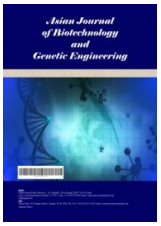

Asian Journal of Biotechnology and Genetic Engineering

4(1): 27-48, 2021; Article no.AJBGE.65304

Fermentative Enzymes Production Efficiency of Ethanologenic Bacteria Isolated from Raphia Palm Sap

O. A. Ayanshina[1,2*], M. N. Igwo-Ezikpe[1], N. O. A. Imaga[1], T. O. Ajasa[1], O. Adeboye[1], I. A. Odjokpa[1], S. E. Agbale[1], O. C. Okafor[1] and F. N. Onyekawa[1]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, P.M.B 12003, Lagos State, Nigeria.
[2]Department of Chemical Sciences, College of Natural and Applied Sciences, Crescent University, Abeokuta, Ayetoro Road, Lafenwa, P.M.B. 2104, Sapon, Abeokuta, Ogun State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author OAA conceived the idea, coordinated the whole experiment and wrote the manuscript. Authors MNIE and NOAI supervised and made substantial contribution to the work entirely. Authors TOA, OA, IAO, SEA, OCO and FNO contributed to sample collection, experimental approaches and writing of the manuscript first draft. All authors read and accepted the final draft of the manuscript.

Article Information

Editor(s):
(1) Dr. S. Prabhu, Sri Venkateswara College of Engineering, India.
Reviewers:
(1) Zuemy Rodríguez Escamilla, Universidad Nacional Autónoma de México (UNAM), México.
(2) Marco César Prado Soares, University of Campinas, Brazil.
Complete Peer review History: http://www.sdiarticle4.com/review-history/65304

Original Research Article

Received 05 December 2020
Accepted 10 February 2021
Published 02 March 2021

ABSTRACT

Background: Consolidated bioprocessing (CBP) candidate construction remains gray in the biotechnology of bioethanol production; and recent lead way involved genetic transformation of competent cells with ethanologenic and/or cellulolytic characteristics from natural microorganisms.
Aim: As part of an ongoing study, the ethanologenic property of natural tropical bacteria population isolated from Raphia palm sap was investigated.
Methods: Freshly tapped Raphia palm sap (PW) sample was obtained from a Raphia palm plantation site at Elemu Bus-stop, Jakande, Isolo, Lagos State, Nigeria. The morphological and biochemical characteristics (MBC) as well as 16s rDNA genotyping of the bacteria isolated from PW were used for identification. A representative α–keto acid decarboxylase (pyruvate decarboxylase -

**Corresponding author: E-mail: o.ayanshina@crescent-university.edu.ng, ayanshinaoluwamuyiwa@yahoo.com;*

PDC) and alcohol dehydrogenase (ADH) productivities of the isolated bacteria in glucose supplemented media were determined by spectrophotometry.
Results: The combination of MBC and 16s rDNA genotyping of the PW bacteria revealed about 6 isolates that were phylogenetically related to *Bacillus pumilus*, *Bacillus subtilis*, *Paenibacillus validus*, *Macrococcus spp.*, *Yokenella resenberghei*; and *Brevibacillus brevis*. Meanwhile, efficient PDC productivity (0.5-1.8U/mL) was observed for *Bacillus pumilus* and *Bacillus subtilis* at pH 10.0, 25°C, 2.0g substrate concentration, $NaNO_3$ as nitrogen source, and 72 hours incubation period. *Bacillus pumilus* and *Bacillus subtilis* efficiently produced ADH (0.5-2.0U/mL) optimally at pH 10.0, 25°C, 1.0g substrate concentration, peptone as nitrogen source, and 96 hours incubation period.
Conclusion: The Raphia palm sap microbiota are efficiently ethanologenic. Thus, their genes can be adapted for genetic transformation in consolidated bioprocessing.

Keywords: Consolidated bioprocessing; bioethanol; genetic transformation; ethanologenic bacteria; 16s rDNA genotyping.

1. INTRODUCTION

Bacteria often wear the tag as friend or foe. Be that as it may, they are organisms of massive economic importance cutting across multiple sectors of human endeavors including environment, health, and biotechnology [1]. The industrial application of bacteria is conspicuously reported in the formation of bioproducts including food and beverage, enzymes, and biofuel [1,2]. Owing to the replication rate of bacteria; their application, for human gain, is notable in the drive towards sustainable production of bioethanol from biomass, particularly, lignocellulosics [3].

Lignocellulosics are biomass composed of lignocellulose, which is a supramolecular assembly majorly in plant cell walls that is chemically made up of cellulose, hemicellulose and lignin [4]. Bioconversion processes, particularly bioethanol production depends on the hydrolytic breakdown of the carbon-rich polymeric cellulose and hemicelluloses into monomeric simple sugars, which are channeled through glycolytic pathways that favor anaerobic fermentation to produce energy and alcohol or organic acid. Dependent on multiple factors including types of substrate, microbial species, and physicochemical factors such as oxygen availability, fermentation of simple sugars may lead to production of several by-products including ethanol.

The utilization of the monomeric simple sugars for ethanol production is characteristic of a wide range of microorganisms including the ethanol-producing bacteria, otherwise known as ethanologenic bacteria [5]. Their ability to produce ethanol as a by-product of their intermediary metabolism is characteristic of their capacity to express certain glycolytic enzymes that are peculiar to the Entner-Doudoroff and Embden-Meyerhof pathways [6]. Worthy of note is pyruvate decarboxylase and alcohol dehydrogenase. Pyruvate decarboxylase, which belongs to the lyase group of enzymes catalytically, cleaves pyruvate to yield carbondioxide and acetaldehyde; and the acetaldehyde is reversibly reduced into ethanol by an oxidoreductase enzyme known as alcohol dehydrogenase [7,8]. It has been reported that many bacteria have enzymatic machinery to flux simple sugars through either/both Entner-Doudoroff and Embden-Meyerhof pathways [9-11].

However, ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the *Saccharomyces* presently used for practical production of fuel alcohol; and with the recent advances in biotechnology, they have the potential to play a key role in making production of ethanol much more economical [12]. Owing to the timetaken and expense of commonly used multistep separate or simultaneous saccharification and fermentation strategies involving more than one microorganism, current researches are focusing on consolidated bioprocessing approach for bioethanol production. Consolidated bioprocessing is a single step bioconversion involving enzyme production, cellulose breakdown and bioethanol production by a single microorganism [13-15]. Although the development of consolidated bioprocessing microorganism remains gray in the biotechnology of bioethanol production; recent lead way involves genetic transformation of competent cells with ethanologenic and cellulolytic characteristics from natural microorganisms [14]. It is therefore imperative to

continue to search for bacteria that are efficiently endowed with these characteristics based on natural selection or biodiversity.

Palm wine, the sap of the Raphia palm tree is adjudged as a natural source of microbial diversity with alcohol fermentation capabilities [16,17]. Palm wine, according to Amoa-Awua et al. [18], serves as a rich substrate that enhances the growth of various types of microorganisms. Previous studies on the microbiology of oil palm tree (*Elaeis guineensis)* and *R. hookeri* have reported several bacterial and yeast flora to be involved in the fermentation process; moreover, *Acetobacter* species were earlier isolated from palm sap [18-20]. Falegan and Akoja [21] reported the presence of *Lactobacillius spp, Acetobacter spp, Leuconostoc spp, Listeria spp and Corynebacterium spp.* in palm wine sample. Chandrasekhar et al. [22] reported the presence of lactic acid bacteria which includes *Streptococcus, Pediococcus, Leuconostoc* and *Lactobacillus*.

It is quite obvious that a handful of previous investigations from different geographical locations revealed the identity of bacteria isolated from Raphia palm sap. However, the bacteria identification methodology was largely based on morphological and biochemical characterization only. Besides, the previous researches have not focused on identifying efficient fermentative and/or glycolytic enzyme producing ethanologenic bacteria. Since growing interest on consolidated bioprocessing exists, it becomes imperative to identify microbial isolates that produce fermentative and/or glycolytic enzymes efficiently for biotechnological application in the construction of CBP candidates for industrial utilization. As part of an ongoing study, the ethanologenic potential of natural tropical bacteria population isolated fromRaphia palm sap was investigated.

2. MATERIALS AND METHODS

2.1 Sample Collection

Freshly tapped Raphia palm sap was obtained from Raphia palm plantation site located in Elemu bus-stop, Bucknor Jakande, Isolo Local Government Area, Lagos state, Nigeria (GPS coordinates: 6°31'5"N; 3°17'14"E). The plantation site selection was based on accessibility by road that ensured ease of Raphia palm sap collection. The Raphia palm sap was collected into a sterile plain laboratory 25mL sample bottles and transported to the laboratory in coolers containing mixture of ice-packs and NaCl. The sap collected from the Raphia palm tree was used as source of bacteria isolation.

2.2 Experimental Design

Bacteria were isolated from the Raphia palm sap collected; and the morphological and biochemical characteristics of the isolated bacteria were analyzed. Each isolate was identified using 16s rDNA genotyping; and used for alcohol dehydrogenase and pyruvate decarboxylase production in separate media that were optimized under several physicochemical conditions. The specific enzymes activities were evaluated to select efficient isolates.

2.3 Isolation of Ethanologenic Bacteria

Raphia palm sap was subjected to serial dilution and traditional pour plate method was used for the isolation of the ethanologenic bacteria. The medium employed was modified Nutrient Agar composed of NaCl (0.5%), agar (1.5%), meat extract (0.1%), yeast extract (0.2%), peptone (0.5%), with glucose (0.5%) at pH 6.8. The medium was autoclaved at 121°C for 15 minutes before inoculation; and treated with Fluconazole to inhibit fungi growth. About 200µL of each diluted sample was poured on the agar plates and incubated at about 37^{o}C for 48 hours. Using the same medium, spread plate technique was used to subculture from the primary culture. About 30 different colonies were isolated by streaking on fresh agar plates to obtain pure cultures that were coded/labeled. The morphology of each pure culture was identified using microscope (Leica DM300®, Germany) at 100x magnification. In addition, biochemical tests including gram stain, motility, catalase, oxidase, methyl red, Voges Prausker, Citrate, Urease, Nitrate reduction, Tripple sugar iron, indole and sugar fermentation tests were performed. Based on the duplication of the coded bacteria isolates with similar characteristics, the number of isolates that were subjected to genotyping reduced to within half of the initial 30 colonies.

