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Growth Enhancement of *Desulfovibrio indonesiensis* NCIMB 13468 and *D. vulgaris* NCIMB 8303 by Aqueous Extracts of *Basella alba*

[1]Oyewole, O. A., [1]Abalaka, M. E., [1]Oyeleke, S.B. and S. S. D. [2]Mohammed. [1]Department of Microbiology, School of Life Sciences, Federal University of Technology Minna, Nigeria [2]Department of Microbiology, Faculty of Sciences, Kaduna State University, Nigeria

Abstract

The influence of aqueous extracts of Basella alba on the growth of Desulfovibrio indonesiensis NCIMB 13468 and D. vulgaris NCIMB 8303 was examined. The active components of B. alba extract was determined by GC-MS. The result obtained revealed 46 components in the extract of B. alba. The 5 - 100% extract of B. alba was tested against serial dilution of D. indonesiensis and D. vulgaris. The results obtained revealed a ten times growth induction in 50-100% extracts after 1 day of growth when compared with untreated D. vulgaris and D. indonensiensis. After 2 days of growth, there was 100 times growth induction in 70 - 100 % extracts, 100 times growth induction in 5-100 % extract after 3 days and 10, 000 times growth induction after 4 days in 5-100% extracts. The biofilms of D. indonesiensis and D. vulgaris was examined using scanning electron microscopy and confocal laser scanning microscopy. The results obtained revealed an enhanced growth of the test organisms treated with extracts of B. alba when compared with untreated organisms. The results of this study suggest that aqueous extracts of B. alba can be used as a rapid detection tool for D. indonesiensis and D. vulgaris.

Key words: *Basella alba*, extracts, induction, *D. indonesiensis* NCIMB 13468 and *D. vulgaris* NCIMB 8303.
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Introduction.

Microorganisms can influence deterioration of metals used in several industrial sectors, such as oil and gas environments, storage tanks, water/sewage industry, ships, nuclear waste containers and marine platforms (Jack, 2002), this phenomenon is referred to as microbial influenced corrosion (MIC). Biofilms are the initiating and propagating agents of microbially influenced corrosion (MIC). In the formation of biofilm, a thin film first developes; then microbial growth and production of exopolymeric substances (EPS) result in the development of a biofilm (Characklis, 1981; Videla and Herrera, 2009; Oyewole, 2011). The nature of the attached surface, properties of microbial cell surface, particularly the presence of extracellular appendages, interactions involved in microbial cell-cell communication and production of EPS are key to biofilms formation and development (Simo˜es *et al*., 2010). Sulphate reducing bacteria (SRB) have long been identified as key contributor to MIC due to their ability to reduce oxidised sulphur compounds to form sulphide that attacks metal and leads to corrosion and pitting of the [illegible] Akn, 2009; Guan *et. al.* 2013). SRB [illegible] chemolithotrophic and [illegible] distinct group of anaerobic bacteria [illegible] oxidise sulphur compounds, as final electron acceptor during anaerobic growth [illegible] [illegible] also been implicated in biofouling, reservoir souring and hydrocarbon degradation (Barton and Fauque, 2009).

To control SRB growth, several strategies have been employed such as nanofiltration techniques, corrosion inhibitors, chemical biocides, protective coating and modification of environment and conditioning of metals. However, rapid detection of the presence of SRB is fundamental to their control because, it takes about 28 days before the complete detection in the field (Cowan, 2005). Focus of plant extracts over past decades has been in the areas of antimicrobial, anti-inflammatory and antioxidant properties but its role in induction of microbial growth has not been assessed.

B. alba is a widely cultivated vegetable belonging to the family Basellaceae. It is a very fast growing plant native to Asia and having a unique climbing characteristic. The colour of the stems and leaves are usually green or purple (Roshan *et. al*., 2012; Oyewole *et. al*., 2012).

Therefore, this study has [illegible] B. alba leaf as a possible [illegible] in the detection of SRB at low [illegible] density [illegible] rapid diagnostic [illegible] [illegible]es.

propidium iodide. The stained were allowed to act for 30 minutes. The excess washing solution was removed using filter paper and sealed using nail varnish after which was viewed using confocal laser scanning microscope, LSM 710 (Zeiss) using fluorophores and excitation wavelengths of Argon 488 nm and Helium-Neon (He-Ne) 543 nm.

