OPTIMUM PARAMETERS IN THE FERMENTATION OF ANTIMICROBIAL COMPOUNDS FROM THE FUNGUS *Stachybotrys* sp. AGAINST *AGROBACTERIUM TUMEFACIENS*

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Abstract

The fungus *Stachybotrys* sp. were studied, glucose as carbon source 2 %, 3 % and 4 % with yeast extract as nitrogen source 0.6 %, 0.7 % and 0.8 % were used to test on *Agrobacterium tumefaciens.* To optimize the fermentation medium, it was found that glucose 4 % with yeast extract 0.8 % yield the best result. There are seven different media in which 4 % glucose is combined with two nitrogen sources. There are also four different media in which 4 % glucose is combined with three different nitrogen sources. In the present study, different types of fermentation media were formulated by using 4 % glucose combined with different types of nitrogen sources and found out maximal antibacterial activity against *Agrobacterium tumefaciens*. In the investigation of ages and sizes of inoculum, 72 hrs ages and 20 % sizes of inoculum were optimized for fermentation. In the fermentation studies, the maximum activity reached at 72 hrs of fermentation for the production of antibacterial compound.

Keywords: fermentation, parameter of carbon and nitrogen ratio

Introduction

Fungi are primarily organisms that cannot synthesize their own food and are dependent on complex organic substances for carbon. Specialized fungi can be pathogenic on the tissues of plants, while others form mutually beneficial relationships with plants and assist in direct nutrient supply to the plants.

Fermentation procedures have to be developed for the cultivation of microorganisms under optimal conditions and for the production of desired metabolites or enzymes by the microorganisms. Several parameters which must be optimized are composition of ingredients, quality, carbon or nitrogen

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relationship, pH value before and after sterilization and changes in the sterilized nutrient solution before inoculation due to increase in temperature and aeration (Malek, 1984).

Media used in the cultivation of microorganisms must contain all elements in a form suitable for the synthesis of cell substances and for the production of metabolic products (Dale and Linden, 1984). Various aspects of microbial media such as carbon and nitrogen sources, minimal salts, trace elements, vitamins and pH have been reviewed (El-Tayeb *et al*., 2004; Rizk *et al*., 2007). Microbial growth kinetics is necessary to understand for the production of primary and secondary metabolites (Omura, 1985 and Crueger, 1989).

Physical factors such as incubation temperature can exert different effects on the growth and production phases of secondary metabolism. The purification of bioactive metabolites from fermented broth of microorganisms largely depends upon the physico-chemical properties of metabolite (Saxena *et al*., 2007).

The main objectives of present study is to investigate to find out pH, size of inoculum, incubation time, effects of carbon and nitrogen sources for the production of antibacterial metabolite.

Materials and Methods

Studies on the microbial growth kinetics of fungus (Omura, 1985, Crueger, 1989)

A loopful of isolated *Stachybotrys* sp. was inoculated into the seed medium (glucose 1.0 %, yeast extract 0.2 %, peptone 0.2 %, DW 100 mL) and incubated for 120 hrs at 100 rpm rotary shaker. The culture samples 5 mL was checked in 12 hr intervals for the growth. The sample 5mL was centrifuged at 2000 rpm for 30 mins and PCV % (Packed cell volume) was calculated.

Sample calculation example

Sample 5mL……..cell volume 0.5 mL

Sample 100mL……..0.5 x 100/5 = 10 %

Packed cell volume……... 10%

(Centrifugation at 2000 rpm/min)

Study on the effects of ages of seed culture on the fermentation (Crueger and Crueger, 1989)

The strain *Stachybotrys* sp. was inoculated into the medium, 48 hrs, 60 hrs, 72 hrs, 84 hr, 96 hrs and 108 hrs were employed with 15 % seed culture in 12 hrs intervals for the growth.

Study on the effects of sizes of seed culture on fermentation (Crueger and Crueger, 1989)

In the investigation of sizes of inoculums, 5 %, 10 %, 15 %, 20 % and 25 % of 72 hrs seed culture were utilized for the fermentation. Fermentation was carried out 5 days and antibacterial activity was tested by paper disc diffusion assay.

The effects of carbon and nitrogen sources on fermentation

In the investigation of carbon and nitrogen sources, the 1.5 % of each carbon source used in these studies glucose, sucrose, fructose, glycerol, glactose, tapioca powder and potato broth.

