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Bacteria Diversity and Bacteria Identification as Elisitor Candidate from Phyllosphere of Medicinal Plant *Ageratum conyzoides* L.

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ABSTRACT

Bacteria of phyllosphere connected to secondary metabolite biosynthesis in plant, especially as elicitor biotic factor. The research aim to study bacteria diversity and to identify bacteria as elicitor candidate from phyllosphere of *Ageratum conyzoides*. Bacteria diversity was studied through morfology analysis, Gram staining, test of protein hydrolitic and chitin hydrolytic. Meanwhile, bacteria was identified employing molecular technique (16S rDNA). Result showed that there are 57 different bacteria isolates, among other things 35 isolates could degrade protein molecule and 10 isolates could degrade chitin molecule. Shape of bacteria colony was dominated by round shape (47%), colour of bacteria colony was dominated by white and yellow, edge of colony was dominated by smooth and shiny, elevated of colony was dominated by flat and curve. Gram staining was dominated by Gram positive (75%) and shape of cell was dominated by bacilli (70%). Molecular identification revealed the best isolate of protein degradable are *Enterobacter sp* strain IGCAR-11/07 (EF523425.1, max identity 100%, E value 4e-97) dan *Ochrobactrum anthropi* strain HAMBI12402 (AF501340.1, max identity 99%, E. value 0.0). The best isolate of chitin degradable are *Bacillus cereus* strain MQ23R (HM241941, max identity 99%, E value 0.0) dan *B. thuringiensis* strain INRS7 (FJ601903.1, max identity 97%, E value 0.0). Bacteria phyllosphere from *A. conyzoides* have physical and chemical diversity.

Keywords : phyllosphere, 16S rDNA, *Ageratum conyzoides*

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Introduction

Ageratum conyzoides L. is medicinal plant and use as antidiuretic, antipyretic, antimicrobes. Leaves extract of *A. conyzoides* from Botanical Garden of Indonesia University of Education could be inhibited growth of *Pseudomonas aeruginosa, Streptococcus pyogenes* (Fitriani *et al.* 2009a), *Staphyllococcus aureus* (Desiarianty 2009), and *Candida albicans, Tricophyton mentagrophytes* (Fitriani *et al.* 2009b). Analysis GC-MS of leaves extract of *A. conyzoides* showed that contained flavonoid compound (1-2H benzopyran), terpenoid compound (β-caryophllene, precocene II, phytol) and alkaloid compound (3', 8', 8'-trimetoksi-3-piperidil-2,2'-dinaftil-1,1',4',4'-tetron, 4-hydroxy-3,5-dimethoxyphenyl)methylene) (Fitriani *et al.* 2009a).

Existence of microorganism, included bacteria in plant has multiple function for living of plant, especially for plant metabolism. Many evidence showed that bacteria has indirect role to secondary metabolite biosynthesis and we recognized as elicitor biotic factors. Elicitors biotic factors influenced metabolism activity through increasing of intracellular reaction in cytoplasm involved vacuole. Other mechanism showed that involving genetic activity as transcription factors to synthesize protein by cis-trans acting mechanism (Fosket 1992). The aim of the research is to study bacteria diversity and to identify bacteria as elicitor candidate from phyllosphere of *Ageratum conyzoides*.

Materials and Methods

Sample preparation

Leaves of *Ageratum conyzoides* took from Botanical Garden in campus of Indonesia University of Education. First, third, and fifth leaves was separated from plant using aseptic apparatus and then put it to ice box.

Isolation of Phyllosphere Bacteria

One gram of leave was put in 10 mL of NaCl 0,85% and vortex for 15 minutes. Dilution was done for 10^{-1} to 10^{-6} respectively, and put 1,0 mL into petridish with Luria Bertani (LB) media. Growth of bacteria was observed for 24 hours at room temperature.

Morphology Observation and Gram Staining

Amount and characteristic of colony was observed for 10 X 24 hours. We observed shape of colony, color of colony, edge of colony, and elevation of colony (Cappuccino & Sherman 1987). Amount of colony was done using colony counter. Identifying Gram was done employing Gram staining procedure.

Hydrolytic Analysis

Bacteria, 48 hours age culture, was analyzed its protein and chitin hydrolytic capability (Dajanta 2009). 2,5 mm of diameter bacteria isolate was growth on Luria Bertani (LB) media with addition 1% skim milk or 0,5% chitin. Positive proteolytic and chitinolitic reaction was showed with clear zone in around colony bacteria (Cappucino & Sherman 1987).

