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(RESEARCH ARTICLE)

Morphological and biochemical characterization of isolated *Aspergillus niger, Saccharomyces cerevisiae* and *Zymomonas mobilis* from local indigenous sources

Tambuwal Amamatu Dahiru, Muhammad Inusa Babanladi, Alhaji Sani, Muhammad Sirajo and Ogbiko Cyril *

Department of Pure and Applied Chemistry, Faculty of Science, Usmanu Danfodiyo University Sokoto, Sokoto State North Western, Nigeria.

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Abstract

Rapid global population growth has increased the demand for food and energy supply from cheap and readily available indigenous sources. Fungi and bacteria species were attempted to be isolated from indigenous sources using standard procedures. The bacteria isolated from *Hibiscus sabdariffa* drink and *Citrus aurantium* were characterized and identified as *Saccharomyces cerevisiae* and *Zymomonas mobilis* respectively while the isolated fungus from maize grains was characterized and identified as *Aspergillus niger*. These results show the enormous potential of these local substrates for bioethanol production.

Keywords: *Aspergillus niger*; Characterization; Isolation; *Saccharomyces cerevisiae*; *Zymomonas mobilis*

1. Introduction

Microorganisms are living heterogeneous creatures that are microscopic in size such as algae, fungi (mould and yeasts) and bacteria [1]. The multiplication of microorganisms in food is greatly influenced by inherent as well as environmental characteristics of the food [2]. Indigenous natural fermentation takes place in a mixed colony of microorganisms such as moulds, bacteria and yeasts [3]. Nearly all food fermentations are the result of more than one microorganism, either working together or in sequence, but growth is generally initiated by bacteria, followed by yeasts and then moulds [4-5]. Compounds formed during fermentation processes includes**;** organic acids, ethanol, aldehydes and ketones [6].

Aspergillus niger is a filamentous ascomycete used mainly in the production of citric acid as well as an expression host for both heterologously and homologously expressed extracellular enzymes [7]. The genome sequences of the two *A. niger* strains each revealed the presence of 16 genes encoding putative chitin-degrading enzymes [8-9]. These chitinolytic enzymes are of great significance to their fungal hosts since they are involved in the degradation of chitin substrates [10]. Chitinases are believed to modify chitin which are the integral structural component in fungal cell wall hence play a key role during morphological changes such as autolysis, hyphal branching or germination of conidia [11-12].

Saccharomyces are the safest and most effective microorganisms employed for fermenting sugars to ethanol [13]**.** Although yeast is ubiquitous; it is most frequently isolated from sugar rich samples as well as from a wide variety of natural habitats as different as leaves, flowers, sweet fruits, tree exudates, grains, roots fleshy fungi, insects, dung, soil among others [14] where its ethanol tolerance, sugar tolerance and invertase activities are important properties specifically considered for its use in industrial ethanol production [15].

* Corresponding author
E-mail address: cyril.ogbiko@udusok.edu.ng, cyrilogbiko@gmail.com

Zymomonas mobilis has long been recognized as a potential biocatalyst for large scale ethanol production close to the maximum theoretical value [16]. However, the substrate range of wild-type strains of *Z. mobilis* is restricted to glucose, fructose and sucrose [17].

2. Material and methods

2.1. Source of samples

Maize grains, *Hibiscus sabdariffa* drink and orange (*Citrus aurantium*) fruits were purchased from local sellers at Kasuwar Daji market, Sokoto South Local Government area of Sokoto State, Nigeria. The samples were identified and authenticated by a taxonomist at the Department of Plant Biology, Faculty of Science, Usmanu Danfodiyo University Sokoto, Sokoto State Nigeria.

2.2. Preparation of growth media

2.2.1. Nutrient agar

28 g of nutrient agar powder was dissolved in 1.0 L of distilled water. The mixture was thoroughly shaken, heated to boil and allowed to cool at room temperature. It was sterilized by autoclaving at 121 ^{o}C for 15 minutes and allowed to cool to 45 ^{o}C before dispensing into sterile Petri dishes [18].

2.2.2. Sabouraud dextrose agar (SDA)

65 g of sabouraud dextrose agar powder was dissolved in 1.0 L distilled water. The mixture was thoroughly shaken and heated to boil on a hotplate. The solution was sterilized by autoclaving at 121 ^{o}C for 15 minutes. There after allowed to cool to 45 ^{o}C before being dispensed into sterile Petri dishes [18].

