

ENDOPHYTIC BACTERIA MANGROVE PLANT *BRUGUIERA GYMNORRHIZA* IN KUALA ENOK INDRAGIRI HILIR- RIAU AS ANTIBACTERIAL PRODUCERNurtanny¹, Anthoni Agustien*² and Akmal Djamaan³¹Post Graduates of Biology, FMIPA, Andalas University, Padang, Indonesia.²Department of Biology, Andalas University, Padang, Indonesia.³Faculty of Pharmacy, Andalas University, Padang, Indonesia.***Corresponding Author: Anthoni Agustien**

Department of Biology, Andalas University, Padang, Indonesia.

Article Received on 09/11/2018

Article Revised on 30/11/2018

Article Accepted on 21/12/2018

ABSTRACT

Bruguiera gymnorrhiza is one of mangrove plants that has potential as a producer of antibacterial compound. To determine the extent of endophytic bacterial activity in *B. gymnorrhiza* then it is necessary to test the activity. This study aims to determine bacterial endophytic bacteria *B. gymnorrhiza* bacteria that produce antibacterial and the extent to which potential antibacterial activity. This research used survey method with purposive sampling and experiment in laboratory. The results of this study obtained eighteen bacterial endophytic bacteria potentially against *Escherichia coli* test bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*. Of the eighteen potential endophytic bacterial isolates were obtained two isolates with very strong inhibitory category and one strong category against test bacteria. Characteristics of these three potential isolates were two groups of Gram negative bacteria and one group of Gram-positive bacteria.

KEYWORDS: *B. gymnorrhiza*, antibacterial, endophytic bacteria.**INTRODUCTION**

Mangrove plant is a tropical forest ecosystem located between land and sea (Zhou H W, Guo C L, Wong Y S, Tam N FY, 2006). Mangrove is resistant to high salinity, acting as a major producer in the food chain of estuary ecosystem. Besides being a highly productive ecosystem along the coast, it has a very high value in terms of economic, ecological, and scientific and cultural resources. The mangrove plant is also capable of producing unique metabolite in order to increase its environmental adaptability (Bandarnayake, 2002).

Ruhe JJ, Monson T, Bradser R W, Menon A (2005) said that the commercial use of antibiotics, which is a synthetic antibiotic, is susceptible to trigger resistance to bacterial pathogenic microbes.

Indragiri Hilir regency is one of the districts located in the coastal region, rich enough with mangrove vegetation that can be seen along the muddy coast. There are still many mangroves potential not optimally utilized in this area, especially making mangroves as one source of drugs or one source of endophytic microbes to produce antibacterial compounds.

Based on this background, it is necessary to conduct research related to the potential of mangrove *B. gymnorrhiza* in Kuala Enok Indragiri Hilir regency as a

producer of antibacterial compounds. The research aims to obtain endophytic bacterial isolate from mangrove *B. gymnorrhiza* plants as antibacterial producer and to know the most potential isolate as antibacterial producer.

MATERIALS AND METHODS**Sampling**

The samples include roots, stems, and leaves of mangrove plant type *B. gymnorrhiza*. Then, samples processing in the laboratory is conducted by an aseptic method. The fresh mangrove leaves are washed with running water and 70% alcohol and then the leaves are divided into two halves right in the middle of the leaf bone. After that, cut to the size of 2 x 2 cm, as well as the stems and roots.

Isolation of Endophytic bacteria

Samples of roots, stems, and leaves that have been cut are inoculated by using tweezers into a Petri dish contained NA medium. Each of the Petri dish is planted 5 pieces of the sample, then incubated at 35⁰ C for 48 hours. After the bacteria growing, the purification is conducted, then the isolates are coded (Zam et al., 2017).

Preparation of Inoculum of Endophytic Bacteria

Prepare 100 ml antibiotic production medium for fermentation with 30 ml meiza immersion water, 30 g sucrose, 5 g CaCO₃, 1 ml Tris (Fe SO₄ 0,1%; Mg Cl₂

0,2% and Zn SO₄ 0,01%). Then, each of the isolates of endophytic bacteria is inoculated in production medium. Furthermore, they are incubated at 35 ° C in a rotary incubator shaker at 120 rpm for 24 hours (Zam *et al.*, 2017).