Isolation of DNA

One loop of the best colony bacteria degradable protein or chitin from cryopreservation buffer was inoculated to LB agar media and growth for 24 hours at room temperature. Then colony bacteria was subcultured to LB media and incubated for 16 hours at 25 °C in shaker with 175 rpm agitation. DNA bacteria was isolated using isolation kit from Fermentas (Lithuania).

Amplification of DNA

DNA from bacteria was amplified by Polymerase Chain Reaction machine (Perkin Elmer), employing primer 16S rDNA referred to Marchesi *et al.* (1998). Mix PCR consist of 10 x buffer enzyme (NEB USA) final concentration 2,5 mM, dNTP's mix final concentration 0,2 mM per dNTP, Taq DNA polymerase enzyme (NEB USA) final concentration 1-2,5 U/µl, primer with final concentration 0,4 µM. DNA bacteria as DNA template, and ddH_2O. Total volume of PCR mix is 25 µl. Condition of PCR is predaturation, 95 °C, 1 min; denaturation, 94 °C, 30 sec; annealing, 55 °C, 30 sec; extension, 72 °C, 30 sec; post PCR, 72 °C, 10 min. Reaction of PCR was done for 30 cycles. Amplicon was runned by electrophoresis in 1,0 % agarose, 1 X TAE buffer and visualized by EtBr on UV light.

Sequencing of DNA

DNA was sequenced by ABI 3130, Genetic analyzer (Applied Biosystems).

Data Analysis

Data was analyzed employing bioinformatics analysis. Sequence DNA was aligned to database in gene bank EMBL (European Molecular Biology Laboratory, http://www.ebi.ac.uk/ebi_docs/embl_db.html).

Results and Discussion

Characteristic morphology of bacteria phyllosphere *Ageratum conyzoides*

Colony bacteria growth on media LB and showed different characteristic morphology. There are 57 isolates that revealed different morphology. Variation of shape of colony could be seen on Figure 1.

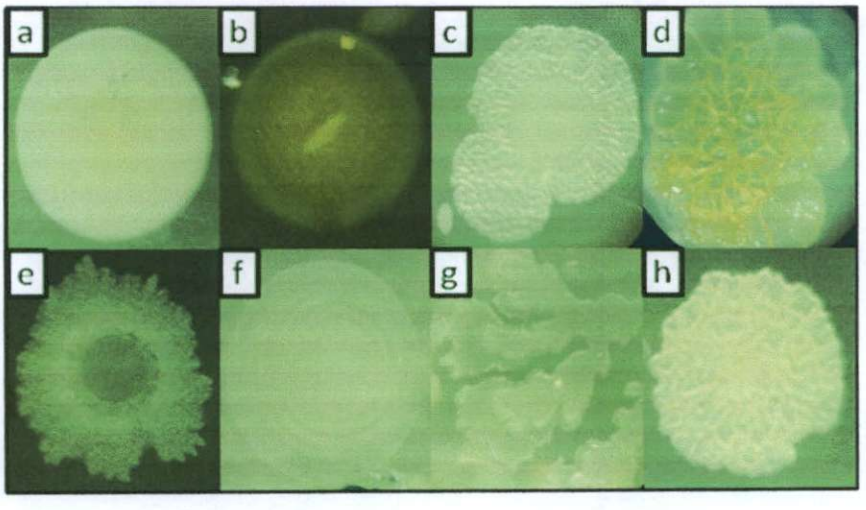
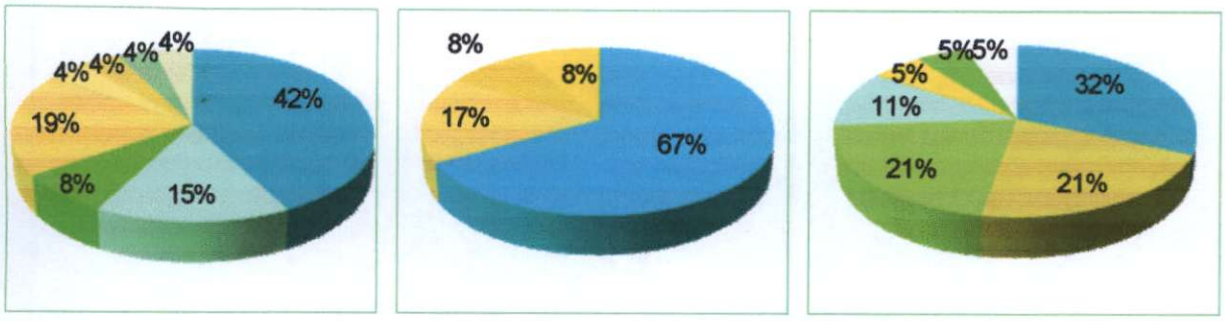