The 0.5 % 0f each nitrogen sources employed in this investigation were yeast extract, cornsteep liquor, KNO_3, peptone, fishcake and peanut cake. Antibacterial activity was examined at 12hrs intervals by paper disc diffusion assay. The choice of carbon source (glucose- 2 %, 3 % and 4 %) with nitrogen source (yeast extract- 0.6 %, 0.7 % and 0.8 %) were

incorporated into fermentation medium for detection of antibacterial activity against *A. tumefaciens* respectively.

Production of antibacterial compound by *Stachybotrys* sp. against *A. tumefaciens*

The fermentation was carried out for the production of antibacterial compound with 20% size of inoculum and 72 hrs age of culture for 120 hrs. The activity was checked 12 hrs interval using *A. tumefaciens*.

Determination of solvents for the extraction of antibacterial metabolite by bio-assay of paper chromatography (Tomita, 1998)

The filter paper and four solvents such as 20% NH_4Cl, *n-butanol* saturated with water, ethyl acetate - acetic acid - water (3:1:1) and ethyl acetate saturated with water were used for preliminary characterization of the compound. The obtained fermented broth samples (100 µL) were applied on the paper and allowed to dry. The chromatographic papers were tested in each solvent. Then, bioautography was done to check the antibacterial activity of each fermented broth. Each paper was placed on assay agar plate. After one hour the paper was taken out and then the plates were incubated for 24 hours. Then, the inhibitory zone was measured and R_f value for the corresponding metabolite was calculated.

Preliminary extraction of the solvent layer with organic solvents

According to the result of paper chromatography, the fermentation broth was mixed with *n-butanol* in the ratio of 12:4. The activity of organic layers obtained (first supernatant and lower sediment; second supernatant and lower sediment) were tested by paper disc diffusion assay.

Determination of solvents for the extraction of antibacterial metabolite by bio-assay of paper chromatography (Tomita, 1998)

The antibacterial activity of each extract was measured and R_f value for the corresponding metabolite was calculated.

Extraction of antibacterial metabolites adjusted pH with organic solvents (James, 1998)

The *n-butanol* layer adjusting pH 5-10 in the ratio of 12:4 (12- fermented broth and 4 - *n-butanol*). The *n-butanol* layer were tested the antibacterial activity compared with original fermented broth.

Results

Study on the microbial growth kinetics of fungus (Omura, 1985, Crueger, 1989)

In the studies of microbial growth kinetics, it was observed that growth phase of fungus *Stachybotrys* sp. between 48 hrs and 108 hrs. According to Omura, 1985 and Crueger and Crueger, 1989, ages of inoculum 48 hr, 60 hr, 72 hr, 84 hr, 96 hr and 108 hr were utilized for the optimization of fermentation as shown in Figure 1.

Studies on the ages and sizes of fungus against on *Agrobacterium tumefaciens*

It was found that growth phase between 48hrs and 108hrs. According to Crueger and Crueger (1989), ages of inoculums (48, 60, 72, 84, 96 and 108hrs) with 20% sizes of inoculum were utilized for the optimization of fermentation. According to the result of age of inoculum, 20% sizes of inoculums were optimized for the fermentation to produce the antibacterial metabolites.

Study on the effects of carbon and nitrogen sources of fungus *Stachybotrys* sp.

In the formation of fermentation medium, the best carbon source is 4 % glucose and in the case of nitrogen source 0.8 % yeast extract provided the highest antibacterial activity as shown in Table 1-8 and Figure 2-7.

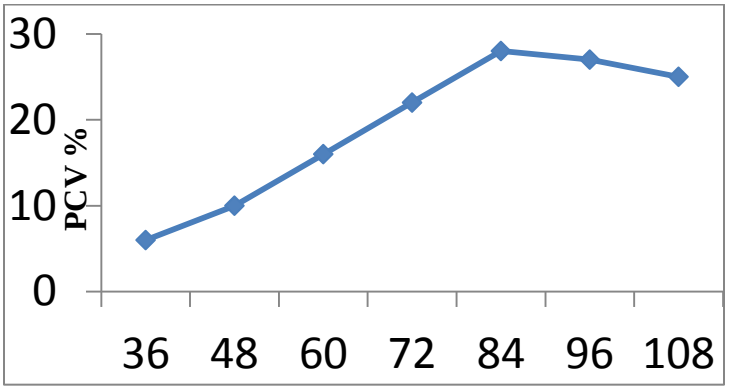


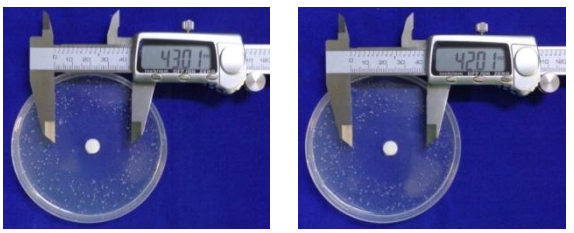
Figure 1. Microbial growth kinetics of fungus