Gas chromatography–mass spectrometry (GC-MS) Analysis of Aqueous Leaf extract of *B. alba*

Sample Preparation.

To a 10 ml aqueous extract of *B. alba* leaf was added 2 ml of hexane and mixed thoroughly for 10 minutes. Extracts in hexane was dried using Na_2SO_4. An aliquot of this dehydrated hexane extract was introduced in amber GC vial for GC-MS analysis.

GC-Method.

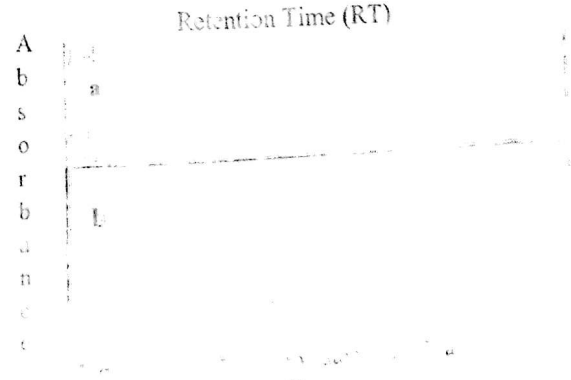
Agilent 6890N GC system coupled to a 5973 Network mass selective detector (GC-MS) (based on a quadruple mass separator) was used to run the *B. alba* hexane extract. A J&W Scientific HP-5MS UI silica fused capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) was used with helium as the carrier gas at a constant flow rate of 1.3ml/min. Splitless injection of 2 µl of the sample extract was automatically done by an injector (injector 7683 series) on the instrument from a syringe10 µl. Purge flow to split vent during the splitless mode was 60 ml/sec of ultra pure helium (99.999%) at for 2 minutes before reverting to gas saver mode of 20ml/min and split delay was 3.50 minutes. Solvent delay of 5 minutes was used. Syringe wash programme was made up of two post injection solvent washes, one sample pre injection wash and two pre injection pressure purges to ensure there was no carry over effect from one sample to the other. The injector and auxiliary temperatures were 290°C and 300°C respectively. The electron impact energy was set at 70 eV. Ion source temperature was 250°C. The mass spectrum was scanned from 50 to 500 Da in the scan mode using a temperature programme for the GC over from 50-300°C. The spectrum was ramped from 20°C/min for Ramp (1-3) to 70°C for Ramp 4. This is because the extract of *B. alba* leaf may contain several compounds and each may have a different boiling point and thus are converted into gas at varying temperature (Table 1). Identification of the compounds in *B. alba* leaf was based on their retention time, peak area and NIST 1998 reference library of match quality of 50 % and above.

Table 1. Program used for the GC-MC analysis.

Ramp	Ramp Rate °C/minutes	Temperature °C	Hold (minutes)	Run Time (minutes)
Initial	2	50.00	3.20	3.20
Ramp 1	0.00	168.00	1.00	10.10
Ramp 2	20.00	231.00	1.00	14.25
Ramp 3	20.00	294.00	1.00	18.40
Ramp 4	70.00	300.00	10	28.70

Results.

GC-MS chromatogram of *B. alba* leaf extracts is shown in Figure 1b while the blanck is shown in Figure 1a. The main peaks are found at retention time 19.03 minutes, 20.08 minutes, 22.80 minutes and 25.65 minutes but the base peak is found in 22.8 minutes. The compounds attributed to the main peaks are tricosane, eicosane, heneicosane at retention time of 20.80, 22.80 and 25.65 minutes and 2,6,10,14,18,22-Tetracosahexaene, 2, 6, 10, 15, 19, 23-hexamethyl-,(all-E), 1,5,9-Undecatriene, 2,6,10-trimethyl-,(z), 2, 7, 11-trimethyl-4-phenylthiododeca-2, 6, 10-triene, 2, 6, 10, 14, 18, 22- tetracosahexaene, 2, 10, 15, 19, 23 hexammethyl-, (all-E), 1, 5, 9-undercatriene, 2, 6, 10-trimethyl, 7,11-Dimethyldodeca-2,6.10-triene-1-ol at retention time of 19.03 minutes (Table 2). Other compounds detected in the leaf extracts of *B. alba* in based on NIST 1998 reference library of 60 % match quality and above are shown in Table 2.