2.4 Bacteria 16s rDNA Genotyping

2.4.1 DNA isolation

Each bacteria isolate was grown as pure culture in an autoclaved and antifungal treated nutrient broth (NaCl (0.5%), meat extract (0.1%), yeast extract (0.2%), peptone (0.5%), with glucose (0.5%) at pH 6.8) for 24 hours. The culture broth was centrifuged at 4000*xg* to allow the cell to

pellet and the supernatant was discarded. The wet cell pellet was subjected to DNA extraction using the Zymo™ Bacterial/Fungal DNA Extraction Kit (Zymo Research, USA). The fidelity of the bacteria isolates DNA extracted was checked quantitatively by Nanodrop spectrophotometer, and qualitatively on 1.5% agarose gel electrophoresis respectively.

2.4.2 l6s rDNA amplification and sequencing

Molecular 16*s* DNA genotyping was performed by polymerase chain reaction (PCR) using universal forward and reverse primers specific for V3-V4 hypervariable region of bacteria genus [23]. The forward primer(5'–GTGCCAGCAGCCGCGCTAA-3') and reverse primer (5'–AGACCCGGGAACGTATTCAC- 3') were synthesized by InqabaBiotec. The PCR was performed in 20µL PCR tube containing 1 µL of 10X PCR buffer, 0.8 µL dNTP mix of 2.5 mM, 1.0 µL of 25 mM $MgCl_2$, 0.5 µL each of forward and reverse primer (5 pmol), 0.1 µL of Taq polymerase, 1.0 µL of DMSO, 2.0 µL of 10ng/µL bacterial DNA and 3.1 µL of injection water. The PCR tubes were fitted in GeneAmp PCR System 9700 (Applied Biosystems™, USA) and amplification was carried out at the following condition: initial denaturation at 94°C for 5 minutes followed by 36 cycles of denaturation, annealing and extension (94°C for 30 sec, 56°C for 30 sec and 72°C for 45 sec) and final extension at 72°C for 7 minutes followed by hold at 10°C for infinity. The amplicons were resolved on 1.5% agarose gel electrophoresis against a 1kbplus DNA ladder (Invitrogene). The amplicons were purified using Gel extraction kit (Zymoclean™ Gel DNA Recovery Kit, ZymoResearch, Irvine, CA, USA). Sequencing services of Bioscience Department of the International Institute of Tropical Agriculture (IITA), Ibadan was sought. Briefly, the purified amplicons were subjected to BigDye thermocycling reaction (BigDye terminator V3.1™ cycle sequencing Kit); and at termination the amplicons were loaded onto ABI 3500 Genetic Analyzer (Applied Biosystems™, USA) for Sanger sequencing.

2.4.3 16s rDNA identification

The 16s rDNA sequences of the bacteria isolates generated were subjected to the 16s rDNA reference database of the Genbank through the Basic Local Alignment Search Tool (BLAST) program(http://blast.ncbi.nlm.nih.gov/). Alignment files were downloaded and exported to MEGA 7 software for phylogenetic tree construction.

2.5 Enzyme Production and Optimization of Factors Affecting Production

2.5.1 Evaluation of fermentative enzymes activities

Isolated ethanologenic bacteria were screened for alcohol dehydrogenase and pyruvate decarboxylase production in liquid medium composed of mineral salts and specific substrate, in this case, glucose. The liquid medium contains 1.0 g KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; NH_4NO_3, 0.3 g; $MnSO_4$, 0.16g; $ZnSO_4$, 0.14 g; $CoCl_2$, 2.0 g; pH 6.8 per liter of distilled water. In a separate flask, 10 g of glucose in 100 mL of distilled water was prepared. The mineral salts medium and dissolved glucose substrate were autoclaved at 121°C for 15 minutes before inoculation. Broth cultures of the bacteria isolates freshly grown overnight were used as inoculum. In triplicate, the liquid medium (50 mL), glucose substrate (5 mL) and 1 mL of the inoculum were aseptically aliquoted into 100 mL Erlenmeyer flasks and incubated on a shaker at agitation rate of 130rpm for 96 hours at room temperature. The broth culture was centrifuged at low speed and the supernatant was used as the source of extracellular fermentative enzymes. The pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were quantified as described by Sobana et al. [7]. At varying pH (3,7 and 10), temperature (4-45°C), substrate concentration (0-3 g) and time (24-120 hours), the production of pyruvate decarboxylase and alcohol dehydrogenase was investigated. Meanwhile, the effect of nitrogen source on these enzymes production was determined by medium supplementation with different nitrogen sources including ammonium nitrate, sodium nitrate, peptone or tryptone.

2.5.2 Determination of pyruvate decarboxylase activity

The activity of PDC was measured in by monitoring the pyruvic acid-dependent oxidation of NADH. Briefly, the reaction mixture consisted of 2.7 mL of 200 mM citrate buffer, 100µL of 1M sodium pyruvate, 50µL of 6.4mM β-NADH, and 100 µL of cell extract. The reaction mixture was mixed and the assay was carried out at 25°C. The enzyme activity was determined by measuring the conversion of NADH to NAD^+ at 340nm using spectrophotometer. One unit of PDC activity is defined as the amount of enzyme required for the conversion of 1µmol of NADH to

NAD^+ per minute under the given assay conditions.

2.5.3 Determination of alcohol dehydrogenase activity

The assay of ADH activity was measured by monitoring the aldehyde-dependent oxidation of NADH, in which the conversion of NADH to NAD^+ was determined spectrophotometrically. The reaction mixture contained 0.5 mL of 6.4 mM β-NADH, 1.0mL of 100mM Tris- HCl, 0.5 mL of acetaldehyde, and 0.2 mL of cell extract and incubated at 40°C for 5 minutes. The alcohol-dependent oxidation of NADH using acetaldehyde was measured at 340nm. One unit of ADH activity is defined as the amount that oxidized 1µmol of NADH/min.

Both enzyme (PDC and ADH) activities were calculated using the following formula:

Unit/mL extract = (ΔA340nm/min test − ΔA340nm/min blank) × (reaction volume)/ (6.22) (enzyme volume)

6.22 is the millimolar extinction coefficient of β-NADH at 340nm [7].

2.5.4 Statistical analysis

All quantitative data are reported in Mean ± standard deviations of triplicate determinations of various assays. The measure of central tendency and line graphs were drawn using Graphpad Prism 6.0.

3. RESULTS

3.1 Morphological and Biochemical Charateristics (MBC) of the Isolates

A total of thirty (n=30) colonies (PW) were isolated from the Raphia palm sap. Among the colonies were gram positive and negative rods and cocci bacteria isolates that were majorly non-motile, possess catalase, oxidase and urease activities, tested positive to indole and methyl red but negative in Voges Prausker reaction (Table 1) alongside their abilities to ferment glucose, fructose and mannitol (Table 2). No single colony was found to ferment cellulose and starch (Table 2). Meanwhile, the alignment of the colonies morphological and biochemical characteristics indicated similarities within some colonies, hence such colonies were merged as one; the total number of colonies became fourteen (n=14) and their probable identity were inferred (Table 3). However, colonies that were microscopically found to have yeast buds were discontinued from this study.

3.2 Identity and Phylogenetic Characteristics of the Isolates

The distinguished bands depicted by the agarose gel electrophoreogram showed, qualitatively, the fidelity of the isolated bacterial DNA extracted (Fig. 1). Given the specific universal primers utilized, the amplification of the 16s rDNA of each bacterial isolate (16s rDNA amplicons) was successfully characterized as indicated by the correlation of the obtained band size with the expected band size of about 850bp on the agarose gel electrophoreogram (Fig. 2). The partial nucleotide sequences of the 16s rDNA amplicons revealed the identity of bacteria specie that were isolated as shown on the phylogenetic tree (Figs. 3-7). Generally, the scale bar of the tree branch lengths measured about 0.0005–0.2 number of substitutions per site of nucleotide for all the bacteria isolated from the Raphia palm sap. The isolate tagged PW1, PW2 and PW11 were closely related to *Bacillus subtilis*; and its 16s rDNA partial sequence was deposited in the GenBank under the accession numberMK503422. Isolates PW3, PW5, and PW12 were closely related to *Macrococcus hajekii*; and tagged with an accession number MK503423, the partial sequence of its 16s rDNA was deposited in the GenBank. Isolates PW4 and PW7 shared very close relation to *Brevibacillus brevis* and *Yokenella resenberghei* respectively; and in turn the accession numbers for their 16s rDNA partial sequences deposited in the GenBank were MK503424 and MK503425.Isolates PW8 and PW13 shared phylogenetic relationship with *Bacillus pumilus*; and its 16s rDNA partial sequence was deposited in the GenBank under the accession number MK530256. Isolate PW9 was related to *Streptococcus dysgalactiae*;and PW10 to *Paenibacillus validus*. The 16s rDNA partial sequence of the *Paenibacillus validus* as deposited in the GenBank was given the accession number MK503425.

3.3 Optimization of Physicochemical Conditions Affecting Ethanologenic Enzymes Production

Pyruvate decarboxylase (PDC) productivity was found to be optimum at basic pH in comparison to acidic pH (Fig. 8) as temperature within 4°C to 25°C enhanced PDC productivity as compared to higher temperature ranges (Fig. 9). However,

recovery in productivity was observed as the temperature increased above 35°C (Fig. 9). The PDC productivity increased linearly as the substrate concentration increased minimally (from 0.0g to 1.0g to 2.0g); however, the enzyme production efficiency decreased as further increase in the concentration of the substrate (>2.0g) was encountered (Fig. 10). Pyruvate decarboxylase productivity increased through 72 hours incubation period beyond which there was decrease the enzyme production as incubation time reached 96 hours; but production slightly increased as the time reached 120th hour (Fig. 11). Increased PDC productivity was encountered by majority of the isolates in the presence of sodium nitrate ($NaNO_3$) as the nitrogen source (Fig. 12).

Majorly, enhanced PDC productivity under each of the physicochemical conditions [pH of 10 (0.8–1.4U/mL), temperature of 25°C (1.4–2.0U/mL), substrate concentration of 2.0g (>1.4U/mL), nitrogen source as sodium nitrate (>0.4U/mL) and time of incubation of 72 hours (1.3–1.5U/mL)] observed was higher for isolates PW1, PW2, PW8, PW11 and PW13 in comparison to other isolates. The isolates codes correspond to *Bacillus pumilus* and *Bacillus subtilis* as shown by the phylogenetic tree.