Table 1. The effects of carbon sources on fermentation

No.	Carbon source	Activity (inhibitory zone, mm)
1	**Glucose**	**43.01**
2	Sucrose	42.57
3	Fructose	40.62
4	Glactose	36.83
5	Glycerol	39.01
6	Potato broth	38.00
7	Tapioca powder	39.01

Table 2. The effects of nitrogen sources on fermentation

No.	Nitrogen sources	Activity (inhibitory zone, mm)
1	KNO_3	40.34
2	**Yeast extract**	**42.01**
3	Corn steep	41.62
4	Peptone	39.04
5	Fishcake	37.13
6	Peanut cake	39.70

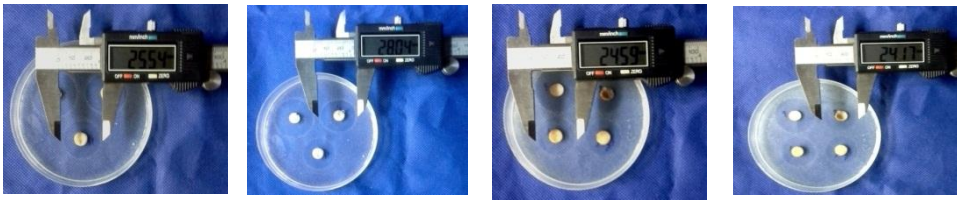


glucose (43.01 mm) Yeast extract (42.01 mm)

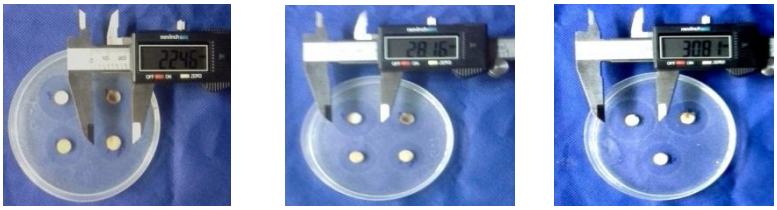
Figure 2. The effects of carbon and nitrogen sources on the antibacterial activity against *A.tumefaciens*

Table 3. The effects of different carbon and nitrogen sources on the antibacterial activity against *A. tumefaciens*

C and N source		Inhibitory zone (mm)
Glucose-2%	Yeast extract-0.6%	25.54
	Yeast extract-0.7%	28.04
	Yeast extract-0.8%	24.59
Glucose-3%	Yeast extract-0.6%	24.17
	Yeast extract-0.8%	22.46
Glucose-4%	Yeast extract-0.6%	28.16
	Yeast extract-0.8%	**30.81**



G-2%,Y-0.6% (25.54mm) | G-2%,Y-0.7% (28.04mm) | G-2%,Y-0.8% (24.59mm) | G-3%,Y-0.6% (24.17mm)



G-3%, Y-0.8% (22.46mm) | G-4%, Y-0.6% (28.16mm) | G-4%, Y-0.8% (30.81mm)

Figure 2. The effect of different carbon and nitrogen sources on the antibacterial activity against *Agrobacterium tumefacien*

Table 4. The effects of 4% glucose with two combination of different nitrogen sources on the antibacterial activity against *A. tumefaciens*

C and N source		Inhibitory zone(mm)
Glucose 4% per 100mL	Peptone, fishcake (Medium 1)	30.14
	Yeast extract, peanut cake (Medium 2)	24.91
	Yeast extract, cornsteep (Medium 3)	**34.73**
	Yeast extract, fishcake (Medium 4)	30.20
	Corn steep, peptone (Medium 5)	34.62
	Peanut cake, peptone (Medium 6)	33.41
	Yeast extract, peptone (Medium 7)	27.15

