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Full Length Research Paper

Screening and molecular identification of indigenous strains of acetic acid bacteria isolated from mango biotopes in Burkina Faso

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Abstract

Acetic acid bacteria (AAB) are useful in industrial production of vinegar. The present study is carried out to characterize indigenous strains of acetic acid bacteria from mangos in Burkina Faso. Samples of fermented mangos were used as potential source of acetic acid bacteria. The AAB strains were isolated and identified phenotypically and using biochemical properties. rRNA 16s gene of Acetobacter genera was used for molecular identification using conventional PCR technic. Results revealed that fifteen strains were routinely identified as acetic acid bacteria strains due to their biochemical and physiological properties. Electrophoresis gel of PCR products demonstrated that the fifteen strains were AAB. Using two primers pairs like 16sF and 16sR, twelve strains among fifteen were confirming to belonging Acetobacter sp. as CRSBAN- BVA1, CRSBAN- BVA2, CRSBAN- BVA3, CRSBAN- BVK1, CRSBAN- BVK2, CRSBAN- BVI1, CRSBAN- BVS1, CRSBAN- BVS2, CRSBAN- BVB1, CRSBAN- BVB2, CRSBAN- BVL2, and CRSBAN- BVP2 which showed each a clear band roughly 1200 Pb. Sequencage of 16s rRNA showed that bacteria strain CRSBAN- BVK2 has presented 100 % similarity with *Acetobacter tropicalis*.

The results suggest that fermented juice of mango was a potential biotope source of indigenous acetic bacteria.

Keywords: Mango fermented juice, Indigenous strains, Acetic acid bacteria, Molecular identification

INTRODUCTION

Acetic acid bacteria (AAB) is a group of Gram-negative bacteria, non-spore forming, catalase-positive, and strictly aerobic (Andres-Barrao *et al*., 2012). The group of AAB are heterogeneous organisms motile or non-with peritrichous flagella rods that carry out incomplete oxidation of alcohol and sugars, leading to the accumulation of organic acids as end products (Sievers *et al*. 2005; Saichana *et al*. 2014). Among the most important acetic acid bacteria, the strains of genus *Acetobacter* are mainly involved in vinegar production (Elijah and Etukudo 2016). Vinegars are popular fermented foods produced by AAB present in natural environments (Nanda *et al*., 2001).

Vinegar is generally produced by inoculation of "seed vinegar", which is a microbiologically undefined starter culture, generally obtained from a previous fermentation. The lack of defined pure starter cultures is mainly due to problems such as isolation, cultivation and preservation of vinegar AAB. Several authors have mentioned that, even after successful isolation and cultivation, it is extremely difficult to handle the isolates and to preserve their high acetic acid resistance under laboratory conditions (Gullo

and Guidici 2008). Several studies have recently been performed to characterize AAB from vinegar using molecular techniques (Muramatsu *et al*. 2009; Mounir *et al*. 2016).

AAB are often involved in the production of fermented foods, either in a beneficial (chocolate products, coffee, vinegar and specialty beers) or detrimental (spoilage of beers, wines and ciders) manner (Kersters *et al*., 2006). These bacteria have been isolated using several natural resources such as grape, coconut, palm and mangos (Kadere *et al*. 2008; Maal *et al*. 2010). In Burkina Faso, mango was produced abundantly and its fermented juice has been demonstrated to be a potential source of AAB (Ouattara *et al*. 2019).

The valorization of this fruit through biotechnological processes is therefore essential to reduce losses. Such valorization brings on the domestic market a new generation of highly prized and often imported products like vinegar (Ndoye *et al*., 2006). The present study aimed to isolate and identify indigenous strains of acetic acid bacteria from fermented juice of mangos in Burkina Faso.