Majorly, a neutral to basic pH enhanced the production of alcohol dehydrogenase (ADH) by the isolates (Fig. 13). The pattern of ADH production under varied temperature showed increase in ADH productivity by all the Isolates (PW1–14) as the temperature increased from 4°C to 25°C. Meanwhile, a steep decrease in ADH productivity was observed as the temperature increased further to within 35°C but minute recovery in productivity was found as the temperature increased to about 45°C (Fig. 14). In terms of substrate concentration, minimal substrate loading of about 1.0g resulted in increased ADH productivity barely across all the isolates. But ADH productivity gradually decreased as the substrate loading was increased further (Fig. 15). Meanwhile, peptone supplementation largely and sodium nitrate sparingly accounted for optimum source of Nitrogen that efficiently enhanced ADH production (Fig. 16). Alcohol dehydrogenase productivity increased through 96 hour period of incubation beyond which the enzyme production sharply declined (Fig. 17).

Finally, all the isolates towed the same pattern of ADH production under each of the conditions observed –a similar pattern observed in the case of PDC production. However, efficient ADH productivity under each of the optimum physicochemical conditions [pH of 10 (0.8–1.4U/mL), temperature of 25°C (1.5–1.8U/mL), substrate concentration of 1.0g (>0.6U/mL), nitrogen source as peptone (>0.4U/mL) and time of incubation of 96 hours (≥1.5U/mL)] observed was higher for isolates PW1, PW2, PW8, PW11 and PW13 in comparison to other isolates. The isolates codes correspond to *Bacillus pumilus* and *Bacillus subtilis* as shown by the phylogenetic tree.

4. DISCUSSION

The chemistry of lignocellulosics, which is polymeric, necessitates the involvement of sustainable cellulolytic machinery of cellulase producing microorganisms in cellulose hydrolysis into monomeric glucose and/or simple sugar units; and simultaneous or separate channeling through intermediary metabolism of ethanologenic microorganisms that culminate in ethanol formation. However, having the ethanol production process occur in a simultaneous and/or separate hydrolysis and fermentation approach is cumbersome and time-consuming; hence a consolidated bioprocessing (CBP) approach wherein the aforementioned processes are performed by a single microorganism has been proposed. Meanwhile, the construction of a CBP candidate (microorganism) in the biotechnology of bioethanol production involves genetic transformation of competent microbial cells with cellulolytic and/or ethanologenic characteristics from natural microorganisms. It therefore becomes imperative for research to identify microorganisms with superior characteristics. Herein, and as part of an ongoing study, the ethanologenic property of natural tropical bacteria population isolated fromRaphia palm sap was investigated.

Characteristic morphology and biochemistry are commonly peculiar to some species of organisms, particularly bacteria; thus these characteristics have been utilized in their taxonomy – the principle on which the Bergey's Manuals were written. In this present study, the observation of fresh palm sap having the predominance of yeast buds; some non-motile bacteria with catalase and oxidase activities alongside capability of simple sugar fermentation and growth at room temperature and above, which is characteristic of mesophilic bacteria, corroborates the findings of some previous studies wherein palm sap was adjudged to be a

rich medium that enhances the cultivation of several microbial species including aerobic mesophilic bacteria, acetic acid bacteria, coliform bacteria, lactic acid bacteria and yeasts [18,19,24]. The sugar fermentation to ethanol majorly observed in Raphia palm sap or palm wine is thought to be an inherent property of the Raphia plant; however, it is also reasoned that this biochemical reaction (fermentation) might as well be attributable to bacteria and yeast that contaminate and thrive in the sap as it is being tapped [25].

Nonetheless, there have been variations as regards the microbial species harboring or being isolated from palm sap; and with reference to several studies, only yeast (*Saccharomyces sp.*) has been found widely among many varieties of palm sap across different countries till date [18,26-32]. In this present study, acetic acid bacteria were not among the palm sap bacteria isolates. Perhaps, the absence of acetic acid bacteria among the list of bacteria being identified by this present study could be attributed to the use of freshly tapped palm sap that was immediately subjected to isolation techniques as it was transported to the laboratory. After all, acetic acid bacteria are reportedly found in palm sap that has undergone fermentation for about 48-72 hours; and this is responsible for palm wine spoilage as acetic acid (vinegar) is found present in the palm wine [33]. Meanwhile, the observed presence of pathogenic bacteria found in palm sap investigated herein is similar to the study of Olawale et al. [25] who isolated pathogenic bacteria from palm wine.

The inconsistency of solely utilizing morphological and biochemical characteristics (MBC) in the taxonomic identification of bacteria made way for an additional through-put molecular biology technique that utilize specific genetic signatures that discriminatorily differentiate among diverse species. The 16s ribosomal DNA (16s *r*DNA) genotyping that revealed partial nucleotide sequences of the individual bacteria species that was putatively identified based on the MBC suggested phylogenetic relationship of the isolates with *Bacillus subtilis*, *Macrococcus hajekii*,*Brevibacillus brevis*, *Yokenella resenberghei*,*Bacillus pumilus*, *Streptococcus dysgalactiae* and *Paenibacillus validus*. In comparison to several studies, there seem to be some common ground while there are divergent opinions as regards bacteria diversity in palm sap/wine [18,26-30,34].

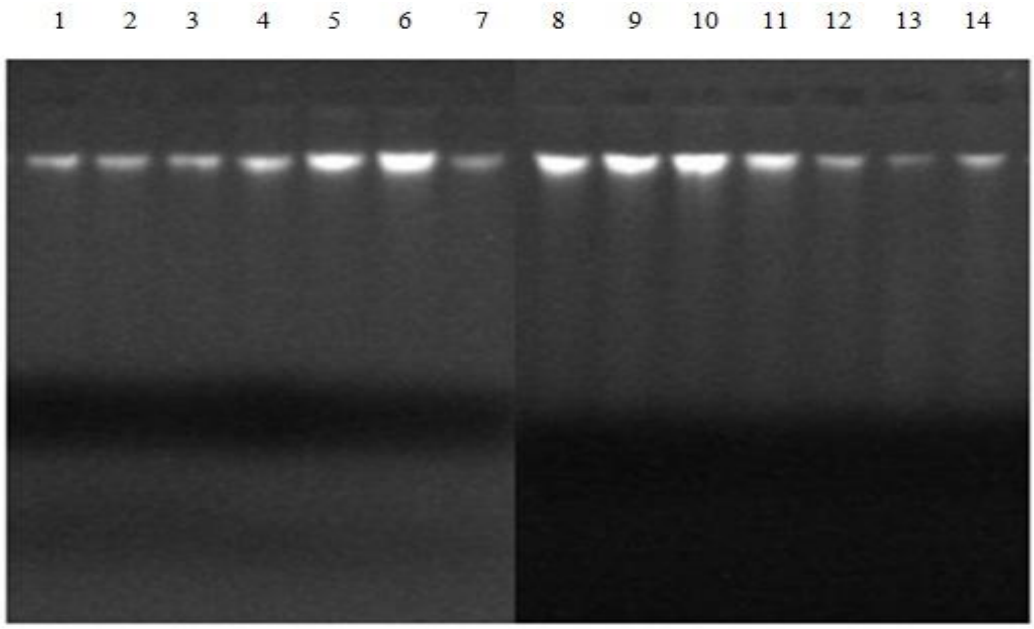

Fig. 1. The gel electrophoreogram of the Raphia palm sap isolated ethanologenic bacteria DNA on 1.5% agarose.Lane 1 = PW1, Lane 2 = PW2, Lane 3 = PW3, Lane 4 = PW4, Lane 5 = PW5, Lane 6 = PW6, Lane 7 = PW7, Lane 8 = PW8, Lane 9 = PW9, Lane 10 = PW10, Lane 11 = PW11, Lane 12 = PW12, Lane 13 = PW13, and Lane 14 = PW14

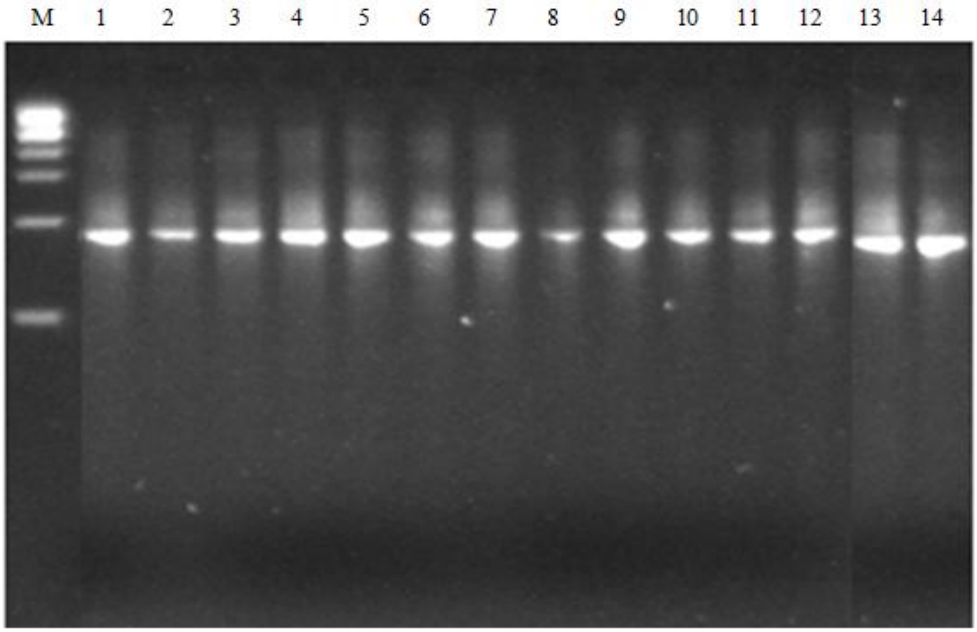

Fig. 2. Gel electrophoreogram of the Raphia palm sap isolated ethanologenic bacteria 16s rDNA amplicons on 1.5% agarose. M-DNA ladder(1kb), Lane 1 = PW1, Lane 2 = PW2, Lane 3 = PW3, Lane 4 = PW4, Lane 5 = PW5, Lane 6 = PW6, Lane 7 = PW7, Lane 8 = PW8, Lane 9 = PW9, Lane 10 =