Screening of Endophytic Bacteria to Produce Antibacteria

Screening is performed by each inoculum of endophytic bacteria inoculated by 5 ml inoculum at 95 ml of production medium. Subsequently incubated at 35⁰ C in a rotary shaker incubator at a rate of 120 rpm for 48 hours. When the incubation is complete, the next stage is fermentation culture centrifuged at 5,000 rpm for 15 minutes (Djamaan *et al.*, 2012). The supernatant obtained is tested for its antibacterial activity against microbes *E.coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa*.

Antibacterial Test of Endophytic Bacterial Isolates

Antibiotic testing of each bacteria is conducted using paper disc method. NA medium is provided in a petri dish. Furthermore, each medium in a petri dish lubricated with test bacteria namely *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. The disc paper is immersed in a supernatant, drifted and positioned over the NA medium aseptically. Then, incubated at room temperature for 48 hours. The diameter of the inhibitory zone is measured with the help of Vernier Kaliper Digital.

Antibacterial Production Time of Potential Endophytic Bacterial Isolates

The most optimal determination of antibacterial production time is conducted against the potential endophytic bacterial isolates or the most significant inhibitory zones. Antibacterial isolation times are 24, 30, 36, 42, and 48 hours (Djamaan *et al.*, 2012). *E. coli* and *S. aureus* are used as test bacteria.

RESULTS AND DISCUSSION

Table 1: Isolation results of antibacterial endophytic bacteria.

No	Sample	Σ Colony	Isolate Code	Σ Isolate
1.	BGR-1	3	EBR-1	1
2.	BGR-2	4	EBR -2	1
3.	BGR-3	5	EBR -3	1
4.	BGR-4	4	EBR -4	1
5.	BGR-5	2	EBR -5	1
6.	BGS-1	7	EBS-1	1
7.	BGS-2	2	EBS -2	2
8.	BGS-3	5	EBS -3	1
9.	BGS-4	6	EBS -4	1
10.	BGS-5	7	EBS -5	1
11.	BGL-1	2	EBL-1	2
12.	BGL-2	2	EBL -2	2
13.	BGL-3	5	EBL-3	1
14.	BGL-4	6	EBL -4	1
15.	BGL-5	5	EBL -5	1
	Total	65		18

BGR= Bruguiera Roots; BGS= Bruguiera Stems; BGL= Bruguiera Leaves

EBR= Endophytic Bacteria Roots; EBS= Endophytic Bacteria Stems; EBL= Endophytic Bacteria Leaves

Table 1 shows the observations of 15 samples of *B. gymnorrhiza* obtained 65 colonies with 18 different endophytic bacterial isolates. Five isolates are obtained from the root, 6 isolates from the stem, and 7 isolates from the leaves. As Gayathri and Muralikrishnan (2013) study have successfully isolated 24 endophytic bacteria from mangrove plants isolated by roots, stems, and leaves. From the total of isolates, nine isolates of endophytic bacteria are pathogenic: *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *P. mirabilis*, *Klebsiella*, *Pseudomonas flourescens*, *P. aeruginosa*, *Salmonella typhi*, and *Streptococcus pyogens*. The best activity is obtained from 8 isolates isolated from roots, stems, and leaves. This shows that *B. gymnorrhiza* proved to produce endophytic bacteria to protect itself from its extreme environmental conditions. According to Gayathri and Muralikrishnan (2013) that endophytic bacteria are capable of producing plant-derived compounds from biotic and abiotic stresses compared with rhizosphere bacteria.

Strobel and Daisy (2003) said that endophytic microbes can be found in almost all plants on earth, microbes growing in the plant tissues can be isolated from the roots, stems, and leaves. Endophytic bacteria enter the plant tissue generally through the roots, but parts of plants that exposed directly to the air such as stems and leaves (stomata) and cotyledons can also become endophytic bacterial entrance. Endophytic bacteria that enter the plant can grow only at one particular point or spread throughout the plant and the microorganisms live in the vascular or intercellular space, roots, stems, leaves, and fruit. (Zinniel *et al.*, 2002; Simarmata *et al.*, 2007; Bacon and Hinton, 2006).

Endophytic bacteria obtained from the isolation of mangroves plant type *B. gymnorrhiza* is a bacterium suspected of having a role against the defense of *B. gymnorrhiza* in facing extreme environmental conditions. According to Widyati (2013), plants have an instinct to optimize their own growth. Plants maximize their growth through root growth thus they have the maximum range of nutrients needed for growth. Endophytic bacteria isolated from the roots, stems, and leaves are bacteria that live on plant root cells that colonize and spread throughout other plant organs.