MATERIAL AND METHODS

Sampling and isolation of bacteria

Isolates of AAB were obtained from seven different mangos varieties collected in four cities of Burkina Faso (Banfora, Bobo-Dioulasso, Orodara and Ouagadougou). These cities were selected according to their annual mangos amount production. After sampling, GYEA (glucose, yeast extract, peptone of caseine, ethanol and acetic acid) medium was used as enrichment media (Shafiei *et al*. 2013). Strains were isolated using GYC standard medium (yeast extract, 10 g/l; D-glucose, 50 g/l; CaCO3, 30 g/l; agar, 25 g/l; distilled water, 1000 ml) and Carr medium (yeast extract, 3 %; agar, 2 %; bromocresol green, 0.002 %; ethanol, 2% (v/v); distilled water, 1000 ml) were used for phenotypical characteristic.

Morphological and biochemical characterization of isolated strains

The strains were characterized and identified using colony morphological characteristics and biochemical tests such as Gram staining, catalase, urea, sugar fermentation, motility, indole, oxidase, citrate utilization, gas and H_2S production from glucose (Holt *et al*. 1994).

Morphological identification of bacteria was done with microscope on isolates from grow on the solid or liquid media. This characterization including shape, size, and arrangement was carried out from cells grown on GYC at 30ºC under aerobic condition (Cleenwerck *et al*., 2002; De Ley *et al*., 1984). Gram staining was done and optical observation was made at G x 100. Motility test was performed in Hanging drop slide. A drop of distilled water was taken in the glass slide and a single colony was taken from the GYC agar with a small tip and mixed it very well. Gas and H_2S production from glucose was monitored using Kliger Iron Agar.

For catalase test**,** a small colony of good growth in GYC medium was smeared on a slide. One-drop catalase reagent (3 % H_2O_2) was added on the smear. The slide was observed for bubble formation (Kowser *et al*., 2015). Ketogenesis from glycerol was determined according modified method described (Aydin and Aksoy 2009). The isolates were inoculated in test tube containing YG medium (3.00% yeast extract, 3.00% glycerol) incubated at 30 ºC for 10 days and adding 8–10 drops of Fehling's solution into the medium. The change of medium color to orange indicated a positive test.

Cellulose production was tested on GYE medium (2 % glucose, 0.50 % yeast extract, and 0. 25 % ethanol 95 %) incubated at 30 ºC for 7 days. Cellulose test was carried out using a Lugol's iodine stain followed by 60 % sulphuric acid on pellicles from liquid culture, the color of cellulose fiber is blue (Romero-Cortes *et al*., 2012).

The sugar fermentation test was done on the liquid medium composed of peptone (3%); K_2HPO_4(0.05 %); KH_2PO_4 (0.05 %); $MgSO_4$ (0.01 %); $(NH_4)_2SO_4$ (0.14 %), with 1 % from sugar compound and 0.0022 % of blue bromotymol like indicator according to the method modified of Soumahoro *et al*. (2015). Acid production was indicated by the colour change reddish to yellow in the medium.

Molecular identification of isolated strains: DNA extraction and PCR

Extraction of genomic DNA:

DNA extraction was conducted from each isolate (preculture of 72 h) using GYC broth medium. A volume of 1 ml bacterial culture was centrifuged (12000 g for 5 minutes) and washed with sterile distilled water. The pellet was suspended in 700 ml sterile distilled, vortexed, to heat with the dry bath with 95°C/30 mn and to centrifuge (12000 g for 5 min). In another tube with Eppendorf were recovered the supernatant and addition 700 ml of pure alcohol to mixed and centrifuge (12000 g for 5mn). The filtrate was eliminated and after drying under the hood, the DNA was stored at -20°C by adding 50 ml sterile distilled water (N'tcha *et al*., 2016).

