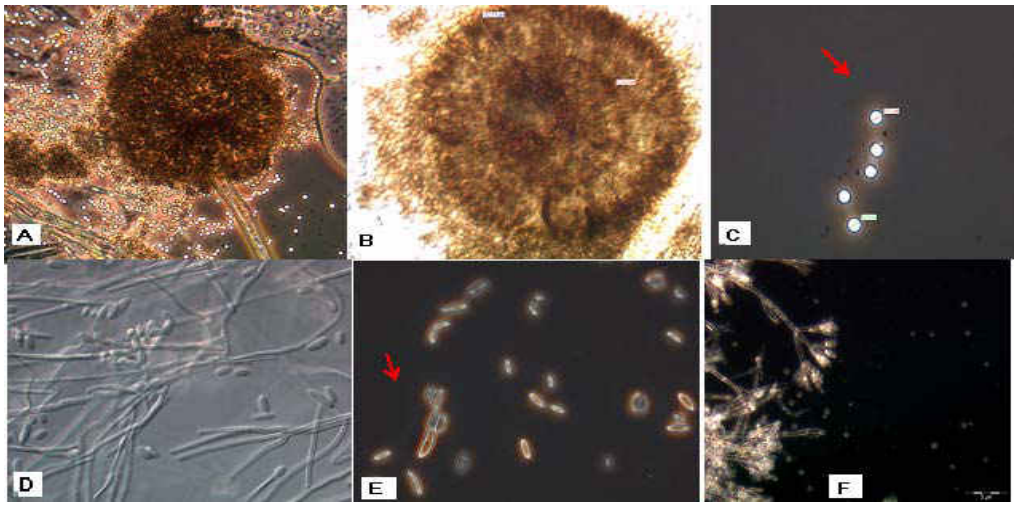

Figure 1: Morphologies ofsome microorganisms of industrial potential from (SBR)

Morphologic differences between Aspergillus niger and other filamentous fungi

Shown in Figure1 are some microorganisms of industrial potential from sisal bole rot stems; (A) *A. niger* Conidia, (B) transect of *A. niger* Conidia, (C) *A. niger* spores, (D) *Fusarium sp*, (E) Banana spores *Fusarium solani* and (F) Septate-conidiophores for *Penicillin sp.*

Macroscopically *Aspergillus niger* colonies on surface Czapeck agar plates at 30°C were initially white, and then blackened with conidial production. On reverse side, colonies were pale yellow and grew radialy. Five days old *A. niger* hyphae became distinctive septate and their respective conidia long and smooth, and hyaline measured (500 to 800 µm). The typical *Aspergilli* featured biseriate characteristic was clearly seen under the

microscope. Presence of a metulae and their corresponding conidiogenous phialides, while metulae and phialides covering entire vesicle to form a rough fruiting conidia head whose diameter measured at 132.78 ± 0.02 µm (A) and (B) in Figure1, was very conclusive also described by head, metula and phialide diameter values which were measured between 3.77 and 3.89 ± 0.02 µm (C). *Fusarium sp* colonies displayed characteristic banana like spores common to *F. solani* (D) and (E) in Figure 1, whereas *penicillin sp* clearly showed their distinct septate conidiophores' with respective metulae and phialides appearing in branched form (Guarro, 1999; Pitt, 1993).

Table 2: Identifiable microorganism and their incidence in (SBR) grab samples (n=10)