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...ials and Methods

...ria Preparation

The medium used for growth of *Desulfovibrio indonesiensis* NCIMB 13463 and *D. vulgaris* NCIMB 8303 was Vitamin medium (VM) following the procedure of Postgate and Beech (2000).

Growth procedure for SRB

One milliliter (1 ml) stock culture of SRB, was inoculated into 9 ml vial of VMI medium using a syringe and needle to prevent introduction of oxygen into the vial. The cultures were incubated at 37°C for at least 28 days. The growth of SRB was indicated by the formation of film and exopolymeric substances (EPS) at the base of the vial and production of hydrogen sulphide (H_2S) from metabolizing cells reacting with the iron sulfate (from the media) to produce insoluble black iron sulfide.

Procedure for Extraction of Bioactive Components.

Dried leaf of *B. alba* was grounded to powder and diluted with sterile water 1:5. It was span in a 40 ml tube (Nalgene) at 18000 rpm, for 35 minutes and temperature of 4°C, using rotor SS-34 and centrifuge SORVALL RC 5C Plus. Supernatant obtained was filter-sterilized into a sterile 20 ml vial using a syringe driven Millex^R sterile filter unit with MF-Millipore^{TM} membrane (0.22 µm) and was stored at 4°C.

Procedure for Testing of Extracts on SRB Growth.

Sterile extracts were degassed for 30 minutes by passing sterile nitrogen gas through a glass microfiber filter with polypropylene housing (Whatman^R USA) fitted to a sterile syringe. The vial was immediately covered with sterile stopper (20 mm) and crimp seals (20 mm) to prevent introduction of oxygen into the vial. 0.5 ml of *D. indonensiensis* strain was inoculated into 5 ml vial containing 4.5 ml of VMR and was serially diluted 5 fold. The 0.5 ml of extracts was introduced into each of the vial using a syringe and needle to prevent introduction of oxygen into the vial and was incubated at 37°C. A control was set up with only pure SRB. Growth of SRB was observed and recorded daily in each dilution by observing film, EPS and insoluble black iron sulfide at the base of each of the vial.

...of ... extracts of *B. alba* ... with ... scanning ... microscopy (...

Effect of aqueous extracts of *B. alba* on the morphology of SRB cells and biofilm formation was determined using a SEM, JEOL 6060LV. Glass coupon (7 x 10 mm) was placed inside 9 ml of VMR medium in 10 ml vial. The medium was de-oxygenated using nitrogen gas for 30 minutes and sterilised by autoclaving for 15 minutes. An aliquot of the 7 days old culture of *D. indonesiensis* and *D. vulgaris* was added into the medium using 1:10 (v/v) ratio and extract was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 4 days. All above procedures were carried out using aseptic technique. Controls were set up without extracts.

Glass coupons containing biofilms were fixed with 4 % Gluteraldehyde in 0.1 M phosphate buffer overnight rinsed in 0.1 'M phosphate buffer twice at ten minutes interval. The glass coupons were then fixed with 3 drops of 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour and mixed gently. The coupons were rinsed twice in 0.1 M phosphate buffer and then 30 %, 50 %, 70 %, 90 %, 100 % ethanol and 100 % acetone. Few drops of hexamethyldisilazane were then placed on each glass coupon and the biofilms were incubated overnight. The glass coupons were set on aluminum stubs and sputter coated with gold using Q150R ES for 5 minutes and viewed at 15 kV accelerating voltage value.

The effects of extracts on the growth of *D. indonensiensis* and *D. vulgaris* using confocal laser scanning microscopy (CLSM).