Amplification of DNA:

Amplification was performed according to the method using par N'tcha *et al*. (2016) and Yang *et al*. (2016). The PCR was performed in a 25 µl mixture consisted of 12.5 µl of 2 x Master Mix, 2.5 µl Forward primer (16sF: 5' AGAGTTTGATCCTGGCTCAG 3') and 2.5 µl Reverse primer (16sR: 5' ACGGCTACCTTGTTTACGACTT 3') and 10 µl of ADN. The amplification consisted of 30 PCR cycles in a Thermocycler (TECHEN). The cycling program was: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation with 94°C for 1 minute, hybridization for 44°C for 30 seconds and of elongation at 72°C for 2 min, final elongation at 73°C for 4 min and the amplified product cooled at 4°C

Electrophoresis

The DNA fragments were separated by loading 15 µl amplified DNA in a 1.5 % agarose gel at 110 V for 30 min. a 100 bp DNA ladder was used. The profiles obtained were visualized with the Ultra-violet trans-illuminator (Ultraviolet radiation type T. 05X20-2A; 254 nm) (N'tcha *et al*., 2016). A positive result is materialized by the presence of bands.

Sequencing

After purification of product PCR, strains were sequenced. Sequences obtained were assembled using software Sequencher (version 4.7) marked by Gene Code Corporation.

Contigs were matched with the data base «NCBI» (http// blast.ncbi.nlm.nih.gov). "Somewhat similar sequences (blastn)" was choosed like programs. Dendrogram after blast was obtained using website BIBI.

RESULTS

Phenotypical characteristics of strains

Acetic acid bacteria were successfully isolated from seven-fermented mango juice. Phenotypical characteristics were used to isolate fifteen (15) AAB strains. Selected strains showed different kind of colonies color such as brownish, yellowish, creamy white and pinkish. The cells were bacilli squat in shape, single, paired Cluster or chain in cell arrangements. The cultural and morphology characteristics of isolated strains were showed in (Table1). Strains were Gram negative, positive for catalase test and negative for indole, H_2S, citrate, ketogenesis and oxidase test. The preliminary identification base on biochemical tests were represented in (Table 2). All the isolates have capacity to ferment glucose and mannitol. A negative reaction was observed for lactose and maltose. Results revealed also that the profil of fermentation of strains vary according the sugar as sucrose, arabinose, saccharose, fructose, galactose, sucrose and meliobiose. The preliminary identification according to morphological, cultural biochemical tests brought about the possibility of selected strains to belong *Acetobacter* genus. Hence, molecular techniques were used to confirm identification.

Isolated strains identified by molecular techniques

The 16S rRNA gene of 15 isolates was amplified by PCR. After amplification, twelve (12) isolates of AAB (CRSBAN-BVA1, CRSBAN - BVA2, CRSBAN - BVA3, CRSBAN - BVK1, CRSBAN- BVK2, CRSBAN- BVI1, CRSBAN- BVS1, CRSBAN-BVS2, CRSBAN- BVB1, CRSBAN- BVB2, CRSBAN- BVL2, CRSBAN- BVP2) presented each a clear band roughly 1200 Pb (Figure1). No band was observed after visualization of freezing with three isolates (CRSBAN- BVP1, CRSBAN- BVA4 and CRSBAN- BVL1) who did not presented a band. These results confirm those provided by the biochemical tests. Result obtained after sequencing made possible to establish the consecutive alignment of the nucleotidic sequences 16s rRNA of CRSBAN- BVK2 (Table 3). Comparaison of the nucleitidic sequence showed a rate homology 100 % with *Acetobacter tropicalis*. This alignment made possible to obtain the phylogenetic tree illustrated by (Figure 2).

DISCUSSION

Not all mediums allow the growth of AAB; they are selective from isolates. GYC medium is the allowing medium of isolation of the majority of isolates in traditional vinegar (Gullo *et al.*, 2006). In GYC agar, colonies presented different types of color (Table 1) and showed a clear halo. (Mounir *et al*. 2016) found the similar result. According (Sengun and Karabiyikli 2011), halo formation was caused by hydrolysis of $CaCO_3$ contained in GYC by production of acid. That is one of the most basic and dominant characteristics that associate an unknown colony with acetic acid bacterial group. The methods based on the presence of a clear zone were not completely, because other strains, such as some lactic acid bacteria, could also form distinct clear zones (Chen *et al*. 2016). Morphological characteristics of bacteria presented

Table 1. Cultural and morphological characteristic.