Screening the Endophytic Bacterial Isolates of *B. gymnorrhiza* as Antibacterial Producer

The screening is obtained from 18 isolates. The crude extract solution from the fermentation product on the antibacterial production medium shows that those 18 isolates have activities as antibacterial. The results show that the isolates have a potential to produce antibacterial compounds characterized by their inhibitory zone

formation in each of the test bacteria (Figure 2). To determine the extent of antibacterial activity of endophytic bacterial isolates, the screening antibacterial

activity is done against the test bacteria. The measurements of the inhibitory zone are presented in Table 2.

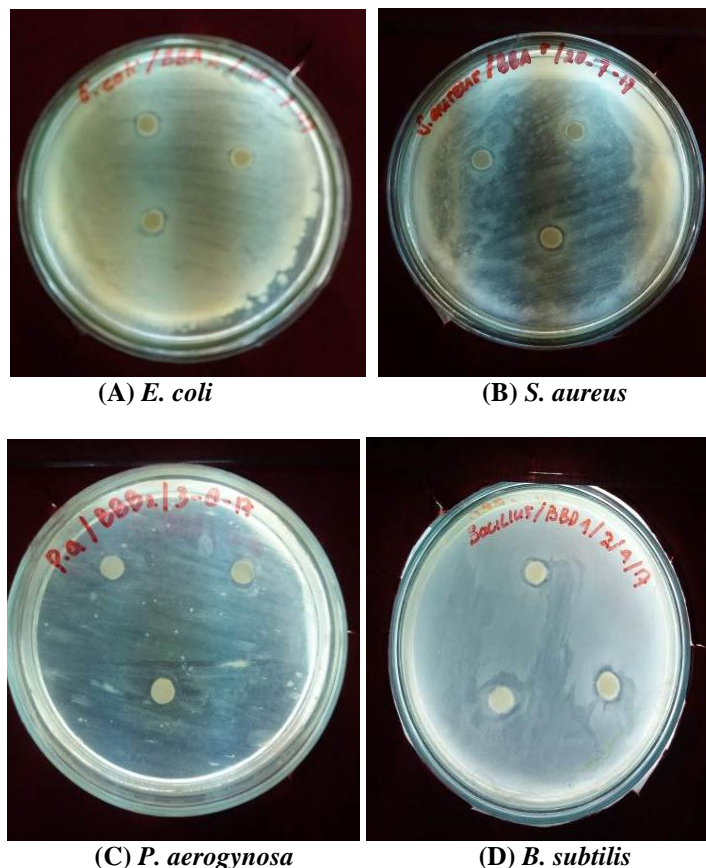


Figure 2: Antibacterial test of isolates EBR-2, EBS-2, and EBL-4 against test bacteria. (A) Potential Isolate EBR-2 against test bacteria *E. coli*. (B) Potential Isolate EBS -2 against test bacteria *P. aeruginosa*. (C) Potential Isolate EBR -2 against test bacteria *S. aureus*. (D) Potential Isolate EBL -4 against test bacteria *B. subtilis*.

Table 2: Inhibitory Zone Diameter Against Test Bacteria (mm).

Isolate Code	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>
EBR-1	15.66	9.33	10.00	11.66
EBR -2	21.80	8.33	23.10	13.66
EBR -3	10.00	9.33	15.00	13.66
EBR -4	15.00	10.00	11.60	13.33
EBR -5	12.30	8.00	12.00	10.00
EBS-1	9.33	9.66	14.00	10.66
EBS -2	8.66	10.66	10.00	11.66
EBS -3	9.00	8.33	11.50	10.66
EBS -4	12.00	8.66	9.33	10.33
EBS -5	9.00	8.33	9.33	9.33
EBS -6	8.66	8.66	9.33	9.33
EBL-1	9.33	9.66	13.30	9.33
EBL -2	11.66	10.33	13.30	12.83
EBL -3	8.00	8.33	10.60	12.66
EBL -4	13.33	7.66	10.00	30.00
EBL -5	9.50	7.33	15.10	11.66
EBL -6	9.33	9.66	13.30	9.33
EBL -7	8.00	8.33	13.30	9.33

EBR= Endophytic Bacteria Roots; EBS= Endophytic Bacteria Stems; EBL= Endophytic Bacteria Leaves