Microscope cover slip (7 x 10 mm) was placed inside 9 ml of VM medium in 10 ml vial. The medium was de-oxygenated using nitrogen gas for 30 minutes and sterilised by autoclaving for 15 minutes. 7 days old cultures of *D. indonensiensis* and *D. vulgaris* were inoculated into the medium. The cultures were incubated with 100 % aqueous extract of *B. alba* leaf. After 4 days incubation period, the biofilms formed on the slides were washed 3 times with 2.5 % NaCl and stained with SYTOX 9 stains and propidium iodide. Live cells are stained green with SYTOX 9 stains while dead cells are stained in red due to

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	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	6.95	0.04	136	94
[illegible]	[illegible]	6.95	0.04	136	64
3	D-Limonene	6.95	0.04	136	68
[illegible]	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2beta.,5 alpha-	13.32	0.18	136	60
5	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	13.32	0.18	296	81
6	3,4-Octadiene, 7-methyl-	13.48	0.63	124	64
7	3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol	13.60	0.07	296	72
8	Dodeca-1,6-dien-12-ol, 6,10-dimethyl-	13.60	0.07	210	64
9	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (alpha.,2 beta.,5. alpha-)	13.60	0.07	138	60
10	Cyclobutane, 1-butyl-2-ethyl-	15.19	0.03	140	64
11	9-12-Octadecadienoic acid (Z,Z)-	15.34	0.46	280	90
12	Tetracosane	18.29	0.58	338	92
13	Tetrapentacontane, 1,54-dibromo-	18.29	0.58	915	90
14	1-Octadecene	18.29	0.58	252	74
15	Ethanol-2-(Octadecyloxy)-	18.29	0.58	314	60
16	Eicosane	18.29	0.58	252	64
17	7-Pentadecyne	18.43	0.44	208	91
18	Tetracosane	18.43	0.44	338	91
19	Octadecanal	18.43	0.44	268	60
20	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-,(all -E)	19.03	2.49	410	90
21	1,5,9-Undecatriene, 2,6,10-trimethyl-,(z)	19.03	2.49	192	83
22	2,7,11-Trimethyl-4-phenylthiododeca-2,6,10-triene	19.03	2.49	314	78
23	2,6,10,14,18,22- Tetracosahexaene, 2,10,15,19,23 hexammethyl-, (all-E)	19.03	2.49	410	90
24	1,5,9- Undercatriene, 2,6,10-trimethyl	19.03	2.49	192	83
25	7,11-Dimethyldodeca-2,6.10-triene-1-ol	19.03	2.49	208	78
26	Tricosane	20.8	2.85	324	96
27	Eicosane	20.8	2.85	282	95
28	Heneicosane	20.8	2.85	296	93
29	Octadecane	20.8	2.85	254	92
30	1-Docosene	20.8	2.85	308	89
31	Tricosane	20.8	2.85	324	94
32	Heptadecane	20.8	2.85	240	93
33	Docosane	20.8	2.85	310	97
34	Cycloeisicosane	20.8	2.85	280	93
35	1- Nonadecene	21.73	1.26	266	60
36	Tetracosane	21.73	1.26	338	91
37	1-Eicosene	21.73	1.26	280	91
38	Z-2-Octadecen-1-ol	21.73	1.26	268	64
39	2,5-Furandione, 3-dodecyl-	21.73	1.26	266	72
40	Tetrapentacontane, 1,54-dibromo-	24.09	1.24	915	87
41	1-Octadecene	24.09	1.24	252	70
42	1-Nonadecene	24.09	1.24	266	70
43	7-Pentadecyne	26.33	0.85	208	94
44	E-11,13-Dimethyl-12-tetradecen-1-ol acetate	26.33	0.85	282	70
45	Z-2-Octadecen-1-ol	26.33	0.85	268	64
46	1-Cyclohexylnonene	26.33	0.85	208	60

The effects of aqueous extracts of *B. alba* on the growth of *D. indonesiensis* NCIMB 13468 and *D. vulgaris* NCIMB 8303 is shown in Table 3 and Table 4. There was no influence of 5% - 40 % of the extract on *D. vulgaris* and *D. indonesiensis* growth after 1 day incubation period, but there was a growth induction with 50 -100 % extracts with 10 times growth difference when compared with untreated cells. At day 2 there was 100 times growth difference of 70 – 100 % extract but after 4 days incubation period, there was 1000 times growth difference of the extracts when compared to the untreated cells in all the concentration of the extracts (50-100 %).

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Table 3: The effects of aqueous extracts of *B. alba* on the growth of *D. indonesiensis* NCIMB 13468 and *D. vulgaris* NCIMB 8303.