Figure 1. Variation of shape colony bacteria phyllosphere A. conyzoides.
(a) Round, (b) round and emerge edge, (c) round and shell edge,
(d) complex, (e) round and spread edge, (f) concentris,
(g) irregular and spread, (h) wrinkled

The first leave of Ageratum was dominated by round shape colony bacteria (42%), the third leave has 67% and the fifth leave has 32%. The highest diversity of shape of colony is possesed by the first leave (8), then the fifth leave (7) and the lowest is the third leave (4) (Figure 2).



(a) (b) (c)

Figure 2. Proportion of shape of colony from phyllosphere *A. conyzoides*. (a) First leave, (b) Third leave,(c)Fifth leave. Blue : round; red : irregular, spread; green : round, emerge edge; purple : round, shell edge; yellow : concentris; orange : complex; grey : irregular, spread; pink : wrinkle; dark pink : rhizoid; brown : round, spread edge.

Round shape of colony bacteria most found on phyllosphere bacteria, example *Pseudomonas syringae* and *Erwinia sp* (Bereswill 1998, Crosse 1973, Ishimaru & Klos 1984). Smiley (1993) described shape of colony bacteria on phyllosphere winter wheat was dominated by round shape (81%) and other shape was irregular with proportion 18%.

Isolate from phyllosphere Ageratum has diversity of colour colony. Variation of colour of bacteria colony was influenced by tolerance to sun light radiation (Beattie & Lindow 2006). Variation of colour colony could be seen on Figure 3.

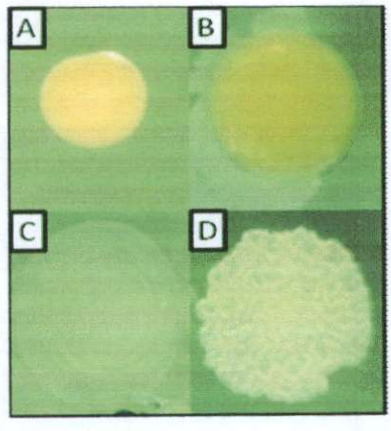


Figure 3. Variation of colour colony bacteria phyllosphere *A. conyzoides*
(a) Pink, (b) yellow, (c) white, (d) white-brown

The first leave showed the highest variation of colour colony and has six different colour, i.e. white (35%), yellow (27%), white-yellow (8%), pink (4%), white-brown (15%) and white-

orange (11%). The variation of colour was caused by exposing to sun light and make into variation of pigmentation. The third leave revealed four different colour,i.e. white (50%), yellow (25%), white-yellow (17%), and pink (8%). The fifth leave has three different colour, i.e. white (63%), yellow (29%) and white-yellow (8%). Meanwhile, phyllosphere winter wheat was dominated by white colony (72%) and yellow (18%) (Smiley 1993).

The edge of colony bacteria from the first and third leave was dominated by smooth colony (46% and 47%), meanwhile colony bacteria from the fifth leave showed was dominated by irregular colony (48%). Appearance of colony was dominated by shiny colony from first, third, and fifth leave (63%, 71%, and 83%, respectively). Elevation of colony from first leave and third leave have curve (42%, 42%, respectively), flat (38%, 33%, respectively), dome-shaped (12%, 17%, respectively), hilly (8%, 8%, respectively). Meanwhile the fifth leave has colony with elevation curve (53%) and flat (47%).

The evidence revealed phyllosphere bacteria from first leave has more variation than bacteria from third and fifth leave. The striking variation is the colour of colony, and is caused by sun light radiation, environment and nutrition. Fluctuation of environment and nutrition on abaxial and adaxial could influenced survival and diversity of bacteria (Lindow and Brandl 2003). Gram staining was done to study shape of cell and kind of Gram. Bacteria phyllosphere Ageratum has two shape of cell, i.e. bacilli and coccus (Figure 4). The most shape of bacteria phyllosphere Ageratum is bacilli with proportion 70%, meanwhile coccus achieved 30%. Gram positive dominated bacteria phyllosphere Ageratum, as Beattie and Lindow (2006).

