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Isolation and characterization of DNA fragments locus *pyr*A (*ivi*I) *Salmonella typhimurium* and expression analysis of carbamoyl phosphate synthetase enzyme

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ABSTRACT

Typhoid fever is a disease caused by infection with gram-negative bacteria Salmonella typhi. iviI gene is one of the genes was found and has homology with carAB operon of E. coli K12. This gene locus contained in pyrA which serves to express the enzyme carbamoyl phosphate synthetase. The specific objective of this research the isolation and characterization of some genes iviI DNA locus pyrA by amplification of the desired part of the gene using the PCR method. The results of analysis of S. typhi, indicating homologous 829 bp and 59 bp do not homologue, which consists of 37 bp mutated transversions and 22 mutated transition. Analysis on carAB operon of E. coli K12 showed 756 and 132 bp homologous not undergo homologous consisting of: 80 bp mutated transitional and 52 mutated transversional. Analysis of amino acid sequence homology of 0.9 kb fragment (295 amino acids) of the amino acid sequence of S. typhimurium LT2 in GenBank showed that 228 amino acids homologous and 7 amino acids are not homologous. Analysis of the amino acid sequence of S. typhi in Genbank showed homologous 256 amino acids and 39 amino acids are not homologous. While the amino acid sequence of E. coli K12 in GenBank showed homologous 277 amino acids and 20 amino acids are not homologous.

Keywords: DNA locus, PyrA, *ivi*I, *S. typhimurium*, typhoid fever

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INTRODUCTION

Typhoid fever is a disease caused by infection with gram-negative bacteria, *Salmonella typhi*. These bacteria enter the human body through the food into the small intestine and into the bloodstream via the lymphatic system and multiply in the liver and gallbladder. To date about 600,000 deaths worldwide per year caused by typhoid fever [1-3]. The results showed that the incidence of typhoid occurred in Indonesia around 350-800 deaths per 100,000 population per year [2,4]. Still caused a high incidence of this disease is caused by a system of therapy and diagnosis that there has not been adequate.

Results of the research that has been done in an effort to deal with typhoid disease has yet to produce optimal therapeutic system. It is caused by a lack of information about the cause of the disease is mainly about the genes responsible for the pathogenicity of these bacteria.

Based on the findings ivi genes in *S. typhimurium* by Mekalanos group, the research team will conduct a study of ivi genes in bacteria *S. typhi* by conducting a study of homology to *ivi* genes in *S. typhimurium* [5]. This is done because both bacteria were in the same genus and symptoms of disease caused by both bacteria similar. The aim of this study was to isolate and characterize the genes of *ivi*I *S. typhimurium*. *ivi*I gene is one of the genes was found and has homology with carAB operon of *E. coli* K12. This gene locus contained in *pyr*A which serves to express the enzyme carbamoyl phosphate synthetase. Have previously performed gene amplification *ivi*I *S. typhimurium* LT2 using the primers CA1 and CA2 and obtained along the 0.6 kb DNA fragment (582 bp).

Amplification of the gene locus part *pyr*A *S. typhimurium* Mutton using CA3 and CA6 primer pairs were designed based on the DNA sequence of the *E. coli* operon *car*AB expected to apply throughout the 0.9 kb fragment. 0.9 kb fragment is an advanced piece of 0.6 kb fragment that had been found previously. Fragments of amplification product subsequently cloned using vectors T pMOS*Blue* and propagated in a host cell. The recombinant plasmid is then isolated from the resulting transformants. Fragments inserted determined restriction maps and the nucleotide sequence determined by Sanger dideoxy method and *Dye Terminator*. Furthermore, the study of homology to sequences of DNA sequencing results in GeneBank are: *S. typhimurium* LT2, *S. typhi*, and *E. coli* K-12.

MATERIALS AND METHODS

Preparation of template solution

Chromosomal DNA is used as template derived from a single colony of bacteria *S. typhimurium* and *E. coli* K12 (PCR positive control). Rejuvenated cells in media Luria Bertani (LB) solid (1% tryptone, 0.5 yeast extract, 1.5% bactoorder, and 1% NaCl). Then lysis in bacterial cells in 1.5 ml eppendorf tube which already contains lysis buffer (50 mM Tris-HCl pH 8.5; 1 mM EDTA pH 8.5; 0.5% Tween 20) and 100 mg/mL proteinase- K. Lysis process takes place by way of incubating the reaction mixture at a temperature of 65 °C using *cell culture incubator ST 5028* (Heraeus, Germany) for three hours. Furthermore, the solution was incubated at 95 °C for five min for inactivation of proteinase-K. Results incubation centrifuged at 12,075 x g for 1 min. The resulting supernatant was used as a marker template in the PCR process [6-8].