	Day 1			Day 2			Day 3			Day 4				
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	-4	-5
Untreated SRB	+	-	-	+	-	-	+	-	-	+	-	-	-	-
+ 5 % extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 10 % extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 20 % extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 30 % extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 40 % extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 50 % extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 60 % extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 70 % extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 80 % extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+
+ 90 % extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+
+100 % extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+

Experiments were done in triplicate. (+) SRB growth indicated by blackening of vials due to production of FeS from medium (-) no SRB growth

Table 4: The effects of aqueous extracts of *B. alba* on the growth of *D. indonensiensis*

	Day 1			Day 2			Day 3			Day 4				
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	-4	-5
Untreated SRB	+	-	-	+	-	-	+	-	-	+	-	-	-	-
+ 5% extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 10% extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 20% extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 30% extract	+	-	-	+	+	-	+	+	+	+	+	+	+	+
+ 40% extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 50% extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 60% extract	+	+	-	+	+	-	+	+	+	+	+	+	+	+
+ 70% extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+
+ 80% extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+
+ 90% extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+
+100% extract	+	+	-	+	+	+	+	+	+	+	+	+	+	+

Experiments were done in triplicate. (+) SRB growth indicated by blackening of vials due to production of FeS from medium (-) no SRB growth

The scanning electron micrograph of *D. indonesiensis* and *D. vulgaris* is shown in Figure 3. The *D. indonesiensis* and *D. vulgaris* supplemented with aqueous extracts of *B. alba* showed increased cell numbers and biofilm formation when compared with untreated cells after 4 days of incubation.

