Study on Antimicrobial activities of soil microorganisms from Pathein Area

Aye Khaing Mon[1]

Abstract

In the study of isolation of soil microorganisms, 20 different fungi, 20 different bacteria and 4 actinobacteria were isolated from ten different soil samples in Pathein Area. In the study of antimicrobial activities of forty four isolated soil microorganisms, four fungi (AA-04, AA-08, AA-13 and AA-15) showed the antibacterial activity against *Agrobacterium tumefaciens* and three bacteria (KK-02, KK-10 and KK-17) against *Staphylococcus aureus*. Among them, only bacteria KK-10 showed the highest antibacterial activity against *S. aureus*. In the studies of time course of fermentation, it was observed that 100 ml volume of bacterium KK-10 started at 24 hrs and ended at 60 hrs. Based on the growth kinetics of bacteria KK-10, 60 hrs age and 2.5 % sizes of inoculum were optimized by using medium FM-1 for the production of metabolite against *S. aureus*. The maximum activity reached at 3 days fermentation (25.91 mm) on *S. aureus*.

Keywords: Soil microorganisms, Antimicrobial activities, Growth kinetics of bacteria

Introduction

Microorganisms are ubiquitous and morphologically diverse, and they have unique physiological and biochemical properties (Ferrman, 1993).The typical materials for microbial sources are soil, living and fallen leaves, leaf litters, dung, insect, fresh water and marine water. The soil sample is the most effective and popular materials for the isolation of microorganisms (Harayama and Isono, 2002).The discovery of antimicrobial agents that possess selective toxicity potential against cells remain an important scientific challenge. Microbial metabolites are biodegradable in nature and giving less stress to the ecosystems than synthetic ones (Phay, 1997). Antibiotics (metabolites) may be more useful than synthetics chemicals in the treatment and control of diseases. These metabolites are produced from microorganisms such as fungi, bacteria and actinomycetes (Kurtzman, 1992). Antibacterial and antifungal antibiotics produced by microorganisms still do not have the required quality in some cases to be used as safe and effective agents (Phay,1997). Although *Staphylococcus aureus* is an important pathogen, many healthy people carry it as part of the normal population of microorganisms associated with the nose, throat and skin. This pathogenic bacterium whose control is one of the most challenging problem in today clinical fields (Bremer *et a*l, 2004).

Materials and Methods

Isolation of microorganisms from soil samples

Ten different kinds of soil samples were utilized for the isolation of soil microorganisms. Sampling places collected samples for isolating soil microorganisms are as shown in field map (Figure 1). And sampling places (address, latitude and longitude), soil type and date were recorded in (Table 1). The isolation of soil microorganisms were undertaken by plating method (Ando

[1] Botany Department, Maubin University

and Inaba, 2004), physical chemical treatment dilution method (Hayakawa and Kobayashi, 2005) and feeding method (Phay and Yamamura, 2005).

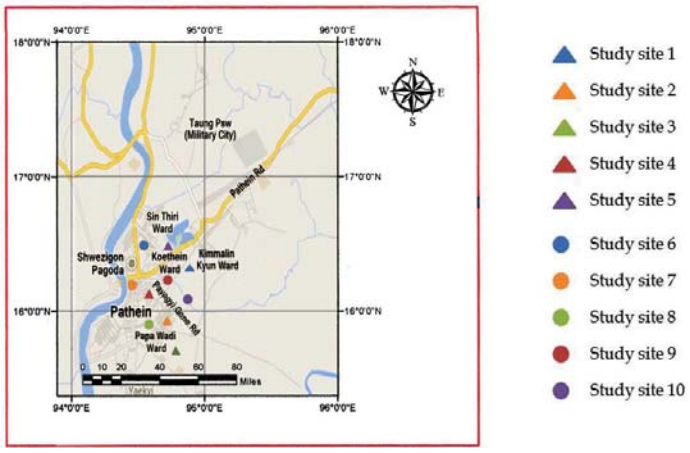


Figure 1 Field Map

Isolation Methods I

Soil Plating Method (Ando and Inaba, 2004)

1.Soil was air dried at room temperature.

2.Ground and sieved.