Amplification of DNA fragment of 0.9 kb *S. typhimurium*

The process of multiplication of the DNA fragment of 0.9 kb *S. typhimurium* conducted using *DNA Thermal Cycler PCR machine* (*Perkin Elmer*). PCR process was also conducted on the template DNA of *E. coli* as positive control. PCR reactions were performed in 0.5 mL eppendorf tube by inserting a 50 mL reaction mixture consisting of 10 mL lysis DNA template; 20 pmol of each primer CA3 and CA6; 1x PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2 and 50 mM KCl pH 8.3) (*Bohringer-Manheim*) and 1.25 units Tag DNA polymerase (Bohringer-Manhein). In each of the Eppendorf tube was added a drop of mineral oil to prevent evaporation during the PCR reaction, then spun and loaded into the machine DNA Thermal Cycler (Perkin Elmer). PCR process takes place within thirty cycles with each cycle consisting of denaturation process at a temperature of 94 °C for one min, annealing to the DNA template (annealing) at 52 °C for 60 sec, and phase of extension primary temperature of 72 °C for 60 sec [9-10].

Analysis of the results of PCR

PCR products were analyzed using 2% agarose gel electrophoresis, a mixture of 0.8 grams of agarose in 40 mL of 1x TAE buffer (40 mM Tris-HCl and 10 mM EDTA, pH 8.0). The reaction mixture was heated to boiling (agarose late) and then cooled to a temperature of 60 °C, pour into molds agarose gels that have a "comb" as forming the gel wells. In the gel wells that have been formed is then inserted 10 mL DNA solution were mixed with 1 mL of loading buffer (50% sucrose, 0.1% brom phenol blue pH 8.0, and 0.1 M EDTA) [11] as an indicator of DNA solution displacement distance. Electrophoresis was performed using a mini sub cell (BioRad) by 1x TAE buffer as the medium of instruction currents at a voltage of 70 volts for approximately 1.5 h. As a standard measure used DNA pUC19 DNA is cut using the enzyme *Hinf*I. To reveal the results of DNA electrophoresis, the gel was soaked in a solution of ethidium bromide (EtBr) 10 mg/mL for 3 min and viewed under UV light. To documentation then the gel was photographed using a Polaroid camera DS34 with the aid of UV light [11-14].

Cloning DNA fragments PCR product and sequencing

The process of cloning research aims to multiply the DNA fragment of 0.9 kb *S. typhimurium*. The process of cloning is done according to the procedures in vector-T pMOS*Blue* kit RPN 1719 (Amersham Life Science, 1994), combined with competent cell manufacturing procedures using Cohen [11,15]. Several stages of work in the cloning process, namely: the purification of the resulting solution PCR, ligation of DNA fragments PCR product to a vector-T *pMOSBlue*, transformation of *E. coli* host cells MOSBlue and isolation of recombinant plasmid. The next stage was sequenced using *Dye Terminator* includes several stages, namely preparation of a template, the PCR process, purification using sephadex G-50 column, electrophoresis, and readings electropherogram using DNAstar program.

RESULTS AND DISCUSSION

Amplification of 0.9 kb fragment of DNA *pyr*A locus *S. typhimurium*

0.9 kb DNA fragment amplified locus *pyr*A S. typhimurium was performed using primers CA3 and CA6. Both primer each containing 21 nucleotides and has a sequence of 5 '→ 3' as follows, for primer CA3: 5'-GGCAAAAGAAGTGACCACCGC-3' and CA6: 5'-CATTGAATCGGCTCGATGTAG-3'. CA3-CA6 primer pair designed based on the nucleotide sequence *car*AB operon of *E. coli* K12 in GenBank [16-17].

The result of amplification of the 0.9 kb fragments were analyzed by electrophoresis using 2% agarose gel. Electrophoresis results showed one band of 0.9 kb size were observed using a standard DNA pUC/*Hinf*I. Fragments of *S. typhimurium* lies parallel to the *E. coli* K12 DNA fragment size 0.9 kb. This shows that the possibility of a DNA fragment desired.