	Cultural and morphological characteristics					
Strains	Shape	Colour	Size	Aspect	Cell Morphology	Arrangements
CRSBAN-BVA1	Round	brownish	3mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVA2	Irregular round	creamy white	2mm	Smooth	Rod	Single,Paired or Cluster
CRSBAN-BVA3	Irregular round	pinkish	3mm	Smooth	Rod	Single, Paired or Cluster
CRSBAN-BVA4	Round	yellowish	1,5mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVK1	Irregular round	creamy white	2mm	Smooth	Rod	Single,Paired, or Cluster
CRSBAN-BVK2	Irregular round	brownish	2,5mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVI1	Irregular round	creamy white	3mm	Smooth	Rod	Single, Paired or Cluster
CRSBAN-BVS1	Round	brownish	3mm	Smooth	Rod	Single, Paired or Cluster
CRSBAN-BVS2	Irregular round	pinkish	2,5mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVL1	Irregular round	creamy white	1,5mm	Smooth	Rod	Single, Paired or Cluster
CRSBAN-BVL2	Round	yellowish	2mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVB1	Round	brownish	3mm	Smooth	Rod	Single, Paired or Cluster
CRSBAN-BVB2	Irregular round	pinkish	3mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVP1	Irregular round	brownish	2mm	Smooth	Rod	Single, Paired or Chain
CRSBAN-BVP2	Irregular round	creamy white	1,5mm	Smooth	Rod	Single, Paired or Chain

Table 2. Biochemical and physiological characteristics.

	Strains														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Biochemical characteristics															
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mobility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxydase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gaz	+	-	-	-	+	-	-	+	+	+	-	+	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellulose Production	+	-	+	-	+	-	-	+	+	-	-	+	+	+	+
Suroxydation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ketogenesis from glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Substrates oxidized															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	-	-	+	-	+	+	-	-	+	+	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-
Saccharose	-	-	-	+	+	-	+	+	+	+	-	-	+	+	-
Galactose	+	-	-	-	-	+	+	-	-	-	+	-	-	+	-
Meliobiose	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+
Sodium Ac	-	-	+	-	+	+	-	+	-	+	+	-	-	+	-

Taxa: 1 : CRSBAN- BVP1 ; 2 : CRSBAN-BVB1 ; 3 : CRSBANBVL2 ; 4 : CRSBAN - BVI1 ; 5 : CRSBAN- BVK1 ; 6 : CRSBAN- BVA7 ; 7 : CRSBAN- BVA5 ; 8 : CRSBAN- BVA2 ; 9 : CRSBAN- BVA1 ; 10 ; CRSBAN- BVB2, 11 : CRSBAN- BVL3 ; 12 : CRSBAN- BVP2 ; 13 : CRSBAN-BVS2 ; 14 : CRSBAN-BVK2 ; 15 : CRSBAN-BVS1

Symbols: + (positive), - (negative), **+/-** (variable), Ac (Acetate).

Table 3. Alignment of 16S rRNA sequences of strain CRSBAN-BVK2.