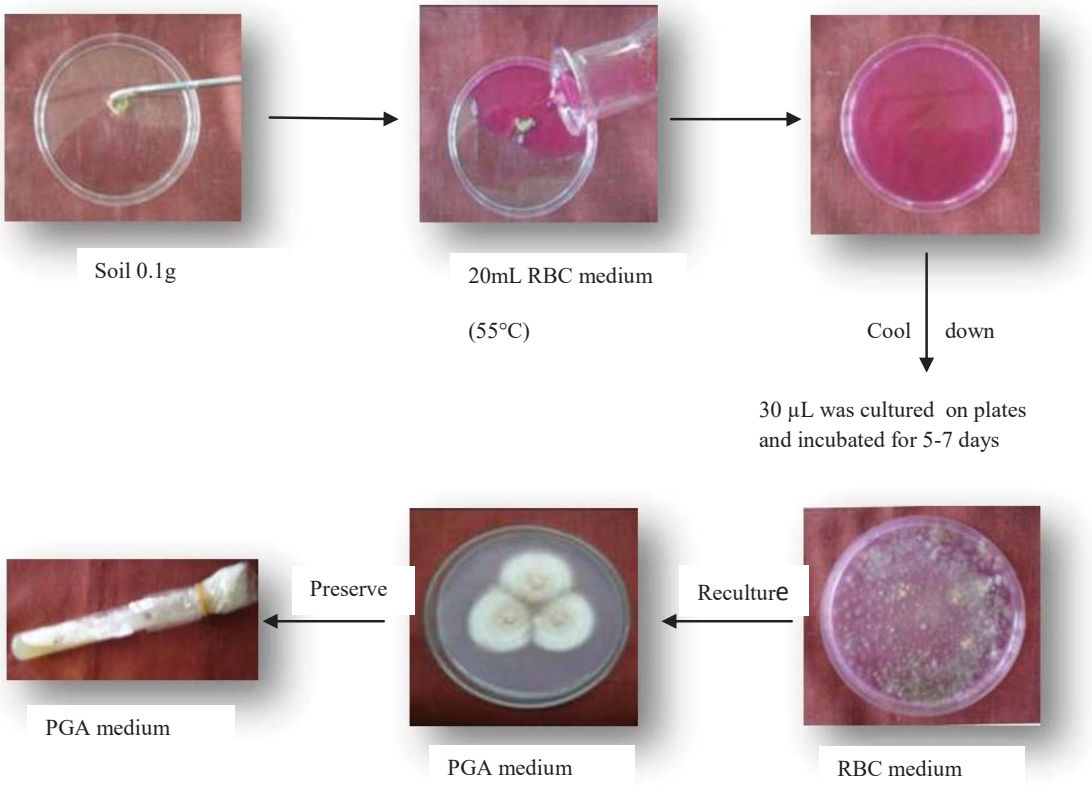


Figure 2 Soil Plating Method

Isolation Methods II

Physical and Chemical Treatment Dilution Method(Hayakawa and Kobayashi,2005)

1 . Air dried .

2. Ground and sieved, then heated at 100 °C for 1 hr.

3. 2g of the sieved soil sample was then put into test tube.

4. 4mL of sterilized distilled water was put into the tube containing soil, and settle for

6 hrs to germinate early- germinating soil fungi.

5. 14mL of 70% Ethanol solution was then added into the tube containing soil suspension, and shaken for 1 minute, and diluted with sterile water.