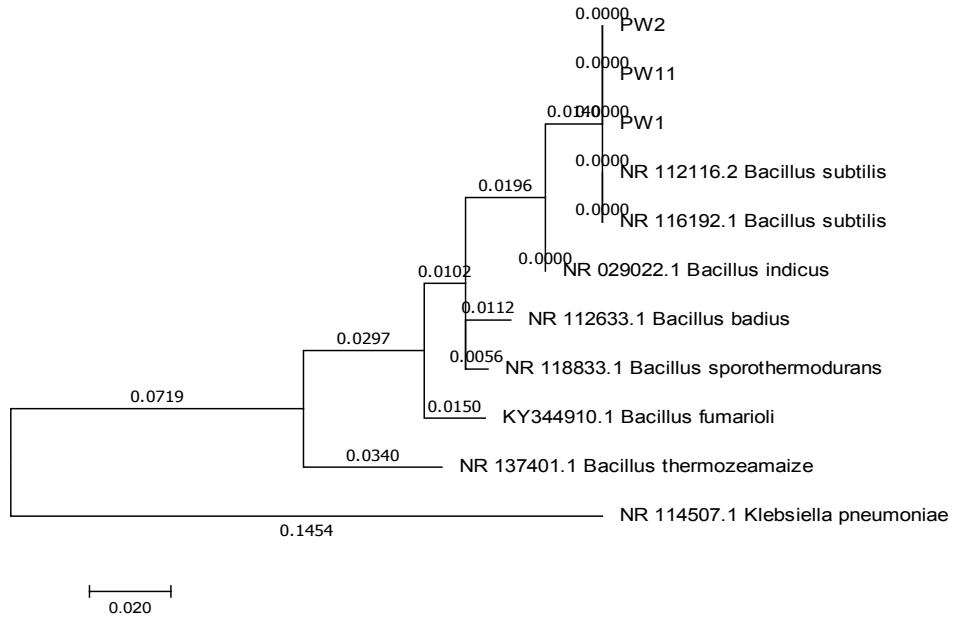

Fig. 3. Maximum likelihood tree depicting the molecular phylogenetic relationship of isolate PW1, PW2, and PW11 with related bacteria specie on the basis of 16s rDNA sequence alignment. The tree with the highest log likelihood (-1008.06) is shown. The scale bar of the tree has branch lengths (= 0.020) that measured the number of substitutions per nucleotide position

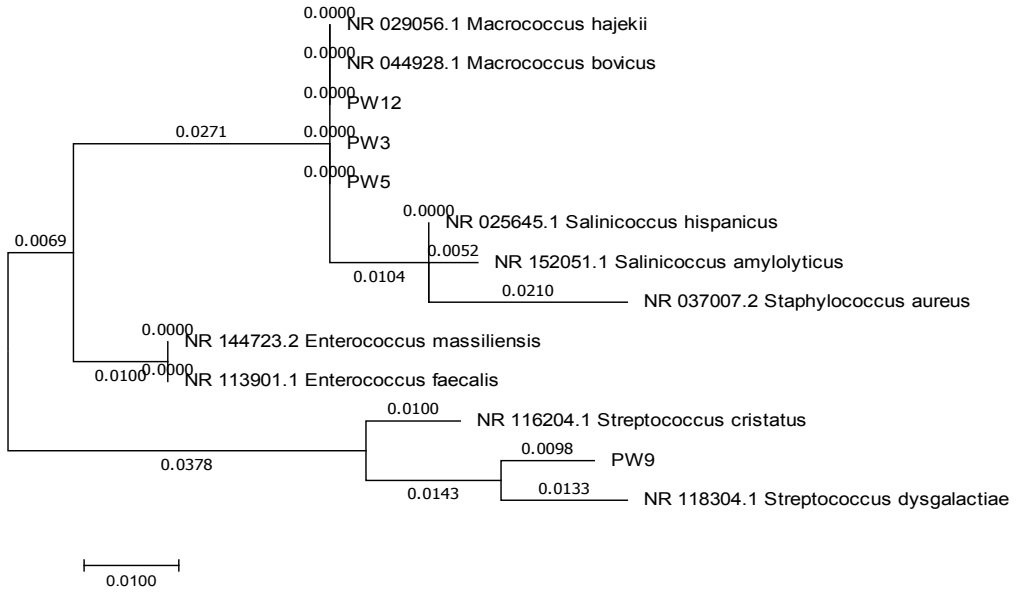

Fig. 4. Maximum likelihood tree depicting the molecular phylogenetic relationship of isolate PW3, PW5, PW9 and PW12 with related bacteria specie on the basis of 16s rDNA sequence alignment. The tree with the highest log likelihood (-446.38) is shown. The scale bar of the tree has branch lengths (= 0.0100) that measured the number of substitutions per nucleotide position

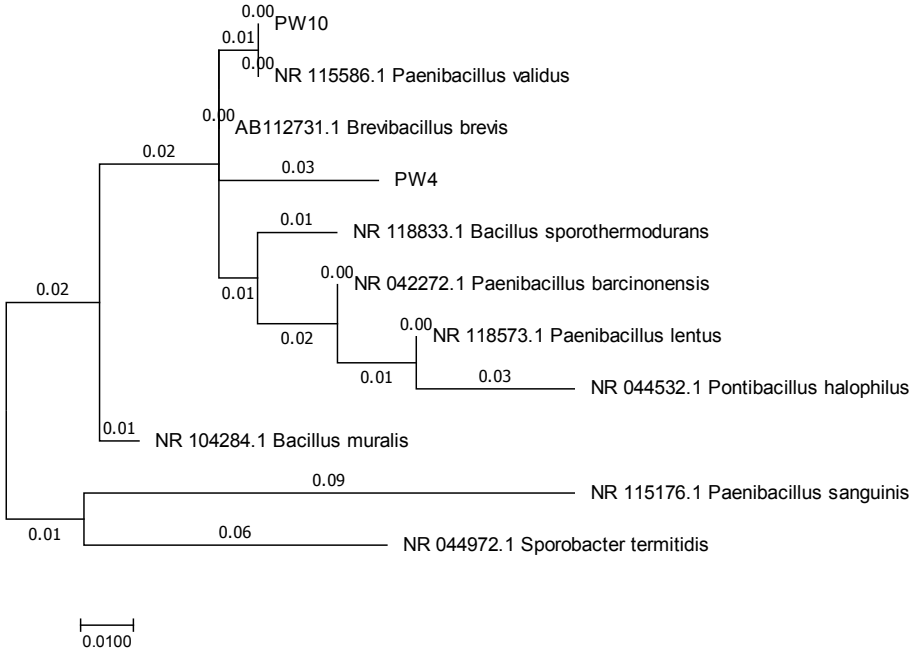

Fig. 5. Maximum likelihood tree depicting the molecular phylogenetic relationship of isolate PW4 and PW10 with related bacteria specie on the basis of 16s rDNA sequence alignment. The tree with the highest log likelihood (-411.91) is shown. The scale bar of the tree has branch lengths (= 0.0100) that measured the number of substitutions per nucleotide position

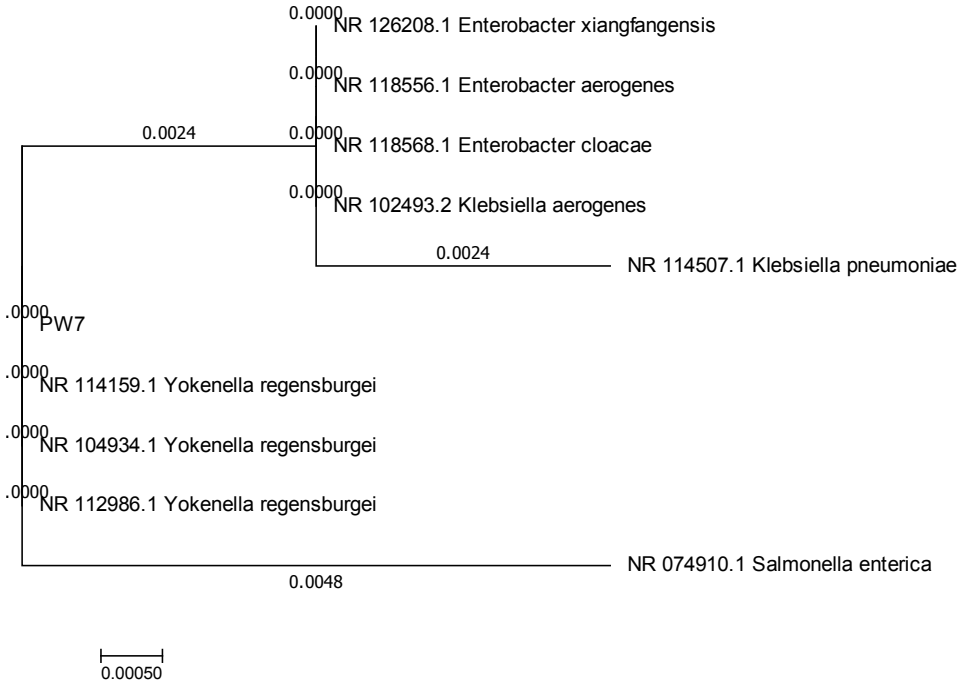

Fig. 6. Maximum likelihood tree depicting the molecular phylogenetic relationship of isolate PW7 with related bacteria specie on the basis of 16s rDNA sequence alignment. The tree with the highest log likelihood (-601.19) is shown. The scale bar of the tree has branch lengths (= 0.00050) that measured the number of substitutions per nucleotide position