Table 2 shows the results of the inhibitory zone measurement produced by 18 endophytic bacterial isolates. The positive control used here is 50 mg/ml ampicillin solution (appendix 3) that generally produces larger inhibitory zones against *E. coli* (29 mm), *P. aeruginosa* (13 mm), *S. aureus* (44 mm) because ampicillin is a tested and standard antibiotic, while the antibacterial compound is still in the phase of its inhibitory activity against the test bacteria and has not been tested for antibiotics. In contrast to the test bacteria namely *B. subtilis* with positive control of inhibitory zone (13 mm), the endophytic bacterial isolate provides better activity than the positive control (30 mm). According to Phoanda *et al.*, (2014) this occurred due to several things such as; the concentration of antibacterial compounds produced is greater than the given positive control concentration; the uneven smearing of bacteria; and the possibility of antibacterial compounds produced by endophytic bacteria more effectively compared to positive control. The negative control used in this study was 0.85% physiological NaCl solution.

4.3 Characteristics of Endophytic Bacteria Isolates EBR-2

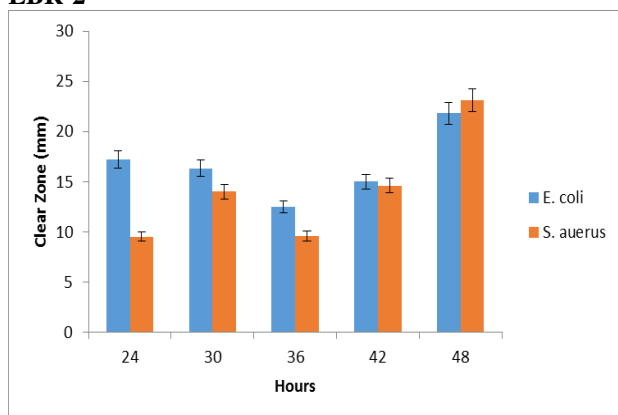


Fig. 1: Influence of Fermentation Time on Inhibitory Zone Formation of endophytic bacterial isolate against test bacteria.

Based on the Fig. 1, endophytic bacterial isolates show different antibacterial activity on both test bacteria after the different time of fermentation. The difference in fermentation time has an effect on the ability of endophytic bacterial isolates to produce antibacterial compounds. This study uses a maximum time of 48 hours fermentation, and the result shows the potential of endophytic bacterial isolates to restrain the test bacteria is very strong. The inhibitory zone obtained in *E. coli* is 21.80 mm while in *S. aureus* is 23.10 mm. The inhibitory zone produced by endophytic bacterial isolates against Gram-positive test bacteria is closely related to a cell wall character of bacteria. The cell wall of Gram-positive bacteria is simpler, only a single layer, thus it facilitates the antibacterial compound produced by endophytic bacterial isolates to penetrate the cell wall of the Gram-positive bacteria.

Djamaan *et al.* (2014) also reported that in the antimicrobial activity of endophytic microbes from bark, leaves, and fruit peels of *Garcinia mangostana L* against Gram-positive and Gram-negative test bacteria results in the best activity of its inhibitory zone against bacteria Gram-positive *S. aureus*.

4.4 Influence of Temperature on Growth of Potential Endophytic Bacteria Isolates EBR-2

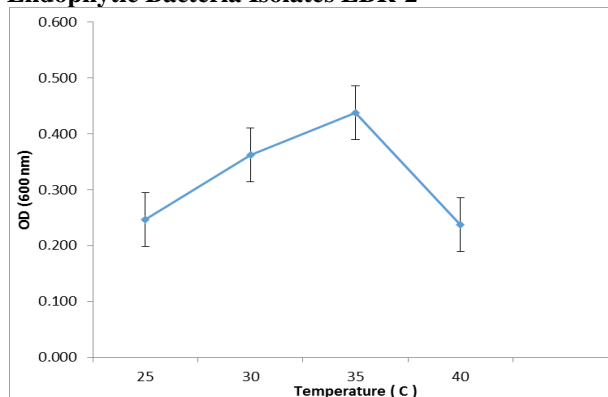


Fig 2: Effect of temperature then endophytic bacteria.