2.2.3. Potato dextrose agar (PDA)

PDA medium was used for culturing the micro-organisms. It is composed of 200 mg peeled Irish potato, 20 g analytical grade dextrose and 20 g analytical grade agar-agar powder. The Irish Potato was cut into small pieces with sterilized knife and boiled with 500 cm^3 of distilled water for 25 minutes. The potato extract was filtered through a muslin cloth into a 100 cm^3 flask after which the measured dextrose and agar-agar powder were added and re-boiled for 10 minutes to obtain complete dissolution. The mixture was made up to 1 L in a volumetric flask with distilled water. The flask was corked properly with an aluminum foil paper, sterilized in an autoclave for 20 minutes at a pressure of 15 psi and temperature of 121 ^{o}C to prevent contamination. The content of the flask was cooled to 45 ^{o}C and 5 cm^3 of prepared streptomycin sulphate solution was added to prevent the growth of bacteria [19].

2.2.4. Cellulose agar

Filter paper was employed as the source of cellulose. 100 g of plane agar dissolved in 1.0 L of distilled water was autoclaved at 121 ^{o}C for 15 minutes, allowed to cool to 45 ^{o}C before dispensing it into properly labeled sterile Petri dishes. Sterilized filter paper (1-each) was placed into each Petri dishes and left until the media solidifies [19].

2.2.5. Starch agar

100 g of plane agar 3 g of starch were dissolved in 1.0 L of distilled water. The mixture was autoclaved at 121 ^{o}C for 15 minutes and allowed to cool at 45^{o}C before dispensing 20 cm^3 portions into properly labeled sterile Petri dishes. The plates were aseptically inoculated with test organism by streaking across the surface of the medium; the plates were then incubated at 37 ^{o}C for 3-5 days. At the end of the incubation, the plates were flooded with lugol's iodine. Positive hydrolysis of starch is indicated by a clear white zone around the colonies of the organisms appearing blue-black while a negative result is indicated by both the surrounding and the organism colony appearing blue-black [20].

2.2.6. Solid standard medium

3 g of yeast extract, 20 g of glucose, 20 g of agar, 5 g of peptone and 3 g of malt extract were dissolved in 1.0 L of distilled water and slightly heated to ensure complete dissolution. The pH of the solution was maintained at 6.8, treated with cyclohexane to inhibit yeast growth before autoclaving it at 121 ^{o}C for 15 minutes [20].

2.3. Isolation and identification of *Aspergillus niger*

Aspergillus niger were isolated from maize grains using direct plating technique. 20 maize grains were picked randomly and surface sterilized by soaking for 1 minute in sodium hypochlorite (2.5%), and rinsed with sterile distilled water. The grains were blotted with sterile filter paper and plated on potato dextrose agar containing 7.5% sodium chloride and 1.0 g of streptomycin sulphate in 1 litre of media [21]. The plates were incubated at 25 ^{o}C and monitored for fungal growth daily for seven days. The resulting cultures were identified based on cultural and morphological characteristics using taxonomic keys [22-23]. Target moulds were sub-cultured to obtain pure single spore cultures. A small amount of the growth colonies were taken and smeared on a glass slide, covered with a slip and heated slightly to remove air bubbles before being viewed under microscope. The organism identified was compared with the standard structure of *Aspergillus niger* as described by Robert and Ellen [24].

2.4. Isolation of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was isolated from fermented *Hibiscus sabdariffa* drink (called sobo drink) from local sobo hawkers at Kasuwar Daji in Sokoto. The fermented drink was soaked in distilled water for three days after which small amount of the fermented solution was inoculated SDA in three different Petri dishes. The plates were incubated at room temperature for 2 to 4 days. Different colorations indicating different bacterial organisms were sub-cultured on different Petri dishes containing SDA to obtain a pure and uncontaminated culture. The pure culture was coded HS.

2.5. Isolation of *Zymomonas mobilis*

Zymomonas mobilis was isolated from rotten *Citrus aurantium* (sweet orange) fruit. The organism was isolated according to the solid standard isolation medium method described by Obire [20]. Three rotten oranges randomly selected were thoroughly washed with water to remove earthy impurities and 1.0 cm^3 juice squeezed out from each orange using sterile syringe. The juice from each sample was diluted with 9 cm^3 of sterile distilled water in a test tube. Serial dilutions were prepared to obtain a 10^{10} dilution factor on the solid standard isolation medium plates. The plates were incubated in an anaerobic jar for two days at 35 ^{o}C. Colonies observed were isolated, purified by streaking on freshly prepared media and incubated for two days at 35 ^{o}C in an anaerobic jar. This procedure was repeated until a pure culture was observed. The pure culture was coded CA.