Analysis of PCR results with restriction enzymes

Analysis of chromosomal DNA fragments of 0.9 kb *S. typhimurium* performed using restriction enzymes to ensure that the fragment was part *pyr*A DNA locus of *S. typhimurium*. The analysis was conducted based on the nucleotide sequence *car*AB operon of *E. coli* K12 which is located between the primar CA3 and CA6, where this *car*AB operon homologous gene locus *ivi*I *pyr*A *S. typhimurium*. Analysis was performed using two kinds of enzymes, ie *Hinf*I and *Sau*3AI. Selection of enzymes is done with the aid of a computer program Genmol version 4.1 to 0.9 kb fragment of DNA of *E. coli* K12. *Hinf*I enzyme recognizes the sequence ↓GATC. ↓ mark indicates the cuts that are recognized by the enzyme [18].

At the 0.9 kb fragment of *E. coli*, the two sides recognize *Hinf*I cuts while cutting *Sau*3AI recognizes one side. Cutting using *Hinf*I yield 0.45 kb sized fragments; 0.3 kb; and 0.1 kb. The cutting results using *Sau*3AI fragment produced a band sized 0.45 kb. This fragment is actually composed of two fragments having a size relatively close to one another, which is 444 bp and 461 bp. Both fragments into one because it can not be separated by agarose gel.

0.9 kb DNA fragment in *S. typhimurium* cutted by enzyme *Hinf*I produces two fragments measuring about 0.3 and 0.1 kb. This suggests the existence of some of the fragments are united. Cutting using enzyme *Sau*3AI showed four fragment measuring about 0.8 kb; 0.5 kb; 0.3 kb; and 0.1 kb. 0.8 kb fragment most likely a fragment uncut perfect because the sum of the four fragments were more than 0.9 kb.

The analysis of the 0.9 kb fragment of *E. coli* K12 and *S. typhimurium* DNA locus *pyr*A cutting results with the enzyme *Sau*3AI showed a fragment measuring about 0.45 kb fragment while the other two are owned by the 0.9 kb fragment locus *pyr*A *S. typhimurium* is not owned by fragments of *E. coli* K12. The analysis of the 0.9 kb fragment of *E. coli* K12 and *S. typhimurium* locus *pyr*A cutted by enzyme *Hinf*I showed the same pattern, namely the fragment measuring 0.3 kb and 0.1 kb. However, size 0.5 kb fragment contained in the 0.9 kb fragment of E. coli K12 is not owned by a 0.9 kb fragment of DNA locus *pyr*A *S. typhimurium*. Different cutting patterns may be caused by the different cuts and fragments of the same pattern of allowing their DNA homology to the 0.9 kb fragment between both bacteria [17-19].

Cloning of fragment of the PCR product

Vector-T pMOS*Blue* containing insert DNA fragment of 0.9 kb *S. typhimurium* propagated *in vivo* in cells of *E. coli* transformants MOS*Blue* produce 399 colonies consisting of 337 white and 62 blue colonies. White colonies showed that cells containing plamid the 0.9 kb fragment was inserted by PCR results. Insertion occurs in the position of a gene *lac-Z*, a gene that encodes the enzyme β-galactosidase that can decipher the substrate similar to lactose ie X-gal (5-bromo-4-chloro-3-indoil-β-D-galaktopiranosa) into galactose and compound blue (5-bromo-4-kloroindigo). While the blue colonies showed that the vector-T pMOS*Blue* not contain the insert DNA in a gene *lac-Z*, causing the *lac-Z* product activity, the enzyme β-galactosidase. The presence of blue colonies is the case with two possibilities: first, in the kit still contains pMOS*Blue* were not cut by *Eco*RV, and secondly, vector pMOS*Blue* been cut with *Eco*RV not entirely plastered by nucleotide T at the 3'-end of its own so that it can undergo ligation on ligation process between the fragments with vectors.