Reference	Morphological Identification	c.f.u	Microbe-count/g-sisal bole rot(% c.f.u)	Remarks
OR5	Yeast budding	$11.12x10^5$	(15.0±1.6)	
OR5 KTH	Yeast budding			For PCR
OR5 NA	Yeast budding			For PCR
4a	Yeast single			
4a KTH	Yeast single			For PCR
Wfus.	*Fusarium solani*	$7.42x10^5$	(10.0±0.12)	For PCR
Wfus KTH	*Fusarium solani*			
BYF KTH	*Aspergillus niger wild*	$26.7x10^5$	(36.0±0.8)	PCR & (citric acid)
BYF	*Aspergillus niger wild*			(citric acid)
BWf KTH	*Aspergillus tamarii*			For PCR& (citric acid)
BWf	*Aspergillus tamarii*			
BfK	*Aspergillus nidulans*			For PCR
Yf_1	*Aspergillus nidulans*			For PCR
Yf_2	*Aspergillus nidulans*			
Pe Green	*Penicillin*	$20.8 x10^5$	(28.0±0.1)	
Pe Gray	*Penicillin*			
White-cream	Endospore coccid	$5.19x10^5$	(7.0±1.5)	API 20 NE TESTS
Off White	Endospore rods	$4.45x10^5$	(6.0±1.5)	API 20 NE TESTS
Shiny White	Coma motile species	$2.97x10^5$	(4.0±1.5)	
Others		$2.89x10^5$	(4.0±1.5)	

Table 3: PCR sequencing confirmation summary

S/No Nadicom	Reference	Identified as	Remarks
449.1	OR5 KTH	*Pichia membranifaciens*	
449.2	4a KTH	*Clavispora lusitaniae*	
449.3	OR5 NA	*Pichia membranifaciens*	
449.6	Wfus.	*Fusarium solani*	
449.7	BYF KTH	*Aspergillus niger*	for CA production
449.8	BWf KTH	*Aspergillus tamarii*	for CA production
449.9	BfK	*Aspergillus nidulans*	
449.10	Yf_1	*Aspergillus nidulans*	
API 20 NE	Endospore coccid		
API 20 NE	Endospore rods		
API 20 NE	Motile species		

Polymerase chain reaction (PCR) Confirmation and *Genomic DNA Results*

Polymerase chain reaction (PCR) sequencing results which are presented in Table 3showed high consistence with morphological identities done using taxonomical keys by (*Guarro et al.,* 1999; Pitt, 1993). Concurrently, the genomic DNA results for Aspergilla fungi shown in Figure 2 indicated that samples 449.7-(BYF KTH) *Aspergillus niger wild*, *Aspergillus nidulans* strains 449.9-(BfK)and 449.10-(Yf_1)seemed to be very close. Concurrently sample 449.8-(BWf KTH) *Aspergillus tamarii* showed a different pattern. These findings thus signified a need for a more intensive identification work at species level, so as to capture genomic evolution (Figure 2).