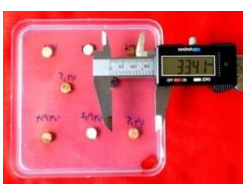
Medium 1(30.14mm)

Medium 2(24.91mm)

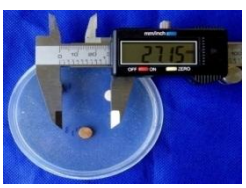
Medium 3(34.73mm)

Medium 4(30.20mm)

Medium 5(34.62mm)



Medium 6(33.41mm)

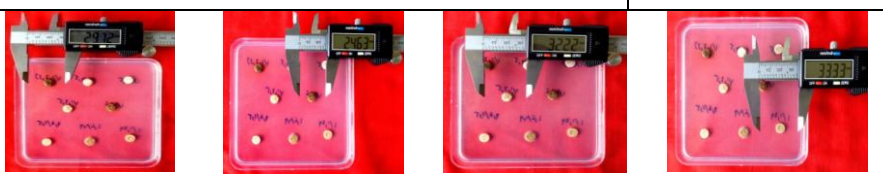


Medium 7(27.15mm)

Figure 3. The effects of different media with 4% glucose various combination of nitrogen sources on the antibacterial activity against *Agrobacterium tumefaciens*

Table 5. The effects of 4% glucose as carbon source and different combination of nitrogen sources on the antibacterial activity against *A. tumefaciens*

C and N source	Inhibitory zone (mm)
Medium 1(glucose with yeast, cornsteep and KNO_3)	29.12
Medium 2(glucose with yeast, cornsteep and fishcake)	24.63
Medium 3(glucose with yeast, cornsteep and peptone)	32.22
Medium 4 (glucose with yeast extract, cornsteep and peanutcake)	**33.33**



Medium 1 Medium 2 Medium 3 **Medium 4**

Figure 4. The effects of 4% glucose as carbon source and different combination of nitrogen sources on the antibacterial activity against *Agrobacterium tumefaciens*

Table 6. The effects of different carbon sources and combination of nitrogen sources on the antibacterial activity against *A. tumefaciens*

C and N ratio		Inhibitory zone (mm)
Glucose and Glycerol	**Yeast** and **cornsteep (Medium 1)**	**34.87**
	Yeast and **peanutcake (Medium 2)**	**34.87**
	Cornsteep and peptone (Medium 3)	30.95
	Peanutcake and peptone (Medium 4)	31.41
	Yeast and peptone (Medium 5)	30.34
	Yeast and fishcake (Medium 6)	31.69
	KNO_3 and peptone (Medium 7)	29.41




Medium1(34.87mm) Medium 2 (34.87mm) Medium 3(30.95mm) Medium 4(31.41mm)



Medium 5(31.69mm) Medium 6 (30.34mm) Medium 7(29.41mm)

Figure 5. The effects of different carbon sources and combination of nitrogen sources on the antibacterial activity against *Agrobacterium tumefaciens*

Table 7. The effects of glucose and potato broth combined with different nitrogen sources on the antibacterial activity against *A.tumefaciens*

C and N ratio		Inhibitory zone (mm)
Glucose and potato broth	KNO_3 and peptone (Medium 1)	31.62
	Yeast extract and fishcake (Medium 2)	29.12
	Yeast and peptone (Medium 3)	34.03
	Yeast extract and peanutcake(Medium 4)	34.88
	Yeast extract and **KNO_3 (Medium 5)**	**34.89**
	Peanutcake and peptone (Medium 6)	32.81

Table 8. The effects of Sucrose and potato broth combined with different nitrogen sources on the antibacterial activity against *A. tumefaciens*

C and N ratio		Inhibitory zone (mm)
Sucrose and potato broth	Yeast extract and fishcake (Medium 1)	25.54
	Peanutcake and peptone (Medium 2)	30.05
	Yeast extract and peanutcake(Medium 3)	29.62
	Yeast extract and **peptone (Medium 4)**	**31.63**

Medium 1 (31.62mm)

Medium 2 (29.12mm)

Medium 3 (34.03mm)

Medium 4 (34.88mm)

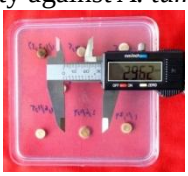
Medium 5 (34.89 mm)

Medium 6 (32.81mm)

Figure 6. The effects of glucose and potato broth combined with different nitrogen sources on the antibacterial activity against *A. tumefaciens*