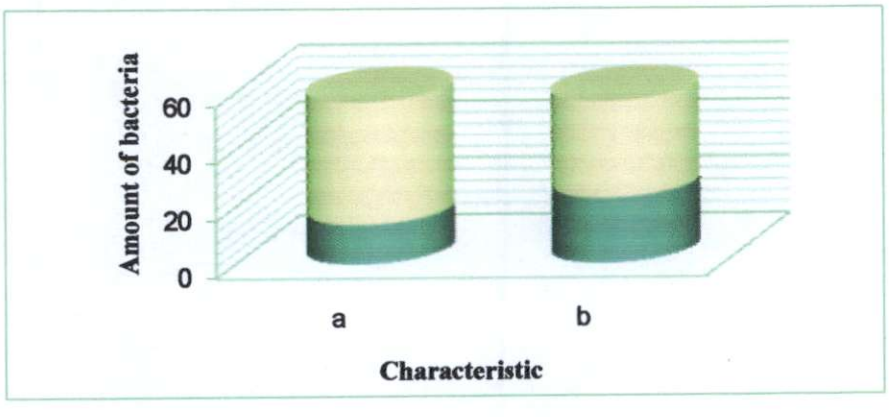


Figure 4. Gram staining. (a) shape of cell, pink : bacilli, purple : coccus;
(a) Gram staining, pink : Gram positive, purple : Gram negative

Dynamics amount of cell per gram leave could be seen on Figure 5. Amount of bacteria in first leave is 5,1 x 10^6 per gram leave and continue increasingly until sixth day reach 5,8 x 10^7, on seventh day, colony bacteria has been uncountable. The third leave, amount of bacteria was 6,8 x 10^7 per gram leave and on fifth day was 1,7 x 10^8. On seventh day, the bacteria has uncountable. The fifth leave has 6,4 x 10^7 and increasingly until fifth day (2,0 x 10^8).

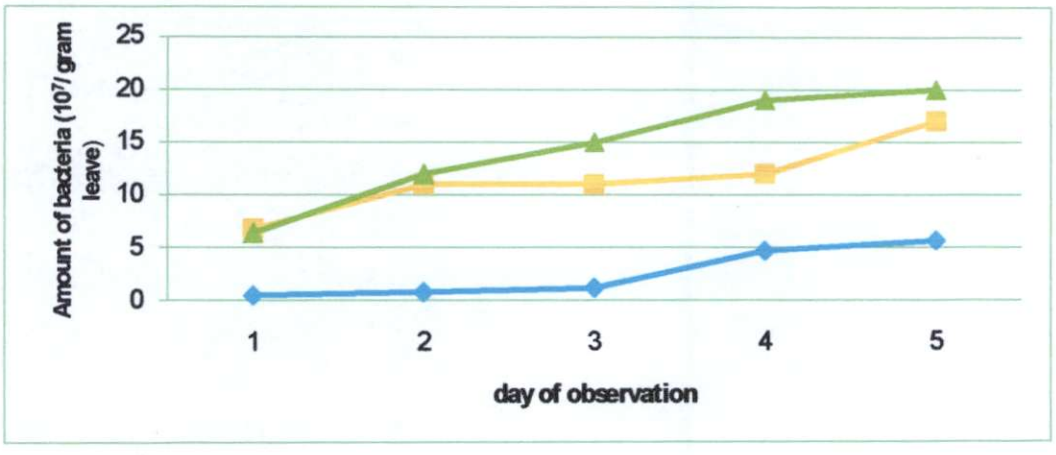
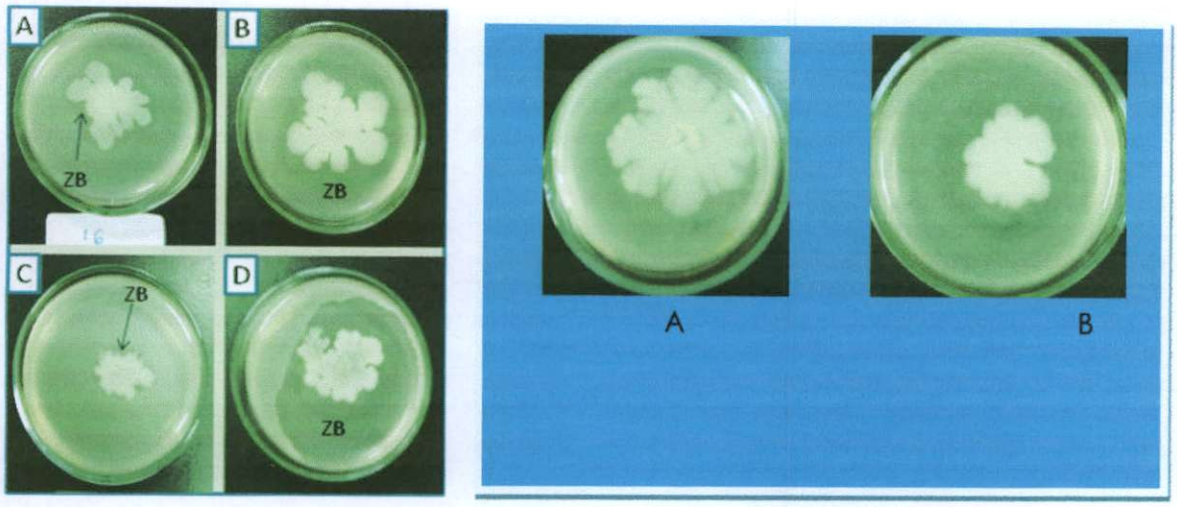