Sequencing DNA fragment of 0.9 kb *S. typhimurium*

Sequencing was performed using the dideoxy method of Sanger and *Dye terminator*. The purpose of determining the nucleotide sequence of this is to ensure that the fragment was part *car*AB operon region *S. typhimurium* Mutton. Sequencing was performed on one of the colonies of the 0.9 kb fragment of DNA loci *pyr*A *S. typhimurium* indicate a nucleotide sequence throughout the 903 bp. This sequence is obtained through three times using three-primer sequencing reaction. This is done because *Dye terminator* sequencing method that is used only capable of reading the nucleotide sequence of 300-400 bp for one reaction. Primer used was M13, T7, and CA4. M13 and T7 primer

region recognize the cloning vector pMOS*Blue*, while CA4 primer recognizing the central part sequence DNA fragments of 0.9 kb *S. typhimurium*. Sequencing using three primer against 0.9 kb DNA fragment of *S. typhimurium* indicate that the fragment contains 903 bp. The sequence of nucleotides along the 903 base consists of 676 bases are part operon *car*A, 212 base part operon *car*B and 15 nucleotides is a nucleotide sequence which is in the region *intercistronic* ie between the termination codon (TAA) to the operons *car*A with the initiation codon (ATG) operon *car*B.

Analysis of sequencing results

Nucleotide sequence homology analysis of 903 bp DNA fragment of *S. typhimurium* Mutton PCR results are applied to both the nucleotide sequence of *S. typhimurium* LT2, *E. coli*, and *S. typhi* [12,17,19-20]. The study of homology fragment of 903 bp of the *S. typhimurium* LT2 showed that the 676 bp sequence of there carA operon has homologous 633 bp, 43 bp do not homologue consisting of 22 bp transitional mutation and 21 bp transversional mutation. In the nucleotide sequence of 212 bp *car*B are homologous and 5 bp do not homologue consisting of 4 bp mutated transitional and 1 bp mutation transversional.

The results of amino acid sequence homology analysis showed that on the *car*A operon there homologous 218 amino acids, 7 amino acids are not homologous, and the operon *car*B there are 70 amino acids homologous.

Table 1. Analysis of nucleotide sequence homology of 0.9 kb DNA locus *pyr*A strains of *S. typhimurium* Mutton with the data GenBank nucleotide sequence of *S. typhimurium* LT2: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (bp)	not homologous	Mutation type	
			transition	transversion
A. *S. typhimurium*	633	43	22	21
B. *S. typhimurium*	207	5	4	1

Table 2. Analysis of amino acid sequence homology of 0.9 kb DNA locus *pyr*A strains of *S. typhimurium* Mutton with the data GenBank amino acid sequence of *S. typhimurium* LT2: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (amino acid)	not homologous (amino acid)
A. *S. typhimurium*	218	7
B. *S. typhimurium*	70	-

Studies homology to the nucleotide and amino acid sequence between strains of *S. typhimurium* LT2 strain Mutton and does not show homology of 100%, but the results of the analysis showed some differences at both the DNA and amino acid. Differences above were then analyzed using Chou-Fasman plots. Nucleotide and amino acid differences are shown in Table 3 below.

Table 3. Differences in nucleotide and amino acid sequences operon of *S. typhimurium* strain Mutton *car*A against *S. typhimurium* LT2

Nucleotide differences	Differences in amino acids
GAA-ACT (284-286)	E(95) → T
AAG-CAG (293-295)	K(98) → Q
CAG-GAA (295-307)	Q(102) → E
GTG-CTA (320-321)	V(107) → L
AAG-GAG (650-652)	K(207) → E
CAC-CAA (653-655)	H(218) → Q
TCT-CAG (662-664)	S(221) → Q

Based on data from amino acid residue difference above, then performed a secondary structure analysis to determine the extent of the conformational differences polypeptide partly *S. typhimurium* operon carA Mutton against *S. typhimurium* LT2. The analysis was performed according to Chou-Fasman Cartoon using Protylize program version 1.0.

The results of homology analysis of the fragments of 0.9 kb *S. typhimurium* Mutton against sequence of nucleotides part *car*A operon of *E. coli* K12 showed that the homologous 556 bp, 120 bp are not homologous with about 72 bp mutated transitions and 48 pb mutated transversion. In the operon *car*B showed homologous 198 bp, 14 bp is not homologous, that there is 10 bp have mutations transition and 4 bp have transversion mutations.

While the analysis of amino acid sequence shows on the *car*A operon there homologous 205 amino acids and 20 amino acids are not homologous, at the *car*B showed 72 amino acids homologous.

