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Characterization and Identification of Probable Halophiles

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Abstract
Halophiliic microorganisms associated with degradation were investigated using a solid-solute solution of degraded wooden-cork samples mixed in saline water. The sodium benzoate sterilized solid samples were air dried, weighed to attain 1gm and added into a saline solution of 4.25g of normal Sodium Chloride (NaCl) dissolved in 500ml of distilled water with pH and temperature at 7.56 and 27ºC respectively. Microbiological analysis using standard laboratory techniques were done to determine the probable isolates. Ten bacteria (Bacillus pumilus, Klebsiella aerogenes, Bacillus laterosporus, Citrobacter amalonaticus, Bacillus megaterium, Xanthomonas campestrii, Bacillus circulans, Enterobacter aerogenes, Staphylococcus aureus and Enterobacter intermedius) and four fungal (Aspergillus niger, Penicillium chrysogenum, Aspergillus flvus, and Rhizopus arrhizus) were confirmed present. The microorganisms demonstrated osmoregulatory ability in minimal salt concentration at pH of 7.56 and temperature of 27ºC respectively. This also shows there is need for improved food preservation while using salt.

Keywords: wooden-corks, Saline-water, Microorganisms, Osmoregulation

Introduction
Microorganims grow in a wide range of environment, from very cold (below 0ºC) to very hot (above 100ºC), from very acidic to very alkaline or high saline concentration, high pressure or any other environment that might not look normal to humans. Microbes living under extreme physical and chemical conditions are referred to as extremophiles (Seckbach *et al*., 2008) [12]. These organisms in recent times have attracted considerable attention due to their peculiar physiology and ecology (González-Toril *et al*., 2003).

The microbes which can tolerate high salt (saline) environment are called halophiles. Gilmour (1990) [3], categorise organisms found in saline habitats into halotolerant (organisms with 0 – 0.3M NaCl optimum growth and a growth range of 0 – 1M NaCl), moderately halophilic (organisms with 0.2 – 2.0M NaCl optimum growth and a growth range 0.1 – 4.5M NaCl) and extremely halophilic (organisms with 3 – 5M NaCl optimum growth and a growth range 1.5 – 5.5M NaCl).These salt – loving organism according to DasSarms and Arora, 2001 have representation in each of the three domains: Archaea, Bacteria, and Eucarya (Oren, 2002) [10]. Some of these halophiles can withstand more than one extreme environment. According to Kushner (1993) [9], haliphilic bacteria could be Gram positive or Gram negative, aerobic or facultative anaerobic, and have been shown to grow well in a variety of salt concentrations ranging from 0.2 to 5.2 M. The salt required by halophiles need not be sodium chloride, but might possess a number of other ions (Johnson *et al*., 2007) [7]. Salts, as recorded by Jensen 1954, was referred to as the main bacteriostatic substance used in meat curing. Its action could be due to the dehydration effect and the alteration of the osmotic pressure of the salt which inhibits microbial growth. The extent to which salt concentration causes changes in bacterial growth depends on the osmotic balance required for such growth (DasSarma, and Arora, 2001) [2]. Microorganisms differ in their salt resisting abilities. Most disease – causing bacteria do not grow below 0.9a_w which is approximately 10% NaCl concentration. However, most molds responsible for food spoilage grow at an a_w as low as 0.8 corresponding to high salt concentration (Parish, 2006) [11]. Some bacteria require an astonishingly high level of salt to begin growth, whereas other bacteria would be immediately killed in high levels of salt (Ara *et al*., 2013) [1]. The factors controlling the survival of enteric bacteria in the marine environment have intrigued scientists for decades. Driven by obvious public health concerns as well as by broader attempts to understand bacterial responses to environmental stress, numerous studies have explored the fate of *Escherichia coli* and other enteric bacteria following their exposure to salt solutions at different concentration. Many of these efforts were motivated by the need to properly evaluate the risk posed by such microorganisms when released into an environment such as recreational waters or to the safety of fisheries or marine agriculture. As pointed out above, in many of the investigations into the survival of *E. coli* and other enteric bacteria in the sea, colony formation ability was used as the main or only viability parameter. When released into the saline solution as the case may be, enteric bacteria are subjected to an immediate osmotic upshock, and their ability to overcome this by means of several osmoregulatory systems