Figure 5. Amount of bacteria per gram leave. Blue : first leave; Red : third leave; Green : fifth leave

Activity of Protein and Chitin Hydrolysis

Not all isolated bacteria from phyllosphere *A. conyzoides* have been possessed protein and chitin hydrolytic activity. Only 35 isolates could be degraded protein in media, it means they produced extracellular enzyme for digested the substrate. While only 10 isolates have capability to digest the substrate chitin in media. Positive reaction was showed through formed clear zone in around colony (Olajuvigbe & Ajele 2008). Every isolate bacteria has differently capability for hydrolytic activity, it was depend on diameter of clear zone formed (Figure 6).



(a) (b)

Figure 6. Test of hydrolitic activity. (a). Activity of protein hydrolisis; A,B : isolate A 1.6; C,D : isolate C 1.18. (b). Activity of chitin hydrolisis; A : isolate 1.8; B : isolate 1.14

Isolate A 1.6 and C 1.18 describe activity hydrolysis of protein. Isolate A 1.6 (A) was incubated for seven days and showed diameter of clear zone was 1,47 $\pm$ 0,08 cm, while isolate C 1.18 (B) showed diameter of clear zone was 1,27 $\pm$ 0,08 cm. Isolate 1.8 (C) and 1.14 (D) showed activity hydrolysis of chitin. Isolate 1.8 (C) has differently characteristic, after incubated for seven days, revealed diameter of clear zone was 1,10 $\pm$ 0,008 cm and isolate 1.14 (D) showed diameter of clear zone was 0,95 $\pm$ 0,008 cm.

Biotics elicitors is very influence to biosynthesis secondary metabolites in plant. Macromolecules in life organism, like protein, peptide, glycoprotein, lipid and oligosaccharide involved to intracellular reaction for biosynthesis secondary metabolite in plant cell (Veit *et al.*, 2001). Several protein and peptide compound has a role as elicitors, example oligogalacturonide, elicitin, harpin, flagelin, protein or peptide toxin (victorin, glutathione, oligandrin and syringoline) (Angelova *et al.* 2006). Elicitin can extracted from *Phytophtora* and *Pythium*, meanwhile harpin, flagelin and syringolin extracted from Gram positif bacteria.

Identification of Isolate Bacteria

Isolate A, B, C and D was identified to study species name and more characteristic bacteria. Identifying of isolate was done by molecular technique, employing amplified of 16S rDNA and analysis of its sequence using bioinformatics. Sequencing of amplicon revealed about

500 nukleotides for four isolates, respectively. Analysis bioinformatics showed that isolate A is *Enterobacter sp* strain IGCAR-11/07 (EF523425.1, max identity 100%, E value 4e-97). Isolate B is *Ochrobactrum anthropi* strain HAMBI12402 (AF501340.1, max identity 99%, E. value 0.0). Isolate C is *Bacillus cereus* strain MQ23R (HM241941, max identity 99%, E value 0.0), meanwhile Isolate D is *B. thuringiensis* strain INRS7 (FJ601903.1, max identity 97%, E value 0.0). Fatimah (2005) and Asano *et al.* (1988) described that *Enterobacter* and *Ochrobactrum anthropi* have protease extracellular until they could degrade protein molecule. Kamil (2007) found 20 isolate bacteria chitin degradable from plant surface, it was only 10% from total isolate.