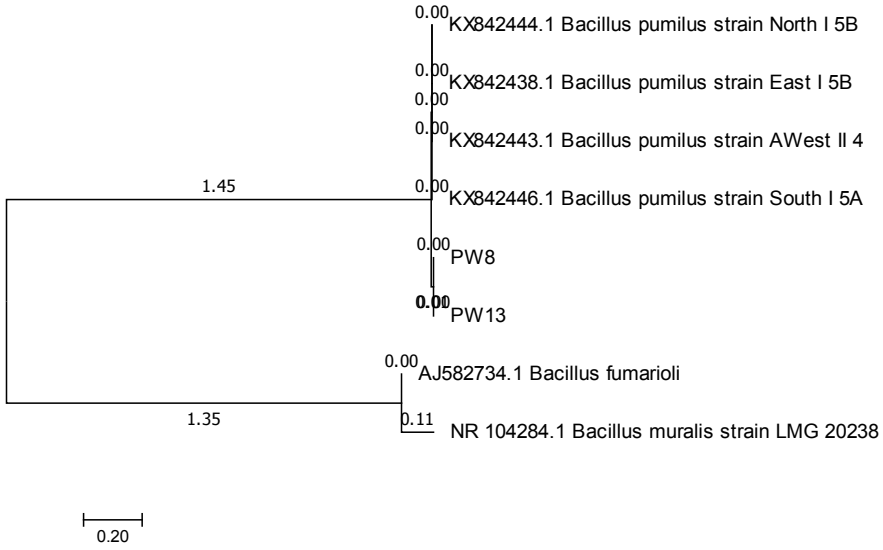

Fig. 7. Maximum likelihood tree depicting the molecular phylogenetic relationship of isolate PW8 and PW13 with related bacteria specie on the basis of 16s rDNA sequence alignment. The tree with the highest log likelihood (-957.34) is shown. The scale bar of the tree has branch lengths (= 0.20) that measured the number of substitutions per nucleotide position

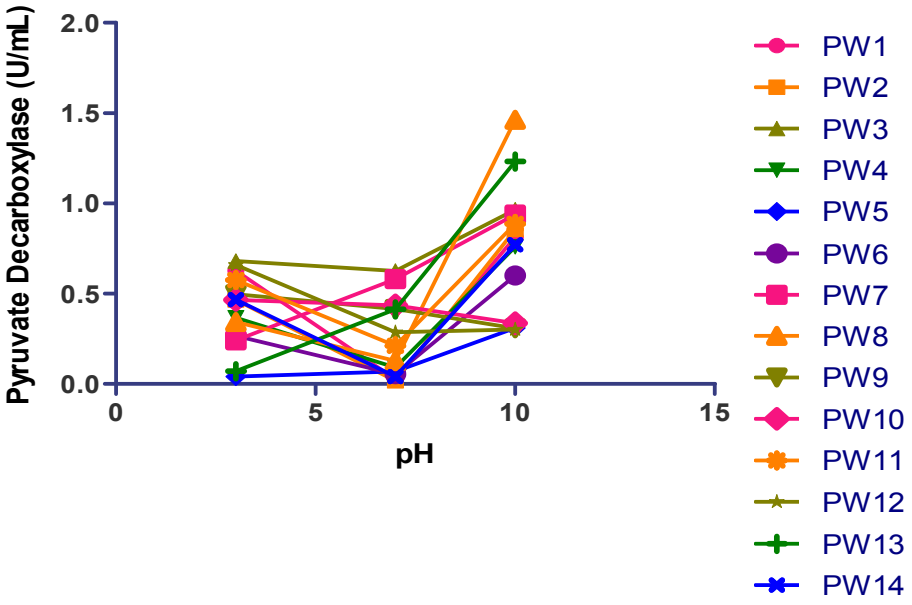

Fig. 8. Optimal pH of the culture media for pyruvate decarboxylase production by isolated bacteria from Raphia palm sap. The optimum pH for endoglucanase production was within the basic range

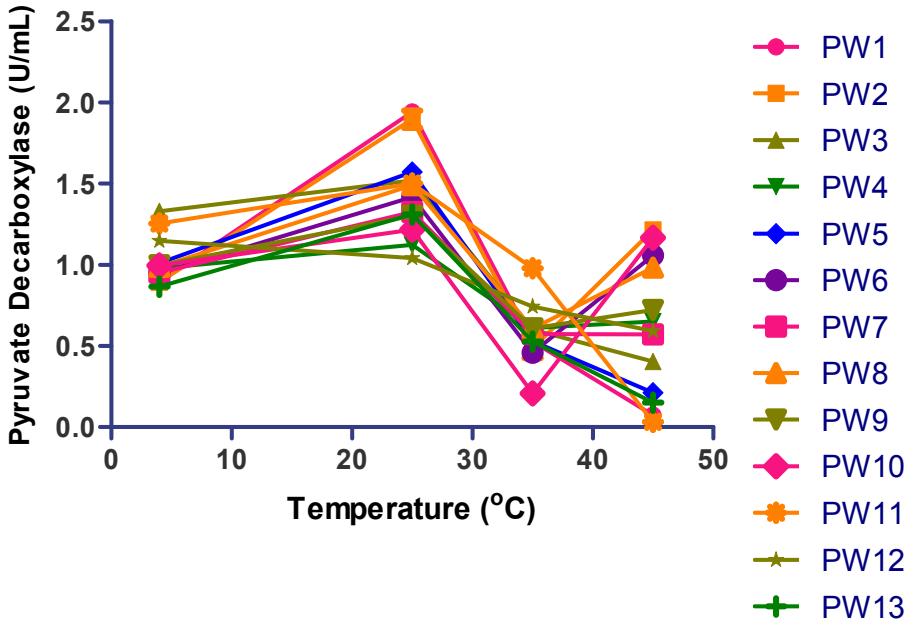

Fig. 9. Optimal temperature of the culture media for pyruvate decarboxylase production by isolated bacteria from Raphia palm sap. The optimum temperature for PDC production was 25°C

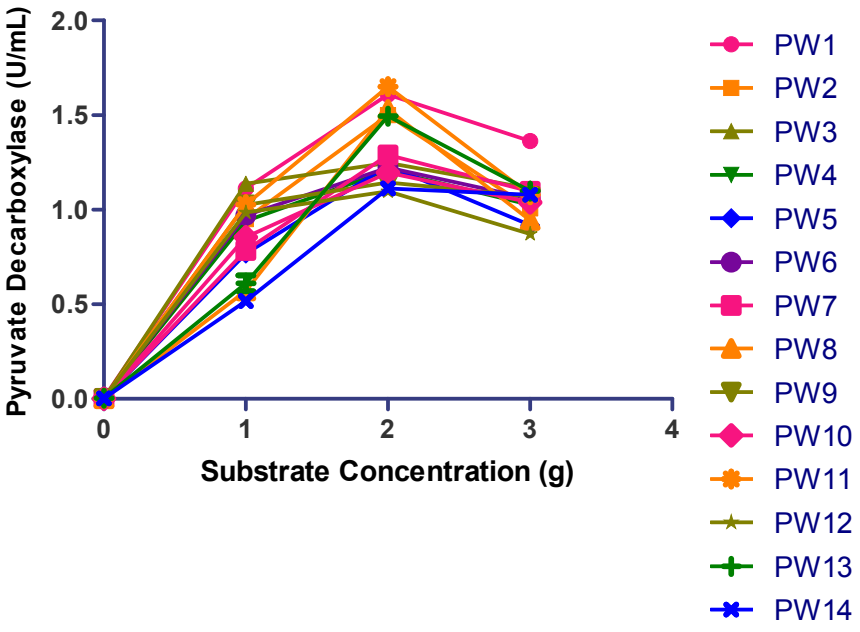

Fig. 10. Optimal substrate concentration of the culture media for pyruvate decarboxylase production by isolated bacteria from Raphia palm sap. Substrate concentration below 2.0g enhanced PDC production

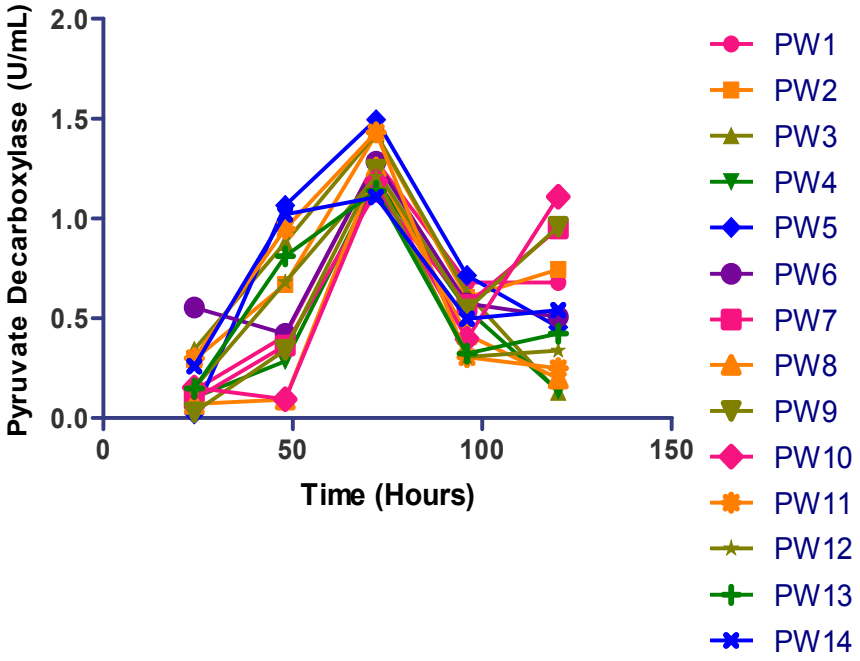

Fig. 11. Optimal time for pyruvate decarboxylase production by isolated bacteria from Raphia palm sap. The productivity of PDC was optimum at 72 hours

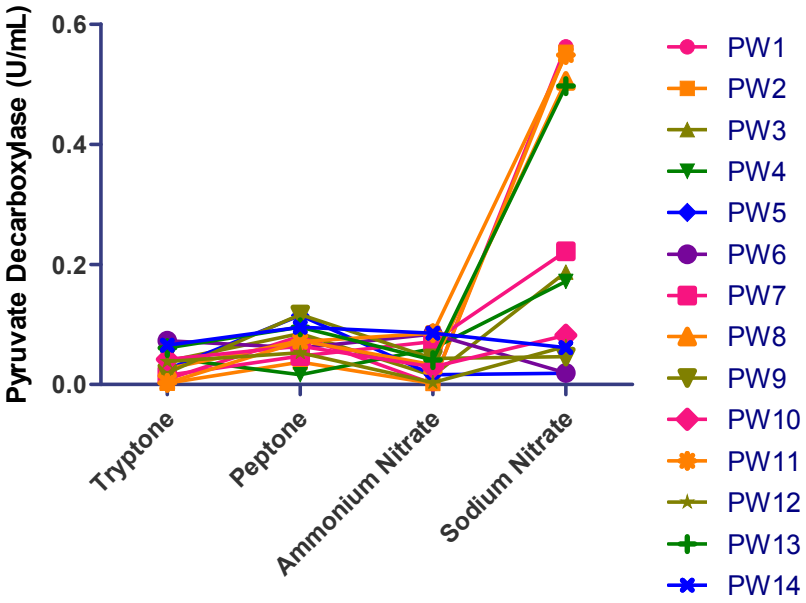

Fig. 12. Optimal Nitrogen source for the culture media for pyruvate decarboxylase production by isolated bacteria from Raphia palm sap. The most suitable nitrogen source that enhanced PDC productivity was sodium nitrate ($NaNO_3$)