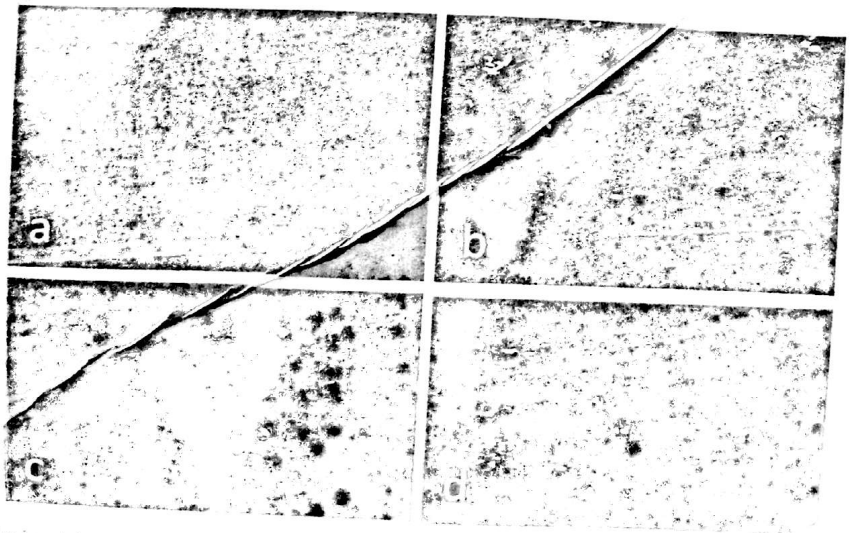

Figure 2. Confocal laser scanning

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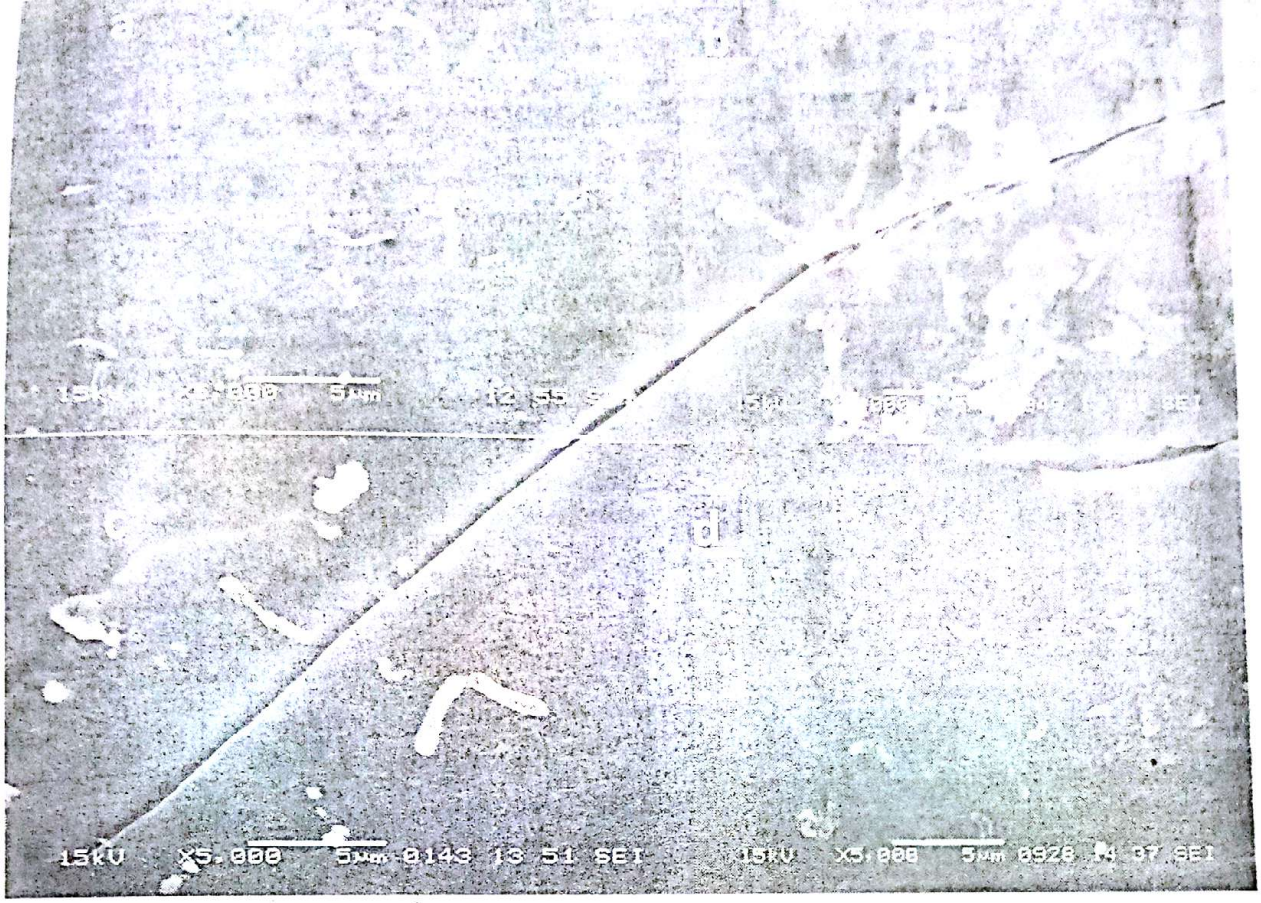

Figure 3. Scanning electron micrographs of (a) *D. indonesiensis* only, (b) *D. indonesiensis* + *B. alba* (c) *D. vulgaris* only, (d) *D. vulgaris* + *B. alba*

Discussion

B. alba aqueous extracts induced the growth of *D. indonesiensis* and *D. vulgaris*. The process for the growth induction may involve a reduction in the time required for cell division in the test organisms. Few approaches have been employed in the induction of SRB growth due to their slow growth rate and difficulty in their growth detection (Cowan, 2005). Example is through the addition into SRB culture medium some growth supplements such as heavy metals or small molecules (Agostini-Costa *et al.*, 2012; Bertrand *et. al.*, 2014).

The presence of the active components in *B. alba* as revealed by the GC-MS (Table 2) indicates that some of the compounds have growth promoting ability. Similarly, folic acid synthesised by probiotic bacteria has been used as growth supplement in bacterial growth media (Rossi *et al.*, 2011). Results obtained in Tables 3 and 4 also revealed that at higher concentrations of aqueous extracts of *B. alba*, there are more induction effects on the growth of the test organisms.

Advantage of the use of extract from *B. alba* is the availability of the plant and its widespread especially in Asia (Roshan *et al.*, 2012; Oyewole *et al.*, 2012).

Conclusions

The 50 - 100% aqueous extracts of *B. alba* had inducing effects on SRB growth, thus may be developed as a growth supplement for SRB detection sensitivity. It might be worth incorporating the extracts in commonly used media for SRB growth in the field.

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