6. Cultured on RBC/SLNA medium and then incubated for 5- 10 days.

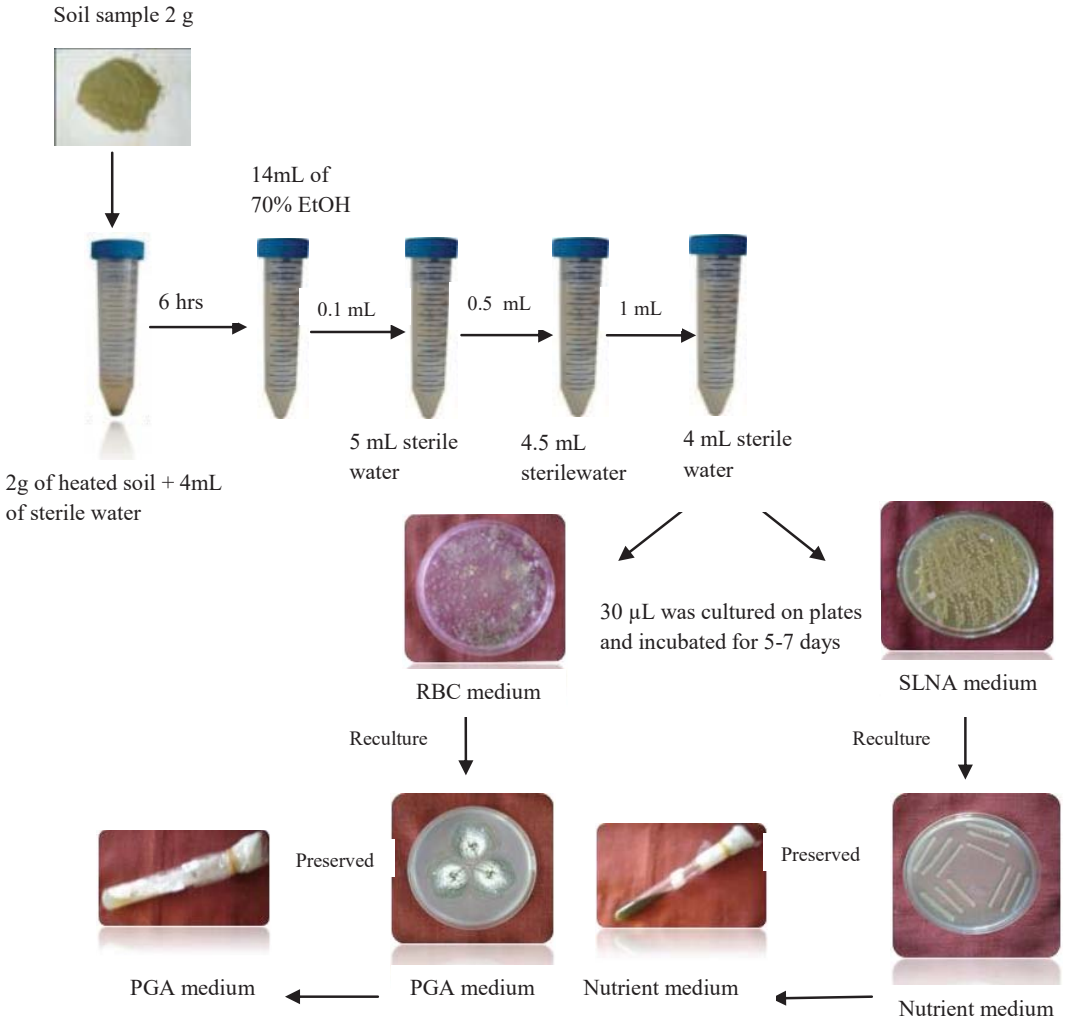


Figure 3 Physical and Chemical Treatment Dilution Method

Isolation Methods III
Feeding Method(Phay and Yamamura, 2005)

1.Soil sample (1.0 g) was poured onto 100 mL SLNA liquid culture medium with 25 µg chloramphenicol and GYR liquid culture medium with nystatin.

2.Then, it was incubated overnight and dilution was carried out.

3. 30 µL sample was cultured on plates containing in SLNA /GYR media and then incubated for 5- 10 days.