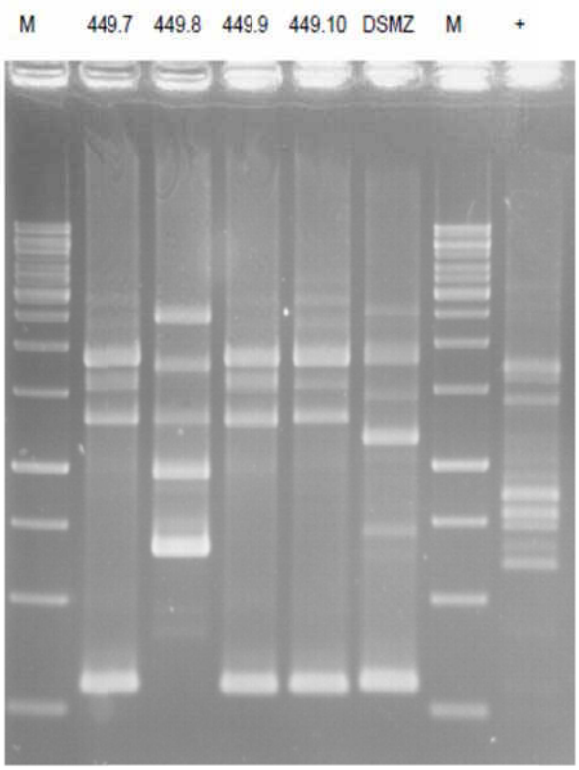

Figure **2**: Genomic DNA results for Aspergilli fungi

Table 4: Biochemical characterization of bacteria colonies viewed from above

ID	13	RTN_{4a}	other	RTC_4	RTC_{6a}	WA_4	7
Media	NA	NA	NA	CZ	CZ	WAYE	NA
Temp ºC	30	30	30	20	20	30	30
Hrs	24	24	24	24	24	24	24
Average diameter of several c.f.u (mm)	2	1.5	2	1.5	3	1.5	3
General Shape:	Spr	Ro	Ro	Ro	Ro	Ro	Spr
Gram Staining	G(-) R	G(-) C	G(-) R	G(-) C	G(-) C	G(-) C	G(-) R
Catalase	(+)	(+)	(+)	(+)	(+)	(-)	(+)
Oxidase	(+)	(-)	(+)	(-)	(-)	(-)	(-)
Endospores	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Oxygen demand	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Minimal nutrient	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Maconkey	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Starch Hydrolysis (+)clear zone indicates starch hydrolysis	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Gelatin	(+)	(-)	(+)	(-)	(-)	(-)	(+)
Casein Hydrolysis	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Litmus milk	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Sulfur Indole Motility Test	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Minimum agar	(+)	(+)	(+)	(+)	(+)	(+)	(+)
MaConkey(-) Negative re confirms G (-) for endo-spores	(-)	(-)	(-)	(-)	(-)	(-)	(-)
FE Negative re confirms G (+) for endo-spore	(+)	(+)	(+)	(+)	(+)	(+)	(+)
FM(-)acid	(-)	(-)	(-)	(-)	(-)	(-)	(-)

KEY:G (-) R = Gram-negative rods; G (-) C = Gram-negative cocci; (Spr) = Spreading; (Ro) = Round irregular

Table **5**: Bacteria colonies reaction to antibiotic (AB Biodisk) after 24 Hrs

ID	Media	Temp ºC	Tetracycline			Penicillin			Aminoglycosides	Sulphonamides
			CL	**TC**	**RI**	**PG**	**AM**	**GM**	**SM**	**SU**
13	NA	30	(R)	(R)	(R)	(R)	(S)	(R)	(S)	(R)
RTN_{4a}	NA		(IS)	(R)	(R)	(R)	(S)	(R)		
other	NA		(R)	(R)	(R)	(R)	(R)	(IS)	(S)	(R)
RTC_4	CZ	20	(IS)	(R)	(R)	(R)	(S)	(R)	(S)	(R)
RTC_{6a}	CZ		(R)	(R)	(R)	(R)	(R)	(IS)	(S)	(R)
WA_4	WAYE	30	(R)	(R)	(R)	(R)	(R)	(IS)	(S)	(R)
7	NA		(R)	(R)	(R)	(R)	(R)	(IS)	(S)	(R)

KEY: (ID) = Colony identity;(IS)-(S) = Intermediate - Sensitive to systemic infection dosage has to be concentrated to organs; (S) = Sensitiveto systemic infection; (R)= Resistantto systemic infection; **CL** =Chloramphenicol 30µg; **TC** =Tetracycline 30mg; **RI**= Rifampicines 2µg; **AM** = Ampicilin 10µg; **GM** =Gentamycin 30µg; **SM** =Streptomycin 30µg; **SU** =Sulphonamide Trimethoprim 250µg, **PG** = Benzyl penicillin 100µg. Shown in Table **5,** bacteria isolates were found to be resistant to most common antibiotics. However in very few cases bacteria reactions to antibiotics was rated resistant to intermediate - sensitive to systemic infections, and colonies moderately reacted to either of the following antibiotic; Chloramphenicol, Ampicilin and Gentamycin. This suggested that if either of these drugs is to be used, the dosages have to be concentrated to organs.

Table **6**: Bacteria coloniesreactions to API 20 NE TESTS

Colony ID	Media	**API 20 NE**			Suggested nomenclature
		Temp ºC	**code-**24 hrs	**code-**48 hrs	
13	NA	30	1510004	1510004	*(B. dim)* synonym, *(P. dim)OR (S. put)*
RTN_{4a}	NA	30	1430000	1430000	*(B. ves)OR (Pas*)
Other	NA	30	1510004	1510004	*(B. dim)*
RTN_{4a}	CZ	20	1430000	1430000	*(B. ves)OR (Pas)*
RTC_{6a}	CZ	20	1600000	1600000	(*Bru*)
WA_4	WAYE	30	400000	400000	*(B. ves)OR (Pas)*
TN_{3b}	NA	30	430000	1430000	*(B. ves) OR (Pas)*