Medium 1 (25.54mm)

Medium 2 (30.05mm)



Medium 3 (29.62mm)

Medium 4 (31.63mm)

Figure7. The effects of sucrose and potato broth combined with different nitrogen sources on the antibacterial activity against *Agrobacterium tumefaciens*

In the optimization of fermentation, the best carbon source glucose-4% was selected. The fermentation media such as glucose with yeast; glucose with yeast, corn steep liquor (Medium 3); glucose with yeast extract, corn steep liquor, peanut cake (Medium 4); glucose, glycerol with yeast extract, corn steep (Medium 1); glucose, potato broth with yeast extract, KNO_3 (Medium 5) and sucrose, potato broth with yeast extract and peptone (Medium 4) were found on the best antibacterial activities on *Agrobacterium tumefaciens* respectively. Among these media, the maximum result indicated by size of clear zone (34.89 mm) revealed in Medium 5 was selected for the further investigation.

Production of antibacterial compound by *Stachybotrys* sp. against *A. tumefaciens*

In the fermentation studies, the maximum activity reached at 72 hrs as shown in Table 9 and Figure 8.

Screening of solvent for the extraction of antibacterial metabolite by bio-assay of Paper chromatography

According to the R_f values, it was considered that solvent 2, *n-butanol* is suitable for the extraction of antibacterial compound. According to the R_f value, it was considered that antibacterial metabolite is soluble also in solvent 2, *n-butanol* and in solvent 4, ethyl acetate. However, solvent 2, *n-butanol* is more suitable for the extraction of antibacterial metabolite than solvent 4, ethyl acetate because of their apparent R_f value (0.9) solvent 2 than (0.7) solvent 4 as shown in Figure 9.

Preliminary extraction of the solvent layers with *n-butanol*

According to the activity of extraction of antibacterial metabolite, it was considered that the first supernatant with *n-butanol* is suitable for the extraction of antibacterial compound. However, the first sediment and the second supernatant extracted with *n-butanol* very little showed antibacterial

activity but the first supernatant and the second sediment resulted from *n-butanol* extraction showed distinct activity indicated by clear zone as shown in Figure 10. However, the first time upper layer was more suitable for the extraction of antibacterial metabolite than the second time lower layer with *n-butanol* as shown in Figure 10.

Extraction of antibacterial metabolite adjusted pH 7.0 with *n-butanol*

In the present work, the total volume of fermented broth (36 liters) with pH 6.0 was adjusted to pH 7.0 and extracted with *n-butanol* in the ratio of 12:4 (broth : *n-butanol*). They were concentrated *in vacuo* and kept in the chamber for further investigation by chromatography as shown in Table 10 and Figure 11and 12.

Table 9. Time course of fermentation for the production of antibacterial compound

Time course (hrs)	Inhibitory zone (mm)
36	+
48	38.45
60	39.67
72	42.00
84	41.17
96	40.54
108	39.04
120	38.36

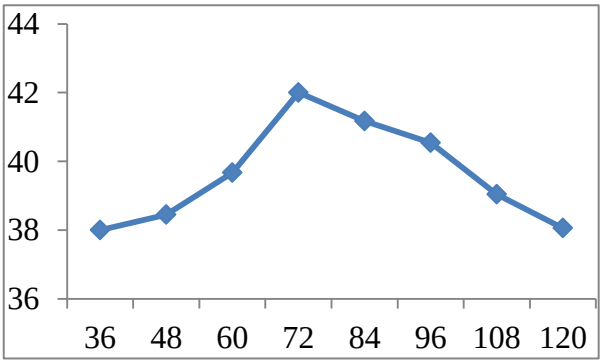
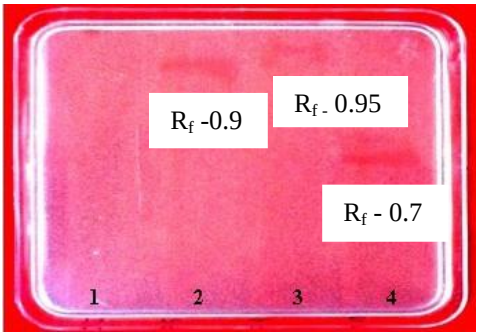


Figure 8. Time course of fermentation for the production of antibacterial compound