could largely influence their subsequent survival in that environment. Some marine bacteria, such as members from the *Vibrio* genera, require a certain amount of salt to grow. Other bacteria, such as *E. coli*, can grow either in the presence or absence of salt (Gilmour, 1990) [3]. The bacterial species that require salt, like *Vibrio* sp., have an internal osmolyte property that requires salt on the outside of the cell to maintain osmotic balance over the cell membrane. Without salt, these organisms would "burst" as water rushes into the cell to try and achieve balance (DasSarma, and Arora, 2001 [2]; Gilmour, 1990) [3]. *E. coli* may have optimal growth in the absence of salt, but in the presence of salt it will grow, just at an attenuated rate as neutral salt has been found to affect the physical nature of the growth of microorganism (Holm and Sheman, 1924) [5]. For bacteria with salt tolerance, growth and salt concentration have a direct correlation. As the amount of salt in the growth medium increases, bacterial growth decreases. For bacteria that require salt, a bell shaped growth curve is observed. Upon an osmotic upshift, bacterial cells accumulate or synthesize specific osmoprotectant molecules, in order to equalize osmotic pressure and avoid drastic loss of water from the cytoplasm. Salt will slow mold growth. Salty solutions will cause the mold cells to become dehydrated through osmosis.

This is why meats such as ham and jerky are traditionally salted to prevent the growth of mold and bacteria. Wooden-corks used to cork bottles containing wine, juices etc. were analyzed in this experiment and studies showed the effect of high concentration of saline water on microorganisms associated with wooden-corks. The aim of the study is to enable identification of microorganisms that can survive in a minimal salty environment associated with wooden-corks found in debris.

2. Materials and Method

2.1 Preparation of Salt Solution:

Zero point one four five molar (0.145M) of normal salt (NaCl) was prepared by weighing 4.5g in 500ml of distilled water. The pH was 7.56 and temperature recorded to be 27ºC.

2.2 Preparation of Wooden Cork Salt Solution (WCSS)

Ten (10) wooden corks were collected from waste dump sites around Oshodi area within Lagos State, Nigeria. These wooden-cork (w-c) samples were washed with sodium benzoate and soaked in the prepared salt solution for 3 days.

2.3 Microbial Analysis

Ten (10) fold serial dilutions of the wooden core salt solution (WCSS) were carried out. Zero point one mililitre (0.1ml) of the diluents 10^2, 10^4, 10^6 and 10^8 were inoculated into Nutrient agar, MacConkey agar, DeMan Rogosa Sharpe agar, Trypton soy agar and Sabraud dextrose agar. The seeded plates of NA, MCA, DMRSA, TSA were incubated at 37^0C for 24 hours while PDA was incubate at 25^0C for 3-5 days

2.4 Characterization

Microbial characterization of isolates after incubation period was carried out using laboratory techniques such as: Total viable counts, gram's reaction, morphology, catalase oxidase and motility tests. Microbial identification was further done with confirmatory tests such as; Nitrate, Citrate, Methyl Red, Voges Proskauer and Sugar fermentation (Fructose, Mannose, Xylose, Sorbose, Sorbitol, Sucrose, Inositol, Lactose, Glucose and Maltose).