Table 4. Analysis of nucleotide sequence homology of 0.9 kb DNA locus *pyr*A of *S. typhimurium* Mutton strains with the data GenBank nucleotide sequence of *S. typhimurium* K12: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (bp)	not homologous (bp)	transition (bp)	transversion (bp)
A. *S. typhimurium*	556	120	72	48
B. *S. typhimurium*	198	14	10	4

Table 5. Analysis of amino acid sequence homology of 0.9 kb DNA locus *pyr*A of *S. typhimurium* Mutton strains with the data GenBank amino acid sequence of *S. typhimurium* K12: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (amino acid)	not homologous (amino acid)
A. *S. typhimurium*	205	20
B. *S. typhimurium*	72	-

DNA sequence analysis of the 0.9 kb *S. typhimurium* Mutton on nucleotides sequence of portion the operon *car*A *S. typhi* showed that homologous 619 bp, 57 bp is not homologous which can be grouped into 20 bp have mutations transitions and 37 pb have mutations transversion. While the operon *car*B showed homologous 210 bp and 2 bp are not homologous (transversion).

Analysis of amino acid sequence homology of 0.9 kb fragment of *S. typhimurium* Mutton against *S. typhi* DNA part *car*A indicate homologous 187 amino acids and 38 amino acids are not homologous, whereas the section *car*B operon showed 69 amino acids and 1 amino acids are not homologous. In the future, the research can be developed on the analysis of mutations associated with nucleotide mutations associated with the disease in an individual, population, and other bacterial organisms [15, 21-32].

Table 6. Analysis of nucleotide sequence homology of 0.9 kb DNA locus *pyr*A of *S. typhimurium* Mutton strains with the data GenBank nucleotide sequence of *S. typhi*: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (bp)	not homologous (bp)	transition (bp)	transversion (bp)
A. *S. typhimurium*	619	57	20	37
B. *S. typhimurium*	210	2	2	-

Table 7. Analysis of amino acid sequence homology of 0.9 kb DNA locus *pyr*A of *S. typhimurium* Mutton strains with the data GenBank amino acid sequence of *S. typhi*: A. part operon *car*A; while the B are part operon *car*B

analysis of homology	homologous (amino acid)	not homologous (amino acid)
A. *S. typhimurium*	187	38
B. *S. typhimurium*	69	1

CONCLUSION

Restriction analysis of DNA fragments of 0.9 kb *S. typhimurium* (PCR product) using two restriction enzymes is *Hinf*I and *Sau*3AI showed banding pattern similar to fragments of DNA of *E. coli* K12. Sequencing DNA fragment of 0.9 kb *S. typhimurium* indicate that it contains 903 bp fragment consisting of 676 bp in the structural gene part *car*A, 212 bp gene structural parts *car*B and 15 bp in the area between the region of *intercistronic* and *car*B, and encoding 295 amino acids. Analysis of nucleotide sequence homology 0.9 bp fragment of the nucleotide sequence of *S. typhimurium* LT2 in GenBank showed homologous 840 bp, 48 bp do not homologue consisting of 21 bp have mutations transversion and 26 bp have transition mutations. The results of analysis of *S. typhi*, indicating homologous 829 bp and 59 bp do not homologue, yan consists of 37 bp have mutations transversions and 22 have mutations transition. Analysis on *car*AB operon of *E. coli* K12 showed 756 homologous and 132 bp not undergo homologous consisting of 80 bp have transitional mutations and 52 have mutations transversional. Analysis of amino acid sequence homology of 0.9 kb fragment (295 amino acids) of the amino acid sequence of *S. typhimurium* LT2 in GenBank showed that 228 amino acids homologous and 7 amino acids are not homologous. Analysis of the amino acid sequence of *S. typhi* in Genbank showed homologous 256 amino acids and 39 amino acids are not homologous. While the amino acid sequence of *E. coli* K12 in GenBank showed homologous 277 amino acids and 20 amino acids are not homologous.