2.6. Identification of bacteria

The identification of bacteria was based on morphological characteristics and biochemical tests carried out on the isolates. Morphological characteristics observed for each bacteria colony after 24 h of growth included colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation. Biochemical characterizations were done according to the method of Fawole and Oso [25]. Some of the key tests for identification include the following:

2.6.1. Gram staining techniques

A thin smear of each of the pure 24 h old culture was prepared on clean grease-free slides, fixed by passing over gentle flame. Each heat-fixed smear was stained by addition of 2 drops of crystal violet solution for 60 sec and rinsed with water. The smear were again flooded with Lugol's iodine for 30 sec and rinsed with water, decolourized with 70% alcohol for 15 sec and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 sec and finally rinsed with water, then allowed to air dry. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pink or red while gram positive organisms appeared purple [25].

2.6.2. Spore staining technique

This test is to detect the presence of bacteria endospores. Heat-fixed smears of the organisms were prepared on separate slides and flooded with 5% Malachite green solution and steamed for a minute. The stain was washed off with water and counter stained with 2 drops of Safranin solutions for 20 sec. The slides were allowed to air dry and examined under oil immersion objective (100) lens. Endospores stain green while vegetative cells stain pink [26].

2.6.3. Motility test

A sterile needle was used to pick a loop of a 24 h old culture and was stabbed onto nutrient agar in glass vials. The vials were incubated at 37 °C for 24-48 h. Non-motile bacteria had growth confined to the stab line with definite margins without spreading to surroundings area while motile bacteria gave diffused growth extending from the surface [27]. A

positive motility test was indicated by red turbid area extending away from the line of inoculation. A negative test was indicated by red growth along the inoculation line [28].

2.6.4. Catalase test

A small quantity of 24 h old culture was transferred into a drop of 3% hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicates the presence of catalase enzyme [26].

2.6.5. Urease test

A small quantity of 24 h old culture was transferred were inoculated in a urease agar and incubated at 30°C for 48 hours, the development of a red-pink color indicate a positive result [28].

2.6.6. Hydrogen sulphide test

A small quantity of 24 h old culture was inoculated into already prepared tubes of triple sugar iron agar and inoculated at 37 °C for 48 hours. After the incubation period the tube were observed for hydrogen sulphide (black coloration) gas production [28].

2.6.7. Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour [26].

2.6.8. Indole test

5 mL of Tryptone broth was placed into different test tubes after which a loopful of the isolated bacterial isolates was inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as control. The test tubes were then incubated at 37 °C for 48 h. After incubation, 0.5 mL of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 min to permit the reagent to rise. A red or red-violet colour at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result [26].

2.6.9. Citrate test

The test is used to detect the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. 10 mL of citrate medium was dispensed into test tubes and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking with the 24 h old culture organisms once across the surface. A change from green to blue indicates utilization of the citrate [26].

2.6.10. Sugar fermentation

Sugar fermentation test was carried out to determine the ability of organisms to ferment sugars with production of acid and gas. Sugar indicator broth was prepared using peptone water medium containing 1% fermentable sugar and 0.01% phenol red. About ten milliliters of sugar broth was dispensed into each of the test tubes, Durham tube which would trap the gas if produced was inverted carefully. The test tubes were autoclaved and inoculated with a loopful of 24 h old culture of the test organisms after then incubated for 2-7 days at 36±1 °C and observed daily for acid and gas production. Yellow colouration indicates acid production while gas production was indicated by displacement of the medium in the Durham tube [25].

2.6.11. Methyl red (MR) test

5 mm of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed into clean test tubes and sterilized. The tubes were then inoculated with the isolated test organisms and incubated at 37°C for 48 h after which few drops of methyl red solution were added to each test and colour change was observed. A red colour indicates a positive reaction [27].

2.6.12. Voges-proskaeur (VP) test

5 mm of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37 °C for 48 h. After incubation, 6% α-naphhtol and 6% sodium hydroxide were added to about 1 mL of the broth culture of the isolated organisms. A strong red colouration formed within 30 min indicates positive reaction [27].

3. Results and discussion

3.1. Isolation of *Aspergillus niger*

A dark brown color, long conidiophores, globule vesicles that is completely covered with biseriate phialides with the phialides borne on brown metulae was observed on the microscopic structure shown in Figure 2a confirms the presence of *Aspergillus niger*. It was compared with the standard morphological strain reported by Robert and Ellen [24] (Figure 2b). The result confirms that *A. niger* is found in association with organisms in plants, soil and decaying vegetation [7] and is in agreement with the findings of Nyongesa *et al.* [29].