Closest species based on 16S rRNA sequence	Acession number of 16SrRNA ref seq	% of 16s rRNA similarity	Closest species based on 16S rRNA sequence	Acession number of 16SrRNA ref seq	% of 16s rRNA similarity
Gluconacetobacter entanii	URS0000594DA5	95,65%	*Acetobacter ghanensis*	URS0000577E5C	97,06%
Gluconacetobacter maltiaceti	URS0000895280	95,75%	*Acetobacter lovaniensis*	URS0000614560	96,77%
Gluconacetobacter persimmonis	URS0000597324	95,83%	*Acetobacter fabarum*	URS0000522087	96,65%
Komagataeibacter hansenii	URS000026533D	96,04%	*Acetobacter okinawensis*	URS00000F16C5	96,64%
Komagataeibacter medellinensis	URS00002AF7AD	96,06%	*Acetobacter lambici*	URS0000183F1B	96,75%
Komagataeibacter kakiaceti	URS00003FC71F	96,15%	*Acetobacter syzygii*	URS000031846E	96,74%
Komagataeibacter sucrofermentans	URS00003E0354	95,84%	*Acetobacter suratthaniensis*	URS0000A11BC6	96,35%
Komagataeibacter xylinus	URS0000100687	95,76%	*Acetobacter pomorum*	URS0000244AC2	96,46%
Komagataeibacter europaeus	URS0000535D4A	95,97%	*Acetobacter pasteurianus*	URS00001956D5	95,16%
Komagataeibacter swingsii	URS000050A2EA	96,04%	*Acetobacter ascendens*	URS00001AB487	96,33%
Komagataeibacter nataicola	URS000021747F	95,94%	*Acetobacter thailandicus*	URS000084C389	97,45%
Komagataeibacter oboediens	URS00002FD334	96,04%	*Acetobacter aceti*	URS00005DFA0B	97,87%
Komagataeibacter intermedius	URS000047B5CA	96,17%	*Acetobacter sicerae*	URS00002AE1F4	97,86%
Komagataeibacter saccharivorans	URS0000184B3D	96,06%	*Acetobacter estunensis*	URS000003DA3E	97,56%
Gluconacetobacter takamatsuzukensis	URS0000353452	96,35%	*Acetobacter oeni*	URS0000603B66	97,18%
Gluconacetobacter sacchari	URS00004A2906	96,27%	*Acetobacter musti*	URS0000A0F72D	98,28%
Gluconacetobacter liquefaciens	URS00005F4503	96,17%	*Acetobacter orientalis*	URS000022E0BE	98 ,59%
Ameyamaea chiangmaiensis	URS0000135F9B	96,25%	*Acetobacter cibinongensis*	URS0000027BEC	98,27%
Tanticharoenia sakaeratensis	URS000007C67C	96,33%	*Acetobacter senegalensis*	URS00004FB952	97,98%
Kozakia baliensis	URS00004A75DA	96,35%	*Acetobacter tropicalis*	URS00005FB346	100,00%
Asaia prunellae	URS00003B1AD4	95,87%	*Acetobacter indonesiensis*	URS0000135937	98,38%
Asaia astilbis	URS00004E59BC	95,96%	*Acetobacter persici*	URS0000358DF0	98,48%
Asaia lannensis	URS0000538C5C	96,15%			

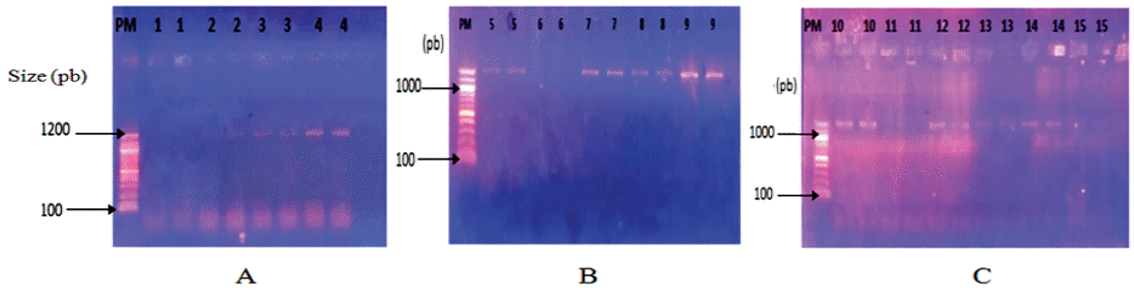

Figure 1: PCR product of the amplified 16s rRNA from the isolates.