Phylogenetic relationship has been formed inter and outer species. It could be seen in Figure 7 and 8.

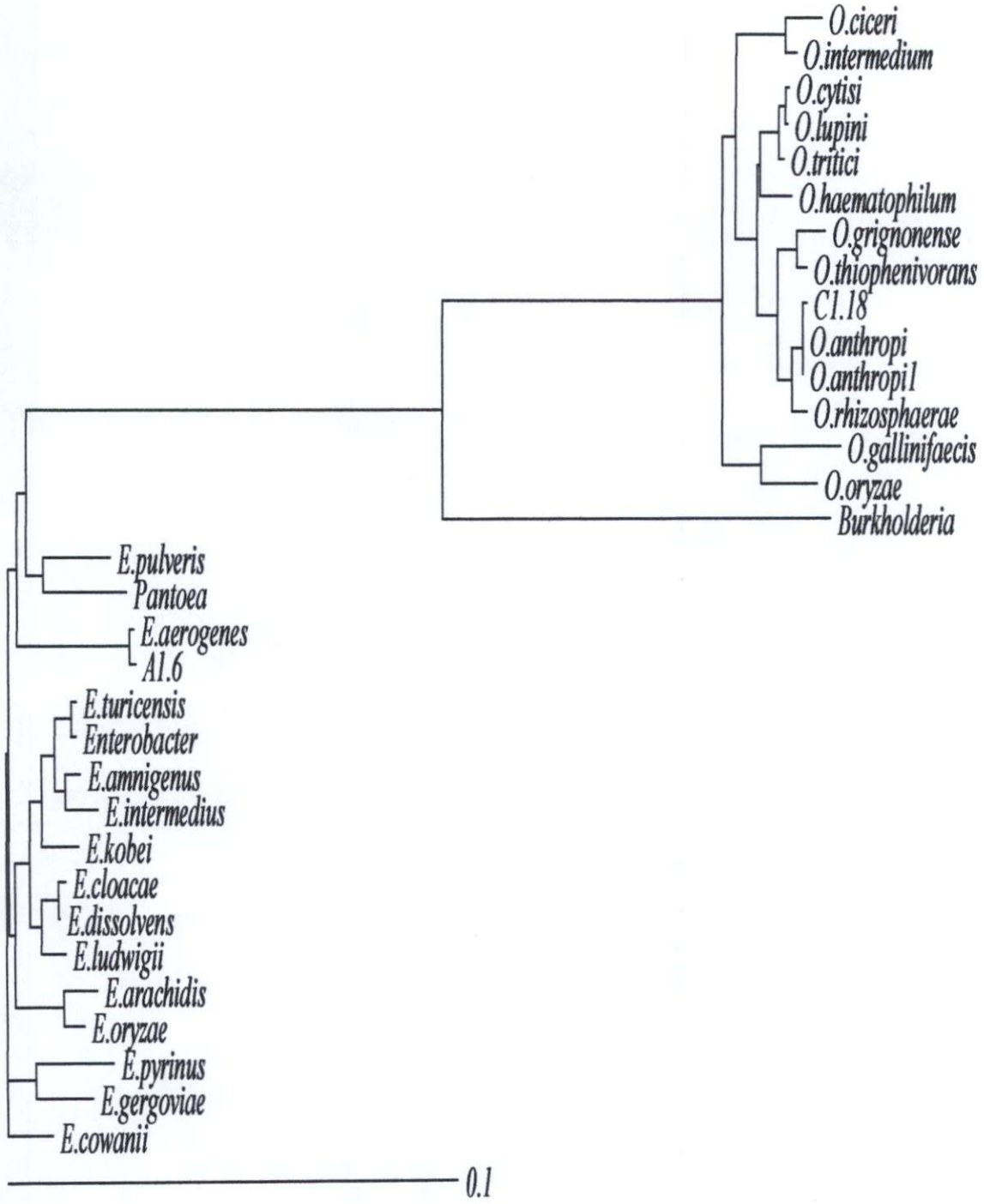


Figure 7. Phyllogenetic tree of isolate A and B

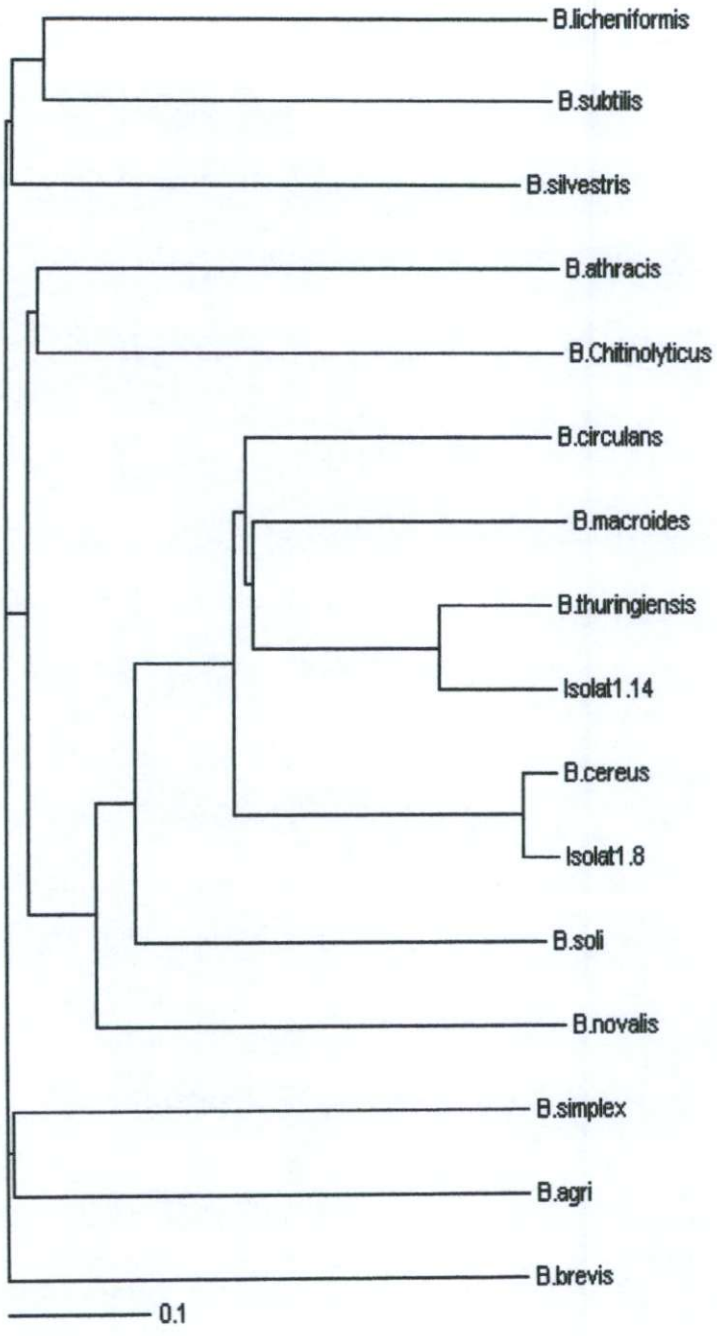


Figure 8. Phyllogenetic tree of Isolate C and D

Conclusions

Phyllosphere of *Ageratum conyzoides* has diversity of bacteria that has diversity characteristic of morphology and physiology. Isolated bacteria showed differently characteristic morphology, i.e. colour, elevation, edge, shape of colony, and shape of cell. Physiology aspect was showed differently characteristic too. Not all isolated bacteria has hydrolytic capability.

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Hydrolytic Analysis

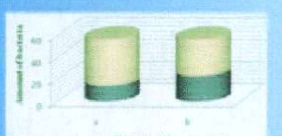
Isolation of DNA

Amplification of DNA

Sequencing of DNA

Data Analysis

Results and Discussion



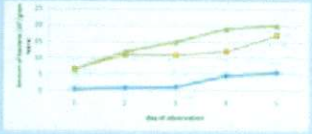


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Figure 7. Phylogenetic tree of isolate A and B

Figure 8. Phylogenetic tree of isolate C and D

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References

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