Results and Discussion

1. A A total number of ten (10) bacteria (Bacillus pumilus, Klebsiella aerogenes, Bacillus laterosporus, Citrobacter amalonaticus, Bacillus megaterium, Xanthomonas campestrii, Bacillus circulans, Enterobacter aerogenes, Staphylococcus aureus and Enterobacter intermedius) and four (4) mold (Aspergillus niger, Penicillium chrysogenum, Aspergillus flvus, and Rhizopus arrhizus) were isolated and screened. Table.1 shows the total viable counts of microbial isolates as 6 × 10^2cfu/ml on nutrient agar as the highest from diluents 2 factor while MacConkey agar had 0.1× 10^1 cfu/ml as the lowest counts from diluent 8 factor. On Sabraud dextrose agar all diluent factors 2 – 8 had a single isolate each different from the other. Table 2 showed bacteria mophorlogical characteristics. The results illustrated that Bacillus spp., Xanthomonas sp., Citrobacter sp., and Enterobacter spp. were rod shaped while Staphylococcus aureus was shaped cocci. Most of the isolates were creamy while Staphylococcus aureus and Xanthobacter sp. were orange and yellow colour respectively.
2. Table 3 shows the mophorlogical characteristics of the isolates. The isolates where characterized and identified as Aspergillus niger, Penicillium chrysogenum, Aspergillus flavus and Rhizopus arrhizus respectively. The results from table 4 illustrated the microbial activity soluble substrates and few activities were observed by the isolates. While isolate 4, a Bacillus sp. demonstrated more activity, others showed less, but isolate 8 which appeared as an unidentified strain of Enterobacter sp. did not demonstrate any activity. From table 5, the isolate was identified based on conventional biochemical tests. The table illustrates the ability of all the isolates to ferment fructose, lactose and glucose It was also observed that Bacillus pumilus and Klebsiella aerogens demonstrated same utilization characteristics on all test samples. The table also showed that Bacillus pumilus, Klebsiella aerogenes, Enterobacter aerogenes and Enterobacter intermedius showed positive effect to Voges Proskauer, fructose, mannose, xylose, and sorbose sugars. It was also observed that the following isolates Bacillus circulans, Enterobacter aerogenes, Bacillus laterosporus and Bacillus pumilus were positive to sucrose, inositol, lactose, glucose and maltose. The results showed isolates that can survive in low saline environment. It has been a known fact from ancient time that salt could be used for food preservation because it has the tendency to inhibit the growth of microorganisms (Koo, 2009) [8], and this form basis for the use of salts in food preservation. Enteric bacteria are halotolerant, hence they can grow in the presence or absence of salt (Gilmour, 1990) [3]. Salmonella sp, E.coli and K. aerogenes are halotoleant enteric bacteria (Gilmour, 1990) [3], these organisms have been found to be food contaminants of public health interest. According to the article "Bacterial Food Poisoning" by Wagner, 2008, Listeria species are able to grow over an extremely large range of salt concentration, up to 30.5 percent. This level of tolerance can affect the food industry's

attempts to use salt as a natural way to control the presence of microorganisms.

4. Conclusion

It is often believed that salt is been used to prevent microbial growth but overtime research has proven there some microorganisms Whom utilize these salts at high concentration for growth. This investigation showed some of these isolates with such ability thus it is necessary to further examine theses microbes. This will help to understand growth pattern of these isolates in different salt water concentration, how they breakdown salt and the byproduct they produce, as well the supporting agents responsible for such activity

Table 1: Total Viable Counts of Microbial Isolates (cfu/ml)

Sample	Dilution	NA	Mac	MRS	TSA	SDA
Wooden Cork Salt Solution	2	1.6×10^2	1.2×10^2	7.0×10^1	1.4×10^2	A single colony
	4	1.6×10^1	1.3×10^1	3.8×10^1	3.0×10^1	A single colony
	6	0.2×10^1	0.1×10^1	2.1×10^1	2.7×10^1	A single colony
	8	0.2×10^1	0.1×10^1	0.8×10^1	1.6×10^1	A single colony

Key: N.A=Nutrient Agar, Mac= MacConkey Agar, M.R.S=de.Man Rogosa Sharpe Agar, T.S.A= Trypton Soy Agar, S.D.A= Sabraud Dextrose Agar.

Table 2: Bacteria Morphological Characteristics of Isolates from Wooden Cork Salt Solution.

Sample Codes	Gram's Reaction	Catalase	Shape	Motility	Morphology	Isolates Codes
WcSs.1	+	+	Rods	+	Cream, dull, low convex, opaque.	Bacillus sp.
WcSs.2	-	+	Rods	-	Creamy, shiny, mucoid, big colony	Klebiella sp.
WcSs.3	+	+	Rods	+	Creamy, watery, flat, big colony	Bacillus sp.
WcSs.4	-	-	Rods	+	Creamy, big colony, raised, entire	Citrobacter sp.
WcSs.5	+	+	Rods	+	Cream, flat, entire, dull, opaque	Bacillus sp.
WcSs.6	-	+	Rods	+	Yellow, watery, spreading	Xanthomonas sp.
WcSs.7	+	+	Rods	+	Creamy, low convex, entire, opaque, umbonate	Bacillus sp.
WcSs.8	-	+	Rods	+	Creamy, watery, slimy, spreading, opaque	Enterobacter sp.
WcSs.9	+	+	Cocci	-	Orange, raised, entire	Staphylococcus sp.
WcSs.10	-	+	Rods	+	Creamy, raised, round, shining	Enterobacter sp.

Key: WcSs = Wooden Cork Salt Solution

Table 3: Mold Morphological Characteristics Isolates from Wooden Cork Salt Solution.