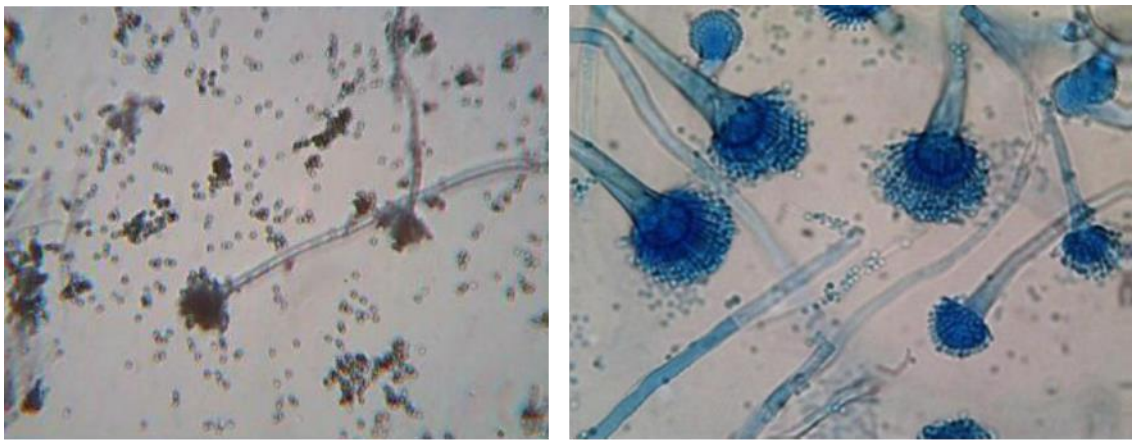

Figure 1 (a) Morphological structure of *Aspergillus niger* x 250; (b)Standard morphological structure of *Aspergillus niger* x 300

3.2. Isolation and morphological characterization of isolated *Saccharomyces cerevisiae* and *Zymomonas mobilis*

The colonial, morphological and biochemical characteristics of the bacteria isolated from *Hibiscus sabdariffa* drink and *Citrus aurantium* is shown in Tables 1 and 2.

Table 1 Colonial and morphological characteristics of isolated microorganisms

Isolate	HS	CA
Colony shape	Fluffy cream colonies	Brilliant white to cream coloured
Cell shape	Purple oval shape	Plump with rounded ends
Gram's reaction	+	-
Endospore formation	-	-
Motility test	-	+

(+) = Positive, (-) = Negative, HS = *Hibiscus sabdariffa*, CA = *Citrus aurantium*

The colonial, morphological and biochemical characteristics of the isolated *Sachromyces cerevisiae* are in agreement with the reports of Rabah *et al.,* [30] who isolation *S. cerevisiae* among other yeasts from palm wine, Ezeogu and Emeruwa [31[who isolated *S. scerevisiae* from palm wine juice in sake-type fermentation and Moneke *et al.* [32] who reported the isolation of *S. cerevisiae* from orchard soil. Similarly, the colonial, morphological and biochemical characteristics of the isolated *Zymomonas mobilis* are in agreement with that of Obire [20] who reported the isolation of *Zymomonas mobilis* from fresh palm wine saps.

Table 2 Biochemical characterization of isolated microorganisms

Isolate/Test	HS	CA
Catalase	+	+
Coagulase	-	-
MR/VP	+/-	-/+
Indole	-	-
Citrate	-	
Urease	-	-
Oxidase	-	-
Hydrogen sulphide	+	+
Starch hydrolysis	-	-
Arabinose	AG	-
D-mannitol	AG	-
Glucose	AG	+
Galactose	AG	-
Fructose	AG	+
Sucrose	AG	+
Lactose	AG	-
Maltose	AG	-
Lactose	AG	-
Suspected organism	*Saccharomyces cerevisiae*	*Zymomonas mobilis*

(+): Positive, (-): Negative, AG: Acid and Gas, HS = *Hibiscus sabdariffa*, CA = *Citrus aurantium*

4. Conclusion

The presence of these organisms during the fermentation periods confirms that they grow in close association with the food substrates and produce extracellular enzymes responsible for the fermentation of most legumes and cereals.

Compliance with ethical standards

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Disclosure of conflict of interest

We the authors of this research work have no conflict of interest and are solely responsible for all the contents and writings presented in this research work.

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