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REFERENCES

[1] Amersham Life Science. Amersham internasional Plc., England. **2005**.
[2] Bhriain, N.N. and Dorman, J. C. *Plenum Press*, New York and London, **1998**. 51-61
[3] Buffery, C. Royal Soc. Chem., 3rd ed., Cambridge. **2001**.
[4] Bussey, B.L and Ingraham, J.L. *J. of Bacteriology*. **1999**.
[5] Cabello, F., Hormaeche, Mastroeni, P., and Bonina, L. *Plenum Press*, New York and London. **2001**. 51-60
[6] Glansdorff, N., Biosyntesis of Arginine and Polyamines, dari Neidhart, F.C. *Am Soc Microbiol*. **1987**. 1: 321-334
[7] Guster. GJ., Zeist. B.A.M. Biologi of *Salmonella*, Prenum Press, New York and London, , **1993**. 405-407
[8] Hackett, P.B., Fuchs, J.A., and Messing, J.W. The Benjamin/Cummings Publishing Company, Inc. 2nd ed. **1988**. California
[9] Innis, M.A. and Gelfand, H. Academic Press, Inc., California. **1990**.
[10] Ivanoff, B., Pasteur Merieux Connaught Rhone – Poulenc Group. **1997**. 5
[11] Kilstrup, M., Liu, C.D., Abdelal, A.H.T and Neuhard, J. *Euro. J. Biochem*. **1988**. 176: 421-429.
[12] Liu, S.L., Wong, K.K., McCleland, and Sanderson, K.E., **1993**, Construction of Physical Map of the Genome of *Salmonella typhimurium*, dalam Cabello, F, *et al*., Biology of *Salmonella*, Prenum Press, New York and London, pp 41-50
[13] Mahan, M.J., Slauch, J.M, and Mekalanos, J.J. *Science*. **1993**. 259: 686-688
[14] Mekalanos, J.J. Minireview, J. of bacteriology. **1992**.
[15] Narain, H. P. Pasteur Merieux Connaught Rhone-Poulenc Group, **2001**. 9
[16] Newton, C.R. and Graham A. BIOS Scientific Publisher, Oxford. **1994**.
[17] Noer, S., dan Rahmayanti, Y. *Proceeding Joint Seminar on Chemistry UKM-ITB*, Malaysia. **1997**.
[18] Promega. Wizzard Genomic DNA Purification Kit, Promega corp. Medison, USA. **1996**.
[19] Pelczar, M.J.Jr., and Chan, E.C.S. 5th ed., McGraw Hill., Singapore. **1986**.
[20] Ubyaan R, Maryuni AE, Sroyer A, Palit EIY, Jukwati, Suaka IY, and Ngili Y. *Int J PharmTech Res*. **2012**. 4(4): 1803-1811.
[21] Tanjung RHR and Ngili Y. *Der Pharma Chemica*. **2016**. 8(6): 165-173.
[22] Bolly, H. M. B., and Ngili, Y. *Der Pharma Chemica*. **2016**. 8(14): 19-24.
[23] Tanjung R.H.R and Ngili, Y. *Int J PharmTech Res.* **2016**. 9(5): 334-341.
[24] Awaludin, K., Lantang, D., and Ngili, Y. *Der Pharma Chemica*. **2016**. 8(10): 187-191.
[25] Siallagan J, Maryuni A, Jukwati, and Ngili Y. *Der Pharma Chemica*. **2015**. 7(9): 334-339.
[26] Bolly, H.M.B and Ngili, Y. *Der Pharmacia Lettre*. **2016**. 8(12): 73-77.
[27] Kawulur, H. S. I., and Ngili, Y. *Der Pharmacia Lettre*. **2016**. 8(14): 85-91.
[28] Ngili Y, Ubyaan R, Palit E.I.Y., Bolly H.M.B., and Noer, A.S. *European J Sci Res*. **2012**. 71(1): 64-73
[29] Ngili, Y., Noer, A.S., Ahmad, A.S., Syukriani, Y.F., Natalia, D., and Syah, Y.M. *Int J ChemTech Res*. **2012**. 4(2): 720-728.
[30] Ngili, Y., Palit., E.I.Y., Bolly, H.M.B., and Ubyaan, R. *J. Appl Sci* Res. **2012**. 8(4): 2232-2240.
[31] Ngili, Y., Bolly, H.M.B., Ubyaan, R., Jukwati., and Palit, E.I.Y. *Austr J Bas Appl Sci*. **2012**. 6(6): 111-118.
[32] Siallagan, J., Maryuni A., Jukwati., Tanjung, R.H.R., and Ngili, Y. *Der Pharmacia Lettre*. **2016**. 8(14): 73-79.