Sample Codes	Macroscopy Morphology	Microscopy Morphology	Isolates
WcSs.1	Colony was white woolly quickly becoming black with conidial production. Reverse was pale yellow.	Hyphae are septate and hyaline were present. Conidial heads were radiate initially, splitting into columns at maturity. Conidiophores were long, smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle. Metulae and phialides were present and covered the entire vesicle. Conidia were brown to black, very rough, globose.	Aspergillus niger
WcSs.2	The colony was flat, filamentous, and velvety, woolly to blue gree. The plate reverse was pale to yellowish	Septate hyaline hyphae, branched conidiophores, metulae, phialides, and conidia were observed. Metulae were secondary branches that formed on conidiophores. The metulae carried the flask-shaped phialides. Round conidia, unicellular, and visualized as unbranching chains at the tips of the phialides.	Penicillium chrysogenum
WcSs.3	Texture is woolly to cottony to yellowish-green. Sclerotia present. Goldish to red-brown in reverse.	Hyphae are septate and hyaline present. Conidial heads were radiate to loosely columnar. Conidiophores were coarsely roughened, uncolored and wide, vesicles globose to subglobose, metulae were present.	Aspergillus flavus
WcSs.4	Colony grew very rapidly, filling the Petri dish in 4 days. The texture was observed with typical cotton pattern. The front color was white initially and turned grey to yellowish brown in time while the reverse is white to pale.	Nonseptate broad hyphae, sporangiophores, rhizoids, sporangia, and sporangiospores were visualized. Sporangiophores are brown in color, unbranched and in clusters. Rhizoids were located at the point where the stolons and sporangiophores meet. Sporangia were located at the tip of the sporangiophores	Rhizopus arrhizus

Table 4: Microbial Activities Isolated from Wooden Cork Salt Solution.

Sample Codes.	Urease	Gelatin	Starch	Casein	Isolates Codes
WcSs.1	-	+	-	+	*Bacillus* sp.
WcSs.2	-	+	+	-	*Klebiella* sp.
WcSs.3	-	+	-	+	*Bacillus* sp.
WcSs.4	+	-	-	-	*Citrobacter* sp.
WcSs.5	-	+	+	+	*Bacillus* sp.
WcSs.6	-	-	+	+	*Xanthomonas* sp.
WcSs.7	-	-	+	-	*Bacillus* sp.
WcSs.8	-	-	-	-	*Enterobacter* sp.
WcSs.9	+	-	+	-	*Staphylococcus* sp.
WcSs.10	-	-	+	-	*Enterobacter* sp.

Key: WcSs = Wooden Cork Salt Solution.

Table 5: Identification of Microbial Isolates from Wooden Cork Salt Solution.

Sample Codes	**N**	**C**	**MR**	**VP**	**Fru**	**Man**	**Xyl**	**Sor**	**Sorb**	**Suc**	**Ino**	**Lac**	**Glu**	**Mal**	**Identified Isolates.**
WcSs.1	+	+	-	+	+	+	+	+	-	+	+	+	+	+	Bacillus pumilus
WcSs.2	+	+	-	+	+	+	+	+	-	+	+	+	+	+	Klebsiella aerogenes
WcSs.3	+	+	-	-	+	+	+	+	-	+	+	+	+	+	Bacillus laterosporus
WcSs.4	+	+	+	-	+	+	-	+	+	-	+	+	+	+	Citrobacter amalonaticus
WcSs.5	-	+	-	-	+	+	+	+	-	+	+	+	+	-	Bacillus megaterium
WcSs.6	+	+	-	-	+	-	+	+	-	+	-	+	+	+	Xanthomonas campestrii
WcSs.7	+	+	-	-	+	+	+	+	-	+	+	+	+	+	Bacillus circulans
WcSs.8	+	+	-	+	+	+	+	+	+	+	+	+	+	+	Enterobacter aerogenes
WcSs.9	-	-	+	+	+	+	+	-	+	+	-	+	+	+	Staphylococcus aureus
WcSs.10	+	+	+	+	+	+	+	+	+	+	-	+	+	+	Enterobacter intermedius

Key: WcSs=Wooden Cork Salt, N=Nitrate, C=Citrate, MR=Methyl Red, VP= Voges Proskauer, Fru=Fructose, Man=Mannose, Xyl=Xylose, Sor=Sorbose, Sorb=Sorbitol, Suc=Sucrose, Ino=Inositol, Lac=Lactose, Glu=Glucose, Mal=Maltose.

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