Taxa: 1: CRSBAN-BVP1; 2: CRSBAN-BVB1; 3: CRSBAN-BVL2; 4: CRSBAN-BVI1;5: CRSBAN-BVK1; 6: CRSBAN-BVA7; 7: CRSBAN-BVA5; 8: CRSBAN-BVA2; 9: CRSBAN-BVA1; 10: CRSBAN-BVB2; 11: CRSBAN-BVL3; 12: CRSBAN-BVP2; 13: CRSBAN-BVS2; 14: CRSBAN-BVK2; 15: CRSBAN-BVS1.

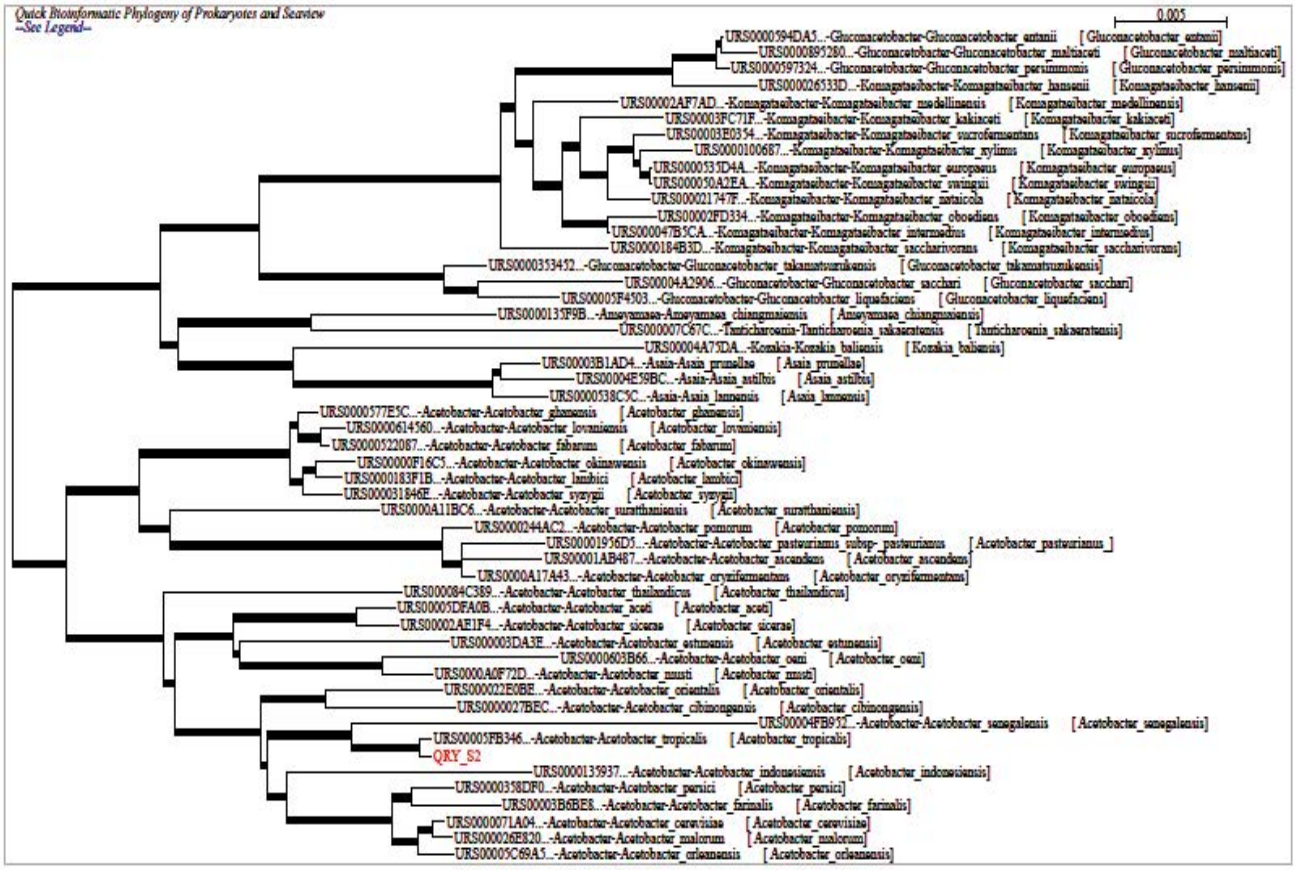

Figure 2: Phylogenetic analyze of the sequence CRSBAN-BVK2.

in (Table 1) are similar to those obtained by (Yamada *et al*. 1999 and Lisdiyanti *et al*. 2001) who isolated *Acetobacter spp* and found this morphologically of cells.