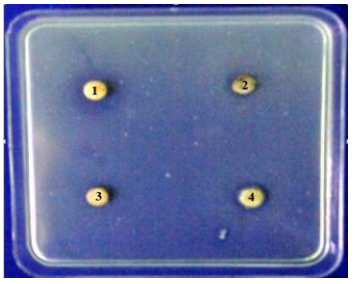
1- 20%NH_4Cl

2 - n- butanol saturated with water

3- ethyl acetate:acetic acid:water(3:1:1)

4- ethyl acetate saturated with

Figure 9. Paper chromatography Bioautographic overlay-assay



1- extract of first supernatant

2- extract of first sediment

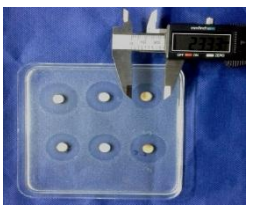
3- extract of second supernatant

4- extract of second sediment

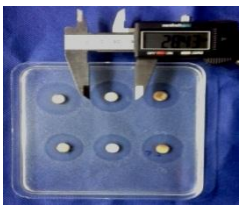
Figure 10. The effect of supernatant and sediment of *n-butanol* extraction on the antibacterial activity

Table 10. The effects of pH on the extraction with *n-butanol* on the antibacterial activity against *Agrobacterium tumefaciens*

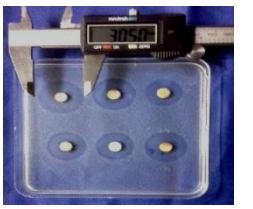
Adjusted pH	Inhibitory zone(mm)
5	23.33
6	28.43
7	**30.50**
8	28.05
9	25.34
10	25.20



pH 5 (23.33 mm)

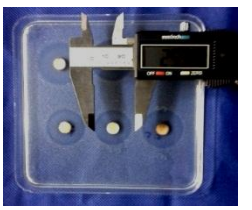


pH 6 (28.43 mm)

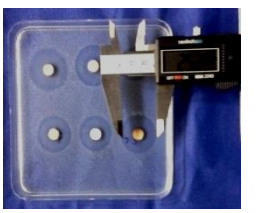


pH 7 (30.50 mm)

pH 8 (28.05 mm)



pH 9 (25.34 mm)



pH 10 (25.20 mm)

Figure 11. The effect of pH on the extraction of compound with *n-butanol* on the antibacterial activity against *Agrobacterium tumefaciens*

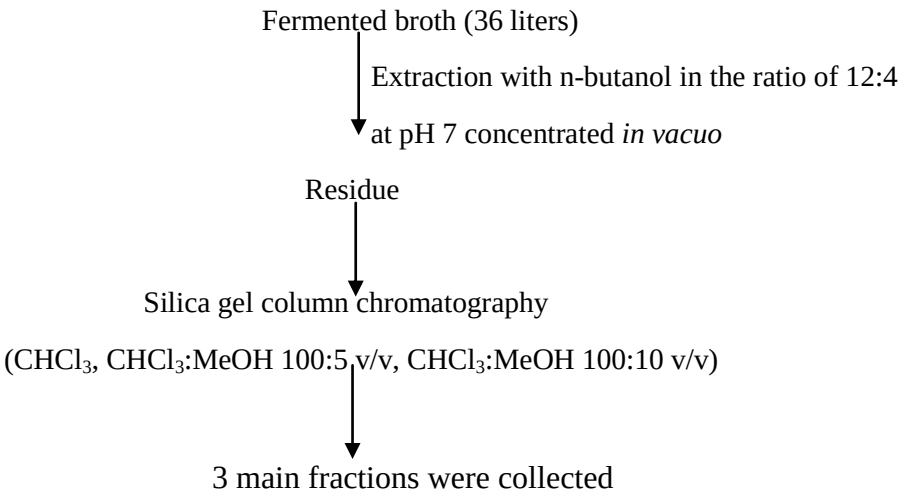


Figure 12. Flow chart of fermentation of medium 5 by using *Stachybotrys* sp.in optimal condition