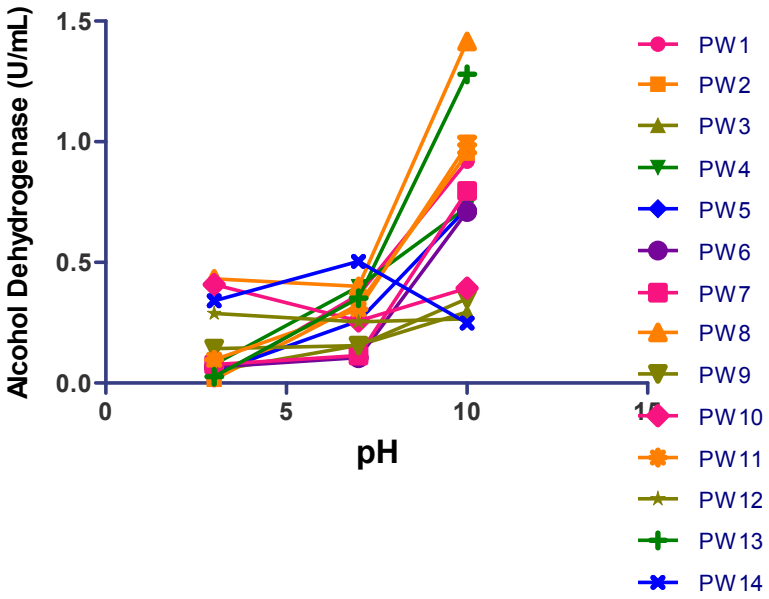

Fig. 13. Optimal pH of the culture media for alcohol dehydrogenase production by isolated bacteria from Raphia palm sap. The optimum pH for ADH production was within the basic range

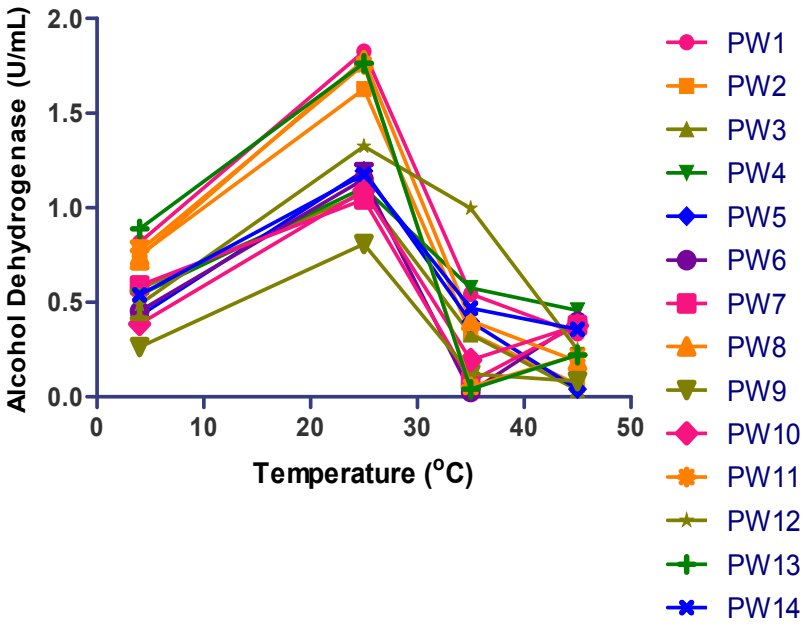

Fig. 14. Optimal temperature of the culture media for alcohol dehydrogenase production by isolated bacteria from Raphia palm sap. Temperature in the region of 25°C was most suitable for the production of ADH

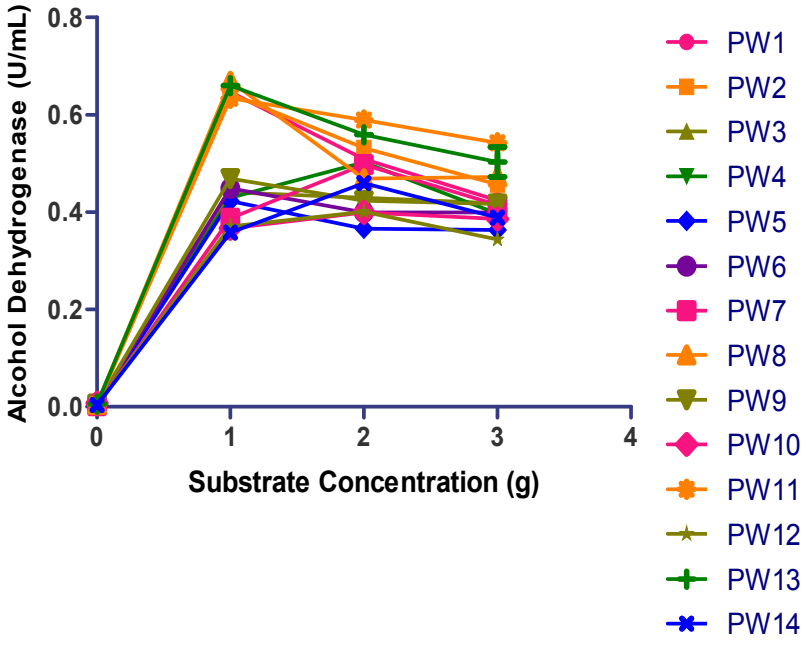

Fig. 15. Optimal substrate concentration of the culture media for alcohol dehydrogenase production by isolated bacteria from Raphia palm sap. Minimal substrate concentration as low as 1.0g was most suitable for ADH production

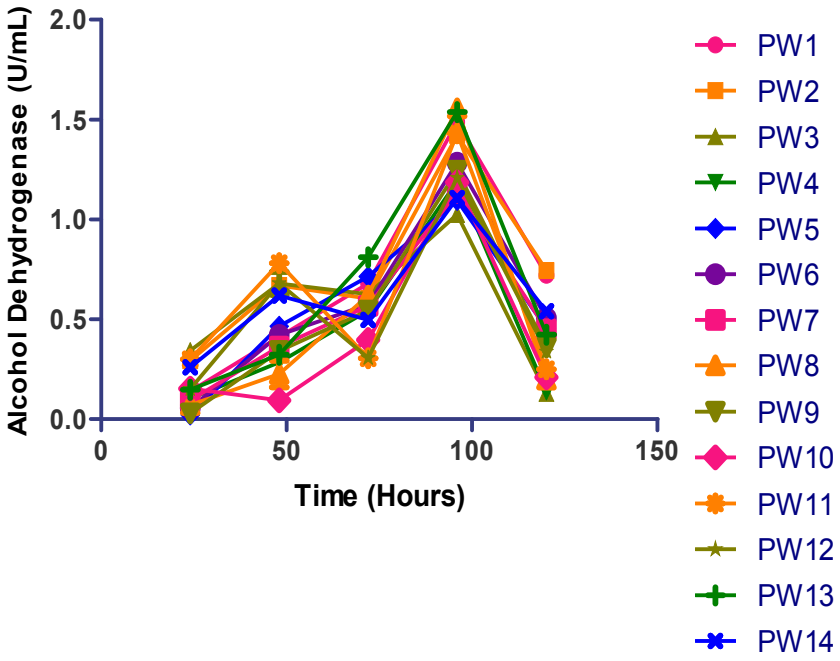

Fig. 16. Optimal time for alcohol dehydrogenase production by isolated bacteria from Raphia palm sap. Incubation time of 96 hours was optimum for ADH production

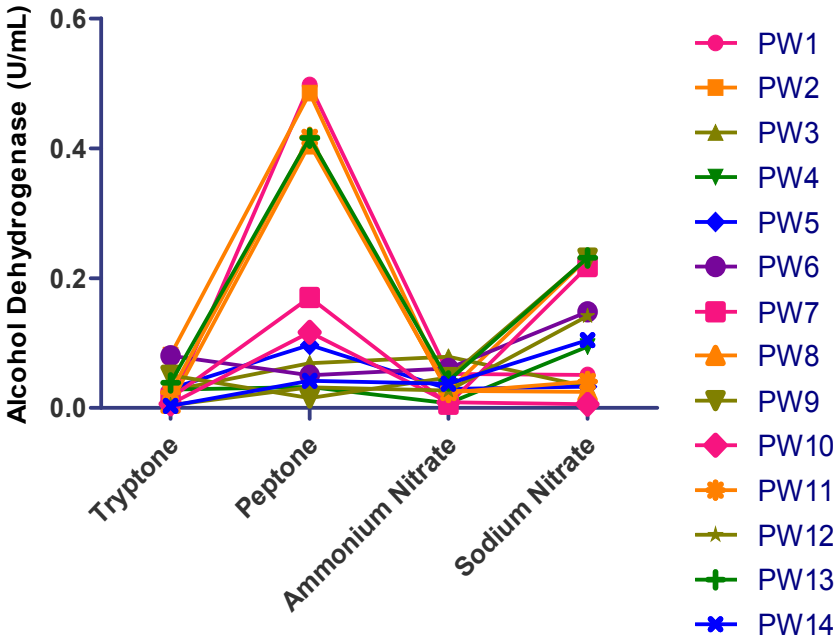

Fig. 17. Optimal Nitrogen source for the culture media for alcohol dehydrogenase production by isolated bacteria from Raphia palm sap. Peptone and partially sodium nitrate, as nitrogen sources, enhanced ADH productivity

Table 1. The morphological and biochemical characteristics of the bacteria isolated from raphia palm sap