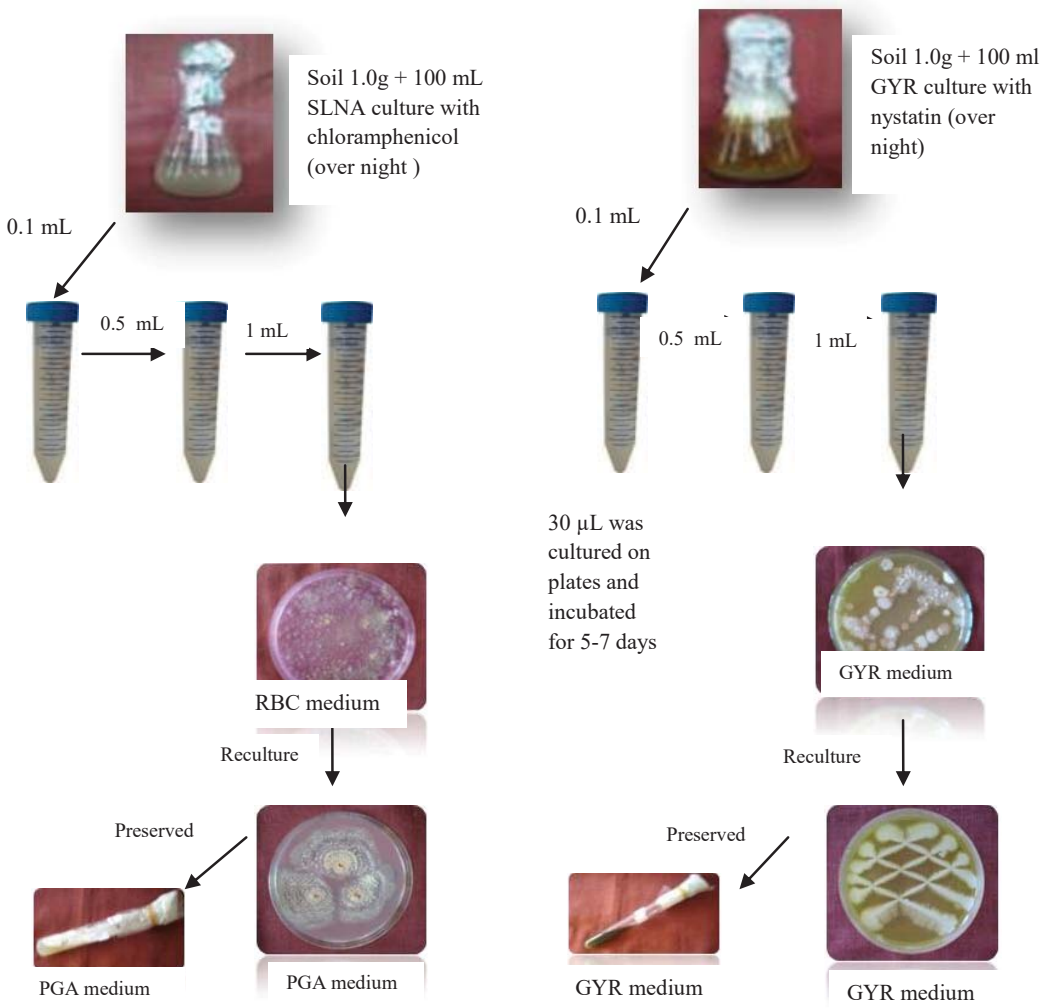


Figure 4 Feeding Method

Table 1 Isolated microorganism from ten different soil samples

Soil No.	Collected Sites	Soil pH	Isolation Methods					Isolated Microorganisms	Total No.
			I	II		III			
			Fungi	Fungi	Bacteria	Fungi	Actinobacteria		
1	PyinKadoo Kong Village	6.3	1	-	3	1	-	AA-01,02; KK -01,02,03	5
2	Saichan Village	3.5	-	-	2	1	-	AA-03; KK-04,05	3
3	Ngagyihto Village	6.2	1	-	2	1	-	AA-04,05; KK-06,07	4
4	Pathein Air Port	3.9	1	1	3	1	-	AA-06,07,08; KK-08,09,10	6
5	KanpatStreet	6.3	-	-	2	1	-	AA-09; KK-11,12	3
6	Myintzu Hostel (PatheinUniversity)	6.0	-	-	2	2	-	AA-10,11; KK-13,14	4
7	Saeyoetan Street	7.6	-	1	2	1	-	AA-12,13; KK-15,16	4
8	Phayargyikone	7.6	1	-	2	1	-	AA-14,15; KK-17,18	4
9	Kuthinaryone Ward	4.5	-	1	1	1	-	AA-16,17; KK,19	3
10	Shwewar Street	3.6	1	-	1	2	4	AA-18,19,20; KK-20 MM-01,02,03,04	8

Preliminary Study on Antimicrobial Activities by Paper Disc Diffusion Assay (NITE, 2005)

The isolated soil fungi were grown on Potato Glucose Agar (PGA) medium for 7 days at 25°C and inoculated into 10 mL of seed medium (Glucose 2.5 g, Yeast extract 0.8 g, $MgSO_4$ 0.2 g, K_2HPO_4 0.001 g and DW 100 mL, pH 6.5) and incubated for 3 days at 27°C. After incubation, the seed culture (5mL) was transferred to the 10mL of fermentation medium (Glucose 1.5 g, Yeast extract 0.6 g, Soluble starch 0.3 g, K_2HPO_4 0.001 g, $MgSO_4$ 0.2 g, and DW 100 mL, pH 6.5). The fermentation was carried out for 8 days by static culture. After the end of fermentation, the fermented broth (20 µL) was used to test the antimicrobial activity against test organisms by paper disc diffusion assay. The isolated soil bacteria were grown on Nutrient Agar medium for 3 days at 25°C. One loopful of