Discussion and Conclusion

This fungus was observed that growth phase between 48 hrs and 108 hrs. It was observed that 20 % sizes and 72 hrs age were suitable for the production of antibacterial metabolites. In the studies of carbon and nitrogen sources utilization, glucose (43.01 mm, inhibitory zone) and yeast extract (42.01 mm, inhibitory zone) were the best for the production of antibacterial metabolites. In the investigation of carbon and nitrogen sources, carbon source (glucose- 2 %, 3 % and 4 %) with nitrogen source (yeast- 0.6 %, 0.7 % and 0.8 %) were used in the fermentation as antibacterial activity on *A. tumefaciens*. Subsequently, carbon source (No. 1 glucose and No. 4 glycerol) with six kinds of nitrogen sources were used in the fermentation tested on *Agrobacterium tumefaciens*. All fungi need organic carbon compounds as a source of energy and for biosynthesis. Most investigations concerning nitrogen metabolism in fungi, cannot fix atmospheric nitrogen. Most fungi can use nitrate as nitrogen source, but ammonium salts and a number of organic

nitrogen compounds ranging from urea and amino acids to proteins are also good nitrogen sources for many fungi (Anna-Lena Sunesson, 1995).

In the investigation of carbon and nitrogen sources, carbon source (glucose - 2 %, 3 % and 4 %) with nitrogen source (yeast- 0.6 %, 0.7 % and 0.8 %) were used in the fermentation of *Agrobacterium tumefaciens*. It was found that carbon source (glucose 4 %) with nitrogen source (yeast extract 0.8 %) the best inhibitory zone (30.18 mm) on *A. tumefaciens*. Therefore, carbon source, glucose 4 % was selected. Subsequently, carbon source (glucose 4 %) with two kinds of nitrogen sources and three kinds of nitrogen sources were takes placed in the fermentation of *Agrobacterium tumefaciens*. It was observed that carbon source, (glucose 4 %) with two kinds of nitrogen sources (Medium 3**,** yeast extract and cornsteep) the best inhibitory zone (34.73 mm) and carbon source, (glucose 4 %) with three kinds of nitrogen sources (Medium 4, yeast extract, cornsteep and peanutcake) the best inhibitory zone (33.33 mm) were occurred.

However, two kinds of carbon sources such as (No. 1 glucose and No. 4 glycerol), (No. 1 glucose and No. 6 potato broth) and (No. 2 sucrose and No. 6 potato broth) with two kinds of nitrogen sources were used in the fermentation tested *A. tumefaciens*.

In two kinds of carbon sources, (glucose – glycerol) with two kinds of nitrogen sources, **Medium 1** (glucose, glycerol with yeast extract, corn steep, inhibitory zone - 34.87 mm), in glucose – potato broth, **Medium 4** (glucose – potato broth with yeast extract and KNO_3, inhibitory zone -34.89 mm), and in sucrose – potato broth, **Medium 4** (sucrose – potato broth with yeast extract and peptone, inhibitory zone – 31.63 mm) were found on the best antibacterial activities against *A. tumefaciens.* According to the result, the effect of carbon and nitrogen sources utilization for the extraction of antibacterial metabolite was found in 5 days period and the maximum activity reached at 72 hrs.

The crude extracts were further investigated on the purification and identification by chromatographic method. Preliminary studies of paper chromatography are required to extract the compound from the fermented broth. The purpose of using paper chromatography is to know how to extract the bioactive compound from fermented broth by which solvent systems (Crueger and Crueger, 1989). In the study of paper chromatography, was carried out by four solvents such as 20 % NH_4Cl, *n-butanol* saturated with water, ethyl acetate -acetic acid -water (3:1:1) and ethyl acetate saturated with water were used for preliminary characterization of the compound. It was found that *n-butanol* is suitable for the extraction of the antibacterial metabolite. Then, preliminary extraction of the solvent was studied with *n-butanol* for the good extraction of the antibacterial compound. In this study, the extraction of the first supernatant was showed the best extraction of antibacterial metabolite.

Antibacterial metabolite extracted was adjusted to pH 5 - 10 with *n-butanol* in the ratio of 12:4 by using the method of James, 1998. The pH level of the growth medium has a marked effect on secondary metabolite production with synthesis fall rapidly either side of an optimal level. The hydrogen or hydroxyl ion concentration may have a direct effect on cell, or it may act indirectly by varying the degree of dissociation of substances in the medium. Therefore, the change of pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk *et al*., 2007). It was found that the best antibacterial metabolite was extracted with *n-butanol* in the ratio of 12:4 at pH 7.0. Thus, the antibacterial metabolite could be extracted with *n-butanol* at pH 7.0. Thus, in this research pH 7.0 is the best for the production of the antimicrobial metabolite by *Stachybotrys* (Jain and Pundir, 2011).

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