Figure 2 showed the growth of potential endophytic bacteria BEA-2 in some temperature treatments. The growth of BEA-2 is measured by opacity (Optical Density; OD). The measurement is made using Spectrophotometer (λ 600 nm). Chart 2 shows that optimal growth of BEA-2 at 35°C resulting absorbance of 0.438 nm with an OD value of 4.36. It can be seen from the bacterial growth curve, at a temperature of 35°C, the BEA-2 enter the logarithmic phase or exponential phase which means a rapid growth phase, characterized by increased activity causing increased turbidity in the medium. In this phase, availability of nutrients needed by bacteria is still available abundantly. Therefore, this causes the growth of BEA-2 also increases. So that, when the calculation with absorbance value becomes high, the turbidity of the medium increases. Also, the light passed becomes little and the OD value becomes small because the amount of light absorbed by the bacteria is quite high. At a temperature of 40°C, the isolate enters a stationary phase approaching the phase of death. It is characterized by reduced turbidity measured from the absorbance of 0.238 nm. This occurs because the endophytic bacterial isolates are not able to adapt to the temperature of 40°C thus the activity is reduced and the availability of nutrients is not utilized optimally. Based on the growth curve of bacteria, there are 4 phases, namely; lag phase is the phase where microbes lose metabolism and enzymes due to unfavorable conditions and there is a process of adjustment with the environment; exponential phase is when the amount of mass of bacterial cell increases because the nutrients are still available; stationery phase occurs when lack of nutrient or accumulation of toxic products so that growth stops; and decline or death phase is a drastic decrease after stationary period (Brooks *et al.*, 2005).

4.5 Influence of Salinity on Growth of Endophytic Bacteria Isolate EBR-2

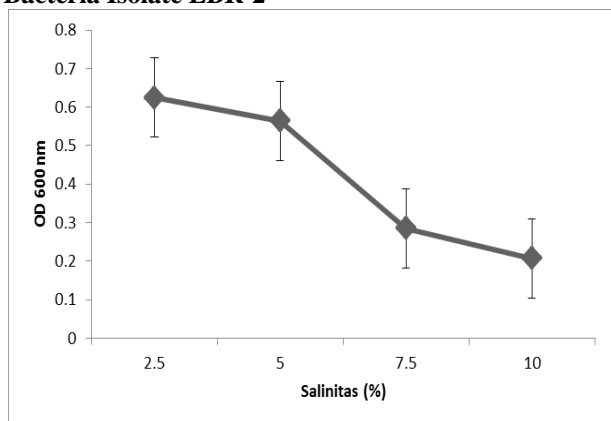


Fig. 3: Effect of salinity then of endophytic bacteria growth.

The most potential bacterial isolates are then tested for growth against salinity variation. Salinity levels used for endophytic bacterial isolates are 2.5%, 5.0%, 7.5%, and 10%. Based on Chart 3, the influence of salinity on growth of endophytic bacteria shows a high yield at the level of 2.5% resulting 0.625 nm of absorbance. From the chart, it is known that endophytic bacteria are halophilic. According to Madigan *et al.*, (2000), the halophilic bacteria are very easy to find in a saline environment. The salt content of halophilic bacteria habitat is from 2 to 30%. Arora *et al.*, (2014) also reported that in general, the growth of endophytic bacteria decreases with increasing NaCl concentration in the medium. Purwoko (2007), also added that bacterial growth can also be seen from the level of turbidity (medium turbidity). The murkier a culture medium, the more the number of cells. The light emitted on the spectrophotometer will hit the cell so that some of the light will be absorbed and partly passed on. The amount of light absorbed is proportional to the number of bacterial cells at certain limits.

4.6 Influence of pH on Growth of Endophytic Bacteria Isolate EBR-2

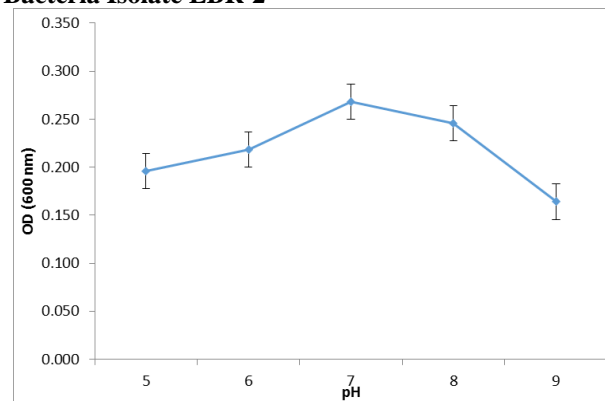


Fig 4: Effect of pH then endophyte bacteria growth.

The pH test is performed to determine the influence of pH on the growth of endophytic bacteria isolate. The pH levels tested are 5, 6, 7, 8, and 9. The best endophytic

bacterial growth at pH 7 resulting 0.268 nm of absorbance. From Chart 3, it is known that the pH concentration of media influences microbial growth because it will affect the proteins of enzymes and transport system of the cell membrane in microbes. The protein structure changes when the pH in the media also changes. Enzymes contained in microbes will function perfectly if the microbes are within a certain pH range. The increase of BEA-2 in pH 7 may show that endophytic bacterial isolates are able to grow optimally at pH 7.