KEY: (*B. dim*) =*Brevundimonas diminuta sp;* (*B. Ves*) = *B. vesicularis;* (*P. dim*) =*Pseudomonas diminuta;* (*S. put*) =*Shewanella putrefaciens sp;* (*B*) =*Brucella sp;* (*Pas*) =*Pasteurella sp*; ID=Identity

Shown in Table **6**, the dominant bacteria sp are *B. Diminuta, S. Putrefaciens, B. vesicularis* and *pasteurella sp*. Bacterial colony ID 13 tested (+Ve)in respect to NO_3 &N_2 test however were identified as *B. Vesicularis*.

Biochemical characterization and API 20 NE identification

A combination of standard biochemical tests and assimilations tests, are supported by the API 20 NE Identification system for non-enteric Gram (-) rods done at 24 and 48 hours. The growth guide for API 20 NE Identification system was adapted in characterization of the non-fermenting-metabolizing bacteria (Tables **4** to **6**). The standardized inoculums picking with a low bacterial concentration using a (0.5 µl) micro pipette tip from a freshly refined cultures, guaranteed reliability of this method, while eliminating the contaminants from mixed cultures and subcultures.

DISCUSSION

Microbial sampling was done during the humid rainy season, under the assumption that the conditions were suitable for the

proliferations of the fungal diseases. Despite to the fact that the black sisal bole rot has been occasional spotted to some injured individual plants and those under extremely stress conditions, the evidence on existence of spontaneous fungal out breaks were infrequently encountered. This results which are supported by literature cited indicate that sisal bole rot is very rich in microbe biodiversity specifically mould fungi and some endo-spore forming bacteria communities. Concurrently under the normal circumstances sisal plants showed a high immunity to microbial diseases (CFC/UNIDO, 2003; Lock, 1969).

In general terms both microorganisms isolates assimilated raw sisal inulin and used it as the sole carbon source. Most of sisal bole rot isolated fungi species were good in processing raw sisal juice, and strains are known to have a high potential for industrial use, for example strains of *Aspergillus niger* are known to produce citric acid (Kubicek *et al.,* 1994). While *penicillin*, *yeast* and *fusarium* strains are used in enzyme and drugs manufacturing (Masih and Paul, 2002). In this study yeast species that were suspected to be responsible for the volatile alcohol stench while creating competitions with other moulds were not comprehensively investigated thus suggesting further considerations for future studies.

The encountered co-existence of moulds and bacteria was probably due to the inherent symbiosis. Among these, the motile and endo spore forming bacteria were common isolates. These were identified by using the API 20 NE TESTS, and named as; *Brevundimonas diminuta sp, Shewanella putrefaciens sp, Brevundimonas vesicularis sp* and *Pasteurella sp* **(**Ngonyani, 2010). The identified strains were able to hydrolyze starch but not glucose(Table 4). The current study suggested that while bacteria strains preferred complex form of carbohydrate, they were able to assimilate starch and were resistant to common antibiotics as indicated in (Table **5**).

CONCLUSIONS

Agave hybrid H 11648 (sisal bole rot) has got high bio-diversity of microorganisms naturally acclimatized to inulin rich environment. The identified four major groups of fungi and yeasts namely *Aspergillus niger*, *Penicillin*, *Yeast* and *Fusarium* suggested further tests on their versatile use in selective fermentation processing of useful metabolites. The existence of endo-spore forming non-enteric Gram (-) rods and coccid bacteria, suggests some possible symbiosis and competition pattern that needs a lengthy investigation. The isolated fungi strains, for example *Aspergilli niger* was considered as the potential citric acid production strain with sisal inulin hydrolysates being used as feed stock. With less strenuously manipulations fungi strains acclimatized to inulin rich saps are presumed to be in better position for future selective industrial fermentation processing of useful metabolites. The same analysis suggests that further rigorous screening is required for the identification of missed strains.

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