Isolate Code	Shape	Gram Stain	Motility Test	Catalase Test	Oxidase Test	Methylred Test	Voges Test	Citrate Test	Urease Test	TSI			Indole Test
										Acid	H_2S	Gas	
P1	Rods (C)	+	_	+	+	_	+	_	+	+	+	+	+
P2a	Yeast buds	+	_	+	+	_	+	_	+	+	+	+	+
P2b	Yeast buds	+	_	+	_	_	+	_	+	+	+	_	+
P3	Rods (C)	+	_	_	+	_	+	+	+	+	+	+	+
P4	Rods (spores)	+	_	+	+	_	+	+	+	+	+	+	_
P5a	Rods (long) (C)	+	_	+	+	_	+	+	+	+	+	_	+
P5b	Rods (C)	+	_	+	+	_	+	_	+	+	+	_	+
P6a	Cocci (C)	+	+	+	+	_	+	+	+	+	+	_	+
P6b	Rods (C)	+	_	+	+	_	+	+	+	+	+	_	+
P7a	Cocci	_	_	+	_	_	+	+	+	+	+	+	+
P7b	Yeast buds	_	+	+	+	_	+	_	+	+	+	+	+
P8a	Rods	_	_	+	+	_	+	+	+	+	+	+	+
P8b	Rods (spores)	+	_	+	+	_	+	+	+	+	+	+	+
P9a	Cocci (D)	+	+	+	+	_	+	+	+	+	+	+	+
P9b	Cocci (C)	_	_	+	+	_	+	+	+	+	+	+	+
P10a	Rods (spores)	+	_	+	+	_	+	_	+	+	+	+	+
P10b	Cocci (C)	+	_	+	+	_	+	_	+	+	+	+	+
P11	Cocci (C)	_	_	+	+	_	+	+	+	+	+	_	+
P12a	Rods	+	_	+	+	_	+	_	+	+	+	+	+
P12b	Cocci (D)	+	_	+	+	_	+	+	+	+	+	_	+
P13a	Yeast buds	+	_	+	+	_	+	_	+	+	+	_	+
P13b	Yeast buds	+	_	+	+	_	+	+	+	+	+	+	+
P14a	Cocci (C)	+	_	+	+	_	+	_	+	+	+	_	+
P14b	Cocci (C)	+	_	+	+	_	+	+	+	+	+	_	+
P15a	Cocci (C)	+	_	+	+	_	+	+	+	+	+	_	+
P15b	Rods (long) (S)	+	_	+	+	_	+	+	+	+	+	_	+
P16a	Rods (long) (S)	_	_	+	+	_	+	_	+	+	+	_	+
P16b	Rods	_	_	+	+	_	+	+	+	+	+	+	+
P17a	Rods (spores)	+	_	+	+	_	+	_	+	+	+	_	_
P17b	Rods (spirallate)	+	_	+	+	_	+		+	+	+	_	+

Key: "+" indicates a positive test; "-" indicates a negative test; (C) indicates cluster; (D) indicates diploid; (S) indicates scattered

Table 2. The Sugar fermentation characteristics of the bacteria isolated from raphia palm sap

	Fermentation test						
Isolate Code	Cellulose	Starch	Sucrose	Lactose	Glucose	Fructose	Mannitol
P1	–	–	+	+	+	+	+
P2a	–	–	+	+	+	+	+
P2b	–	–	–	–	+	+	+
P3	–	–	–	–	+	+	+
P4	–	–	+	+	+	+	+
P5a	–	–	–	–	+	+	+
P5b	–	–	+	+	+	+	+
P6a	–	–	+	+	+	+	+
P6b	–	–	–	–	+	+	+
P7a	–	–	+	+	+	+	+
P7b	–	–	–	–	+	+	+
P8a	–	–	+	+	+	+	+
P8b	–	–	+	+	+	+	+
P9a	–	–	+	+	+	+	+
P9b	–	–	–	–	+	+	+
P10a	–	–	+	+	+	+	+
P10b	–	–	–	–	+	+	+
P11	–	–	–	–	+	+	+
P12a	–	–	–	–	+	+	+
P12b	–	–	–	–	+	+	+
P13a	–	–	–	–	+	+	+
P13b	–	–	+	+	+	+	+
P14a	–	–	–	–	+	+	+
P14b	–	–	–	–	+	+	+
P15a	–	–	+	+	+	+	+
P15b	–	–	+	+	+	+	+
P16a	–	–	+	+	+	+	+
P16b	–	–	+	+	+	+	+
P17a	–	–	–	–	+	+	+
P17b	–	–	–	–	+	+	+

Key: "+" indicates a positive test; "-" indicates a negative test

Table 3. The raphia palm sap bacteria isolates with similar biochemical characteristics alignment and their putative identity

Primary Isolation code	New Code	Probable Identity
P3	PW1	*Bacillus spp.*
P4	PW2	*Bacillus spp.*
P5a, P6b, P12b, P14a, P17b	PW3	*Staphylococcus spp.*
P5b	PW4	*Enterobacteriaceae*
P6a	PW5	*Enterobacteriaceae*
P7a	PW6	*Enterobacteriaceae*
P8a, P16a	PW7	*Enterobacteriaceae*
P8b, P9a	PW8	*Bacillus spp.*
P9b	PW9	*Enterobacteriaceae*
P10a	PW10	*Bacillus spp.*
P10b, P12a	PW11	*Bacillus spp.*
P11, P14b	PW12	*Enterococcus spp.*
P15a, P15b	PW13	*Enterobacteriaceae*
P17a, P16b	PW14	*Bacillus spp.*

Bacteria isolates including *Staphylococcus aureus*, *Lactobacillus spp*., *Bacillus cereus*, *Streptococcus spp*. and *Escherichia coli* were isolated from palm wine using traditional MBC culture approach [26]. Likewise, Olawale et al. [25] who used the traditional MBC culture approach reportedly isolated some *Bacillus* and *Enterococcus* species from Raphia palm wine. Applying similar traditional MBC culture approach, Ogbulie et al. [34] revealed the presence of *Bacillus, Lactobacillus, Brevibacterium,Staphylococcus,Escherichia coli and Micrococcus* species in Raphia palm wine. In a study that employed the use of uncultured approach, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, *Bacillus*, *Enterobacterium*, *Lactobacillus* and *Acetobacter* were among the bacteria diversity in palm wine/sap [35]. The divergent opinion of bacteria diversity in palm wine could be due to a number of reasons such as the kind of culture media (agar/broth) utilized for isolation, the sole use of traditional MBC technique (culture approach), the use of culture approach coupled with molecular and phylogenetic identification technique, or the utilization of unculture approach with molecular and phylogenetic identification technique. However, the type of molecular and phylogenetic identification technique adopted can bring about diversity as well as environmental factors. Majorly, the molecular technique involved is polymerase chain reaction based (PCR-based). This can be coupled with agarose gel electrophoresis of the amplicons (PCR-AGE) or restriction fragment length polymorphism cum gel electrophoresis (PCR-RFLP-GE) for the culture approach-molecular identification technique. Likewise it can be coupled with denaturation gradient gel electrophoresis (PCR-DGGE), metagenomic cloning technique or next generation sequencing for the unculture approach [19,35,36].

Microorganisms have been isolated from diverse sources on the background of harnessing their capabilities to produce enzymes of biotechnological importance [2]. The alcohol fermentation capability in Raphia palm sap/wine is reportedly linked to microbial fermentation of simple sugars [35]. This metabolic process in bacteria commonly involves the glycolytic pathways including Entner-Duodoroff (ED) and/or Embden-Meyerhof (EM) pathways [9,11]. These pathways utilize the catalytic function of glycolytic enzymes such as alcohol dehydrogenase and/or pyruvate decarboxylase, on which ethanol fermentation is dependent. In this study, all the identified bacteria isolates possess both alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) producing capabilities in the presence of their substrate. This suggests that the identified bacteria employ both Entner-Duodoroff (ED) and Embden-Meyerhof (EM) pathways in their simple sugar metabolism.

However, the enzymes (ADH and PDC) production efficiency was observed to vary by physicochemical factors employed. Although variation exists with the microbial enzymes production under different ranges of physicochemical factors investigated, there exists optimum condition conformity by majority of the microbial species investigated. Sarrazin et

al. [37] reported that microbial community structure and function is often shaped by physicochemical factors, and microbial diversity exists due to growth under specific ranges of physicochemical factors. The ADH and PDC productivity found to be optimum at basic pH by majority of the bacteria species might be attributed to the adaptation of the bacteria isolates to the pH of the Raphia palm sap medium, which according to Amoa-Awua et al. [18] is reported to be near neutral. In corroboration was the study of Inoue et al. [38] who reported optimum alcohol dehydrogenase activity at pH 9.5. Temperature and substrate concentration as well as pH are very crucial to enzyme activities. Enzymes are specific in nature and can be distorted by high temperatures. This might be the reason for the optimal enzyme productivity that was noted at mild temperature as such within room temperature (25°C). However, the observed recovery in PDC productivity as the temperature increased above 35°C might be due to nature of adaptation of microorganisms to fluctuating environmental factors. Previous studies highlighted the role of gene regulation in microbial adaptation to fluctuating physicochemical conditions [39,40]. Since our study did not investigate the enzymes productivity beyond 45^{o}C and PDC productivity recovery was found at temperature above 35°C, there arises a limitation making express deductions about optimum temperature for PDC production. Future studies may evaluate PDC productivity under ranges that include much higher temperatures. Minimal substrate loading observed for optimum production of both PDC and ADH suggests that high substrate concentration might bring about cellular osmotic imbalance that could affect the bacteria cells. Sootsuwan et al. [41] reported that high concentration of sugar creates osmotic stress in bacteria cells. Moreover, the decrease in the enzymes productivity beyond 96 hours might be due to the depletion of substrate that may have down-regulated the expression of genes encoding for ADH and PDC, thereby affecting their secretion. The optimum enzymes (ADH and PDC) production recorded through the utilization of peptone and sodium nitrate as sources of nitrogen suggests that the bacteria isolates can adapt organic and inorganic sources of nitrogen for their metabolic and ultimately, cellular processes. Bearing the two enzymes –ADH and PDC- from the perspective of each identified bacteria isolates, the pattern of optimal enzyme production is observably in tandem; however with variations in production efficiencies that highlight the prowess of the *Bacillus sp.* (*pumilus* and *subtilis*) in comparison to other identified bacteria isolates.