cultured bacteria were inoculated into 10mL of seed medium (Peptone 0.5 g, NaCL 0.5 g, Yeast Extract 0.2 g and DW 100mL, pH 7.0) and incubated for 3 days at 27°C. After incubation, the seed culture (5mL) was transferred to the 10ml of fermentation medium (Glucose 0.5 g, Yeast Extract 0.2 g, NaCL 0.5g and DW 100ml, pH 7.0) (Figure). The fermentation was carried out for 7 days by static culture.After the end of fermentation, the fermented broth (20 µL) was used to test the antimicrobial activity against test organisms by paper disc diffusion assay.The assay medium (Glucose 1.0 g, Polypepton 0.3 g, KNO_3 0.1 g, Agar 1.8 g, DW 100 mL, pH 7) was used for the antimicrobial activity test. These test organisms were inoculated into 25 mL of assay broth in conical flasks respectively and incubated overnight. Paper disc having eight millimeter diameter (Advantec, Toyo RoshiJaisha Co., Ltd., Japan) were utilized for antimicrobial assays. The fermented broth were impregnated and allowed to dry. One percent of test organism was added to assay medium, then poured into plates. After solidification, paper disc impregnated with fermented broth and applied on the assay agar plates. These plates were incubated for 24 – 36 hours at 27°C. The inhibitory zones surrounding the paper disc indicated the presence of bioactive compounds which inhibit the growth of test organisms. The antimicrobial activities were investigated using nine kinds of test organisms such as *Agrobacterium tumerfaciens, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens, Saccharomyces cerevisiae, Salmonella typhi, and Staphylococcus aureus*.

Results

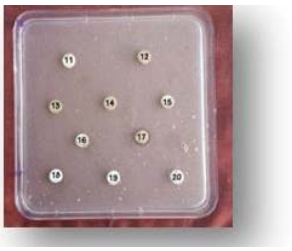
Fermentation Studies for the Production of Antibacterial Metabolites against *Staphylococcus aureus* (Omura, 1985; CruegerandCrueger, 1989)

According to the results of preliminary study on the antimicrobial activity, it was observed that isolated soil bacteria KK-10 showed the highest activity against *S.aureus*than others. Therefore, bacteria KK-10 was selected for fermentation optimization.

Fungi AA-04 (10.25 mm) and AA-08 (10.37 mm) showed activity against *A. tumeficiens*

Fungi AA-11 (13.35 mm) and AA-15 (14.03mm) showed activity against *A. tumeficiens*

Bacteria KK-02 (14.21 mm) and KK-10 (22.31 mm) showed activity against*S. aureus*



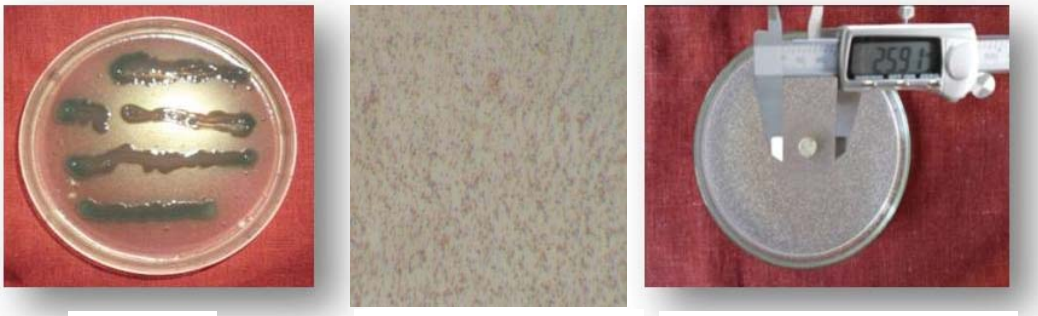
Bacteria KK-17 (11.52 mm) showed activity against *S. aureus*

Figure 5 The antimicrobial activities of isolated microorganisms.

Studies on Growth Kinetics of KK-10

Microbial growth kinetics of bacterium KK-10 was investigated by Omura, 1985; Crueger and Crueger. The bacterium KK-10 was inoculated into 100 mL liquid medium (Glucose 10.0 g, Glycerol 10.0 mL, Yeast extract 6.0 g, Peptone 4.0 g, $MgSO_4.7H_2O$ 0.01 g , K_2HPO_4 0.01 g, DW 1L, pH 7) and incubated for 84 hours. The culture sample was checked in 12 hrs intervals for the growth.