According to Brooks *et al.*, (2005), most neutralophilic microorganisms grow well in the pH range of 6.0 - 8.0. To grow optimally, microbes need the right pH in their medium. In general, bacteria grow well in not-too-acidic and not-too-alkaline conditions. Pathogenic bacteria commonly grow at neutral pH (pH 7) or slightly alkaline (pH 7.4), this relates to the result obtained in this study, also pH levels in the field around the sampling site measured at pH 7.2.

4.7 Biochemical Test of Endophytic Bacteria Isolate

Table 3: Result of Biochemical test of three potential endophytic bacteria isolates.

Examination	Isolate Code		
	EBR-2	EBS-2/	EBL-4/
Motility	+	+/-	
Biochemistry			+/-
Glucose	+	+	
Lactose	+	-	-
Sucrose	+	+	-
Mannitol	+	+	-
TSIA	K/K	K/K	-
Gas	-	+	K/K
H-2S	-	-	-
Indol	-	-	-
Urea	-	-	-
Citrat	+	-	-
MR	+	+	-
VP	+	+	-
OF	+	+	+
KCN	+	+	-
Arginine	+	+	discontinued
Lysine	-	-	
Ornithin	+	+	
Phenylalanine	-	-	
Aesculin	-	-	
Arabinose	+	+	
Raffinose	+	+	
Sorbitol	+	+	
Xylose	+	+	
Dulcitol	-	-	
Nitrate			-
Gelatin	-	-	

(+) = positive test reaction; (-) = negative test reaction
 Further tests for isolate BED-4are not performed due to negative results on the OF test.

CONCLUSIONS

Based on the results of research, it can be concluded that:

1. There are 18 isolates of endophytic bacteria from mangrove plants *B. gymnorrhiza* have potential as the antibacterial producer and broad spectrum character, isolate BGA-2, BGB-2, and BGD-4 are the most potential isolates to restrain bacterial growth.
2. Based on the results of characterization and biochemical test, endophytic bacterial isolate BEA-2 and BEB-2 are Gram-negative bacteria, while BED-4 is Gram-positive, aerobic, motile, hemolytic, it gives a negative result in TSIA test and a positive result in VP test.

REFERENCES

1. Anulika P. N, Ignatius O. E, Raymond S. E, Osasera I. O, Abiola H. A., The Chemistry of Natural Product: Plant Secondary Metabolites. *International Journal of Technology Enhancements and Emerging Engineering Research*, 2016; 4(8): ISSN-2347-4289.
2. Arora S, Patel P. N, Vanza J. M, dan Rao G. G., Isolation and Characterization of Endophytic Bacteria Colonizing Halophyte And Other Salt Tolerant Plant Spesies From Coastal Gujarat. *frical Journal of microbiology research*, 2014; 8(17): 1777-1788.
3. Arunanchalam, Gayathri P., Studies On Bioprospecting Of Endophytic Bacteria From The Medical Plant Of *Andrographis paniculata* For Their Antimicrobial Activity And Antibiotic Susceptibility Pattern. *International Journal Of Current Pharmaceutical Research. PO And Departement Of Microbiology*. Sri Sankara Arts And Science College. India, 2010.
4. Gayathri P., Muralikrishnan V., Antibacterial Activity of Endophytic Bacteria Isolated From Mangrove Plant. *International Journal of Research In Pharmaceutical And Nano Sciences*, 2013.
5. Madigan T. M, dan Matinko, J. M., *Brock Biology of Microorganisms* 11th edition. Pearsons Prentice Hall, London, 2006.
6. Ruhe JJ., Monson T., Bradsher RW., Menon A., Use Of Long- lasting Tetracyclines For Methicillin-Resistant *Staphylococcus aureus* Infections: Case Series And Review Of The Literatur. *Clinical Infection Disesases Epub Review*, 2005; 40(10): 1429- 34.
7. Zhou H. W, Guo C. L, Wong Y. S, Tam N. F. Y., Genetic Diversity Of Oxygenase Genes In Polycyclic Aromatic Hydrocarbon-Degrading Bacteria Isolated From Mangrove Sediments. *FEMS Microbiology Letter*, 2006; 262: 148- 157.