Isolates were biochemically negative oxidase, positive catalase and negative Gram. Similar characteristics was found by (Zahoor *et al*. 2006 and Mamlouk and Gullo 2013). The fermentation of carbohydrate was variable among the isolates. This result was similar to that of (Kadere *et al.* 2008) of *Acetobacter* identification isolated in coconut. According to (Mamlouk and Gullo 2013), AAB are known for their ability to partially oxidize a variety of carbohydrates and to release the corresponding metabolites (aldehydes, ketones and organic acids) into the media. They are also characterized by the ability to oxidize alcohols or sugars incompletely, and a common feature to most of them is the ability to oxidize ethanol to acetic acid. All isolate about in Carr medium supplemented with bromocresol blue produce acid to convert medium color from blue to yellow. This media was used to differentiate *Acetobacter* form other genus of AAB that turns the media colour to yellow

and then to green. (Mounir e*t al.* 2016) who had found isolates obtained from apple and date fruits, cactus and vinegar can able convert Carr medium color from blue to yellow obtained the similar result.

According to this biochemical characterization, all isolate were classified to *Acetobacter* genus. In addition to these results, molecular identification was performed this morphology and biochemical test.

The 16S rRNA gene of isolates was successfully amplified by PCR. After amplified, twelve isolates were further proven genus *Acetobater sp*. The amplified PCR products of isolates were roughly 1200 bp (Figure 1). (Sharafi *et al*. 2010) obtained similar results using the same primer and to identify the strain of *Acetobacter* like *Acetobacter pasteurianus* (1339 bp). (Bellankimath *et al*. 2017) have used the same primer pairs to identify the genus *Acetobacter* and the amplified PCR products were roughly 1454 bp and 1473 bp. The difference between amplified PCR products could depend on subtracts. These results indicate the twelve constraints used in this study with the bacillary form belong to these results of the *Acetobacter* genus. Hence most of the authors reported molecular procedures based on restriction fragment analysis like 16s rRNA as a leaving appropriate technique for characterization of microorganisms (Poblet *et al.,* 2000; González *et al*., 2006). Twelve (12) isolats of AAB that were classified to *Acetobacter* genus, only one of these isolates (CRSBAN-BVK2) were identified to be *Acetobacter tropicalis* after sequencing. The result of rate identification was

similar to those obtained by Ghariani *et al*. (2017) during alignment of nucleotidic sequence 16S rRNA of *Acetobacter tropicalis* isolated from sap palm but

higher to those obtained by Ouattara *et al*. (2019) who found 99.90 % of rate homology with *Acetobacter tropicalis* from sequencing of CRSBAN-BVA1. (Ndoye *et al*. 2007) in their study on the diversity of AAB (*Acetobacter senegalensis*) isolated from mango fruit (*Mangifera indica*) in Senegal found a similarity range 93.3 % with *Acetobacter tropicalis.* The results showed that bacteria strain named CRSBAN-BVK2 belongs to *Acetobacter tropicalis.*

CONCLUSION

This study demonstrated the possibility to isolate and identify the indigenous *Acetobacter* strains in local fruit as mango. Twelve isolats were identified like *Acetobacter* after morphological, biochemical and molecular characteristics. One isolate was identified to *Acetobacter tropicalis.* These isolated strain could be used for biotechnological application. These results are suggested to study applicability of the isolated strain in industrial production of acetic acid.

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