5. CONCLUSION

The Raphia palm sap microbiota, particularly the *Bacillus species* (*pumilus* and *subtilis*), are efficiently ethanologenic. Their ethanologenic capabilities owing to their ADH and PDC is characteristic of optimal production at basic pH, room temperature, minimal substrate concentration, organic or inorganic nitrogen source, and about 3-4 days incubation period. Thus, genes encoding for ADH and PDC in *Bacillus species* (*pumilus* and/or *subtilis*) can be adapted for genetic transformation in consolidated bioprocessing for bioethanol production. In order to aid gene transfer in genetic transformation, characterization of plasmids and antibiogram of these microbial strains would be quite essential. However, future studies could also explore the physicochemical factors effect on the gene expression of alcohol dehydrogenase and pyruvate decarboxylase in ethanologenic bacteria.

AVAILABILITY OF DATA AND MATERIALS

Raw experimental data can be provided if required.

ACKNOWLEDGEMENT

The authors appreciate Professor Abberton and the Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan for providing laboratory space for the genomic experiments. We also appreciate Mr. Usman of Pharmaceutics Microbiology Laboratory of the Faculty of Pharmacy, University of Lagos, for providing guidelines for microbial culture. The technical staff of the Department of Biochemistry, University of Lagos are also acknowledged for providing laboratory space for the enzymes assays.

COMPETING INTERESTS

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as apotential conflict of interest.

REFERENCES

1. Begum K, Mannan SJ, Rezwan R, Rahman M, Rahman S, Nur-E-Kamal A.Isolation and characterization of bacteria with biochemical and pharmacological importance from soil samples of dhaka city. Dhaka Univ. J Pharm Sci. 2017; 16(1):129-136.
2. Nigam PS. Enzymes with special characteristics for biotechnological applications. Biomolecules. 2013;3:597-611.
3. Jiangshan M, Zhang K, Liao H, Hector SB, Shi X, Li J, et al. Genomic and secretomic insight into lignocellulolytic system of an endophytic bacterium Pantoea ananatis Sd 1. Biotechnol Biofuels. 2016;9:25.
4. Glasser WG, Kaar WE, Jain RK, Sealey JE. Isolation options for noncellulosic heteropolysaccharides (Hetps). Cellulose. 2000;7:299-317.
5. He MX, Bo W, Han Q, Zhi YR, Fu RT, Jing LW, et al. Zymomonas mobilis: a novel platform for future biorefineries. Biotechnology for Biofuels. 2014;7::101.
6. Jojima T, Inui M. Engineering the glycolytic pathway: A potential approach for improvement of biocatalyst performance. Bioengineered. 2015;6(6):328-334.
7. Sobana PP, Thirumalai VP, Padma VS, Vidhyadevi U, Archana K, Vennison SJ. Cellulosic ethanol production by recombinant cellulolytic bacteria harbouring pdc and adh ii genes of zymomonas mobilis. Biotechnology Research International. 2012;(817549):1-8.
8. Buddrus L, Andrews ESV, Leak DJ, Danson MJ, Arcus VL, Crennell SJ. Crystal structure of pyruvate decarboxylase from Zymobacter palmae. Acta Cryst. 2016;(F72):700–706.
9. Selig M, Xavier KB, Santos H, Schönheit P. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium Thermotoga. Arch Microbiol. 1997;167:217–232.
10. Liu H, Sun Y, Ramos KRM, Nisola GM, Valdehuesa KNG, Lee W, et al. Combination of entner-doudoroff pathway with mep increases isoprene production in engineered escherichia coli. PLoS ONE. 2013;8(12):83290.
11. Chen X, Schreibera K, Appel J, Makowka A, Fähnrich B, Roettger M, et al. The Entner–Doudoroff pathway is an overlooked glycolytic route in cyanobacteria and plants. PNAS Plant Biology. 2016:1-29.
12. Yanase H, Sato D, Yamamoto K, Matsuda S, Yamamoto S, Okamoto K. Genetic engineering of zymobacter palmae for production of ethanol from xylose. Appl Environ Microbiol. 2007;73(8):2592-2599.
13. Linger JG, Adney WS, Darzins A. Heterologous expression and extracellular secretion of cellulolytic enzymes by Zymomonas mobilis. Appl Environ Microbiol. 2012;76:6360–6369.
14. Linger JG, Darzins A. Consolidated Bioprocessing. In, Advanced Biofuels and Bioproducts. Springer. 2013:267–280.
15. Yee LK, Miguel RJ, Olivia AT, Chunxiang F, Zeng-Yu W, Brian HD, et al. Consolidated bioprocessing of transgenic switchgrass by an engineered and evolved Clostridium thermocellum strain. Biotechnology for Biofuels. 2014;7:75.
16. Ohimain EI, Tuwon PE, Ayibaebi EA. Traditional fermentation and distillation of raphia palm sap for the production of bioethanol in bayelsa state, Nigeria. Journal of Technology Innovations in Renewable Energy. 2012;1:131-141.
17. Assiri PK, Koutoua S, Diallo HA, Ake S. Antifungal activity of bacteria isolated from palm wine against postharvest rot fungi on yam (Dioscorea spp.). International Journal of Advanced Research. 2015;3(1):231-240.
18. Amoa-Awua WK, Sampson E, Tano-Debrah K. Growth of yeasts, lactic and acetic acid bacteria in palmwine during tapping and fermentation from felled oil palm (Elaeis guineensis) in Ghana. Journal of Applied Microbiology. 2007;102(2):599–606.
19. Santiago-Urbina JA, Verdugo-Valdez AG, Ruíz-Terán F. Physicochemical and microbiological changes during taping of palm sap to produce an alcoholic beverage called Taberna, which is produced in the south east of Mexico. Food Control. 2013;33(1):58-62.
20. Ngọc NM, Minh NP, Dao DTA. Different processing conditions affect palm (THOT NOT) wine fermentation. American Journal

of Research Communication. 2014;2(1): 142-157.

21. Falegan CR, Akoja SO.Microbiological and physicochemical studies of two Nigerian fermented alcoholic drinks (Palm wine and Burukutu) in Ekiti State, Nigeria. European Journal of Agriculture and Forestry Research. 2014;2(3):14-24.
22. Chandrasekhar K, Sreevani S, Seshapani P, Pramodhakumari J. A review on palm wine. International Journal of Research in Biological Sciences. 2012;2(1):33-38.
23. Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea using next-generation sequencing. PLoS ONE. 2014;9(8):105592.
24. Karamoko D, Djeni NT, N'guessan KF, Bouatenin KMJ, Dje KM. The biochemical and microbiological quality of palm wine samples produced at different periods during tapping and changes which occurred during their storage. Food Control.2012;26(2):504-511.
25. Olawale AK, Akintobi AO, David OM. Evaluation of microbial quality and alcoholic improvement of natural and fermented raphia palm wine (Ogoro). New York Science Journal. 2010;3(2):35-39.
26. Obi CN, Nwani I. Microbiological and nutritional status of palm wine from umudike and its environs. International Journal of Bioinformatics and Biomedical Engineering. 2018;4(4):62-69.
27. Ouoba L, Kando C, Parkouda C, Sawadogo-Lingani H, Diawara B, Sutherland JP. The microbiology of Bandji, palm wine of Borassus akeassii from Burkina Faso: identification and genotypic diversity of yeasts, lactic acid and acetic acid bacteria. Journal of Applied Microbiology. 2012;113(6):1428-144.
28. Alcántara-Hernández RJ, Rodriguez-Alvarez JA, Valenzuela-Encinas FA, Gutiérrez-Miceli FA, Castañón-González H, Marsch R, et al. The bacterial community in taberna a traditional beverage of Southern Mexico. Letters in Applied Microbiology. 2010;51(5):558-563.
29. Stringini M, Comitini F, Taccari M, Ciani M. Yeast diversity during tapping and fermentation of palm wine from Cameroon. Food Microbiology. 2009;26(4):415-420.
30. Ezeronye OU, Okerentugba PO. Genetic and physiological variants of yeast selected from palm wine. Mycopathologia. 2001;152:85–89.
31. Shamala TR, Sreekantiah KR. Microbiological and biochemical studies on traditional Indian palm wine fermentation. Food Microbiology. 1988;5(3):157-162.
32. Atputharajah JD, Widanapathirana S, Samarajeewa U. Microbiology and biochemistry of natural fermentation of coconut palm sap. Food Microbiology. 1986;3(4):273-280.
33. Kadere T, Miyamoto T, Oniang'o RK, Kutina PM, Njoroge SM. Isolation and identification of the genera Acetobacter and Gluconobacter in coconut toddy (mnazi). African Journal of Biotechnology. 2008;7(16):2963-2971.
34. Ogbulie TE, Ogbulie JN, and Njoku HO. Comparative study on the microbiology and shelf life stability of palm wine from Elaeis guineensis and Raphia hookeri obtained from Okigwe, Nigeria. Afri J Biotechnol. 2013;1(2):015-022.
35. Okolie PI, Opara CN, Emerenini EC, Uzochukwu SVA. Evaluation of bacterial diversity in palm wine by 16S rDNA analysis of community DNA. Nigerian Food Journal. 2013;31(1):83-90.
36. Wang C, Dong D, Wang H, Müller K, Qin Y, Wang H, et al. Metagenomic analysis of microbial consortia enriched from compost: New insights into the role of Actinobacteria in lignocelluloses decomposition. Biotechnol Biofuels. 2016;9:22.
37. Sarrazin J, Juniper SK, Massoth G, Legendre P. Physicaland chemical factors influencing species distributions on hydrothermal sulfide edifices of the Juan de Fuca Ridge, Northeast Pacific. Marine Ecology Progress Series. 1999;190:89-112.
38. Inoue K, Makino Y, Itoh N. Purification and characterization of a novel alcohol dehydrogenase from leifsonia sp. strain S749: A promising biocatalyst for an asymmetric hydrogen transfer bioreduction. Applied and Environmental Microbiology. 2005;71(7):3633-3641.
39. Fernandez L, Mercader JM, Planas-Felix M, Torrents D. Adaptation to environmental factors shapes the organization of regulatory regions in

microbial communities. BMC Genomics. 2014;15:877.

40. Bleuven C, Landry CR. Molecular and cellular bases of adaptation to a changing environment in microorganisms. Proc R Soc B. 2016;283:20161458.

41. Sootsuwan K, Thanonkeo P, Keeratirakha N, Thanonkeo S, Jaisil P, Yamada M. Sorbitol required for cell growth and ethanol production by Zymomonas mobilis under heat, ethanol, and osmotic stresses. Biotechnology for Biofuels. 2013;6:180.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/65304