Antibacterial activity of bacteria KK-10 on *S. aureus*



KK-10 Photomicrograph (X600) Clear zone (25.91 mm)

Figure 6 Antibacterial activity of bacteria KK-10 on *S. aureus*

The Growth Kinetics of Bacterium KK-10

In the study of microbial growth kinetics of bacterium KK-10, it was observed that growth rate was between 48hrs to 84 hrs. It was considered that growth rate increased during this time (Figure 7).

Figure 7 The microbial growth of isolated bacteria KK-10

The Effects of Age of Inoculum for Fermentation

In the investigation of the effects of age of inoculum (48hrs, 54 hrs, 60hrs, 66 hrs, 72hrs, 78 hrs) for the fermentation, it was found that 60 hrs age of culture was the best inoculum according to the results of Table 2 and Figure 8. Therefore, 60 hrs age of culture was selected for the fermentation.

Table 2 The effects of age of inoculums against *S. aureus* for fermentation

Ages of inoculum (hrs)	Antibacterial Activity (Clear Zone, mm)
48	16.51
54	16.67
60	**25.02**
66	21.49
72	20.47
78	17.05

48 hrs (16.51 mm)

54 hrs (16.67 mm)

60hrs (25.02 mm)

66 hrs (21.49 mm)

72 hrs (20.47 mm)

78 hrs (17.05 mm)

Figure 8 The effect of age of inoculum against *S. aureus* for fermentation

It was found that size of inoculum (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%) utilized in this investigation exhibited the antibacterial activity against *S.aureus* as shown

in Table 3 and Figure 9. The size of inoculum 2.5% gave the highest activity than other size of inoculum.

Table 3 The effects of size of inoculum against *S. aureus* for fermentation

Sizes of inoculum (%)	Antibacterial Activity (Clear Zone, mm)
0.5	14.21
1.0	15.26
1.5	17.60
2.0	20.20
2.5	**25.91**
3.0	21.38

0.5% (14.21 mm) **1.0% (15.26 mm)** **1.5% (17.60 mm)**

2.0% (20.20 mm) **2.5% (25.91 mm)** **3.0% (21.38 mm)**

Figure 9 The effects of size of inoculum against *S. aureus* for fermentation

Discussion and conclusion

Hundreds of drugs available today were derived from chemicals first found in microbes. Scientists can use the amazing variety of chemical microbes naturally

produced to create new medicines. When the study of antimicrobial activities from soil microorganisms, there were twenty different fungi, twenty different bacteria and four different actinobacteria that were isolated from ten different soil samples in Pathein Area. These microorganisms were isolated by soil plating method, physical and chemical treatment dilution method and feeding methods. In this study, five fungi were isolated by soil plating method, three fungi and twenty bacteria isolated by physical and chemical treatment dilution method and twelve fungi and four actinobacteria isolated by feeding method . Isolation methods of microbes are classified into two categories: direct isolation and indirect isolation methods. According to Ando, *et al.*, 2004, "A soil dilution method is the typical indirect isolation method for isolating microbes in soil samples. Pretreatments of substrata for isolation microbes are also useful for the reliable isolation. If the media and techniques are changed the results may be different". The investigations of antimicrobial activities were done at least 5 times to get the results confirmation of the results. The fermentation was carried out with 60 hrs age and 2.5% size of inoculum using selected media for 3 to 8 days. The observation of pH and fermentation period for the production of metabolite at pH 6.8 was the best activity and the maximum activity reached at 3 days fermentation as 26.0 mm inhibitory zone against *S.aureus*. We ought to know that pH level high or low at fermentation conditions which need to adjust nearly their conditions to produce metabolites. Atlas, (1988) said that "Microorganisms vary in their pH tolerance range. Most bacteria are unable to grow at low pH values. Bacteria grow well over a range of pH 6-9". According to Omura and Cruger (1989), "The proper cultivation (ages) and transfer (sizes) of inoculum were essential for the production of both primary and secondary metabolites. The pre-culture (seed culture) media and culture conditions often have to be designed for optimal yields. Nutrient media for production also have to be optimized not only the ingredients used but also in how the nutrient medium were prepared". In the study of growth kinetics of bacteria KK-10, it was observed that growth phase of bacteria KK-10 was between 48 hrs and 84 hrs. Based on the growth kinetics of bacteria KK-10, 60 hrs ages of seed culture was the best inoculum for the fermentation and 2.5% sizes of inoculum was optimized for the production of the antibacterial metabolite. The antibiotics that help to protect plant roots also were used to treat infections in humans.

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