# FERMENTATION STUDIES OF *CEPHALOSPORIUM* SP. ISOLATED FROM *HESPERETHUSA CRENULATA* (ROXB.) ROEM.

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## Abstract

Endophytic fungal strain *Cephalosporium* sp. was isolated from the wood of *Hesperethusa crenulata* (Roxb.) Roem. For the fermentation conditions such as various carbon and nitrogen sources, culture media, age of inoculum, size of inloculum and pH utilization of this strain *Cephalosporium* sp. were conducted at Microbiology Lab, Department of Botany, University of Yangon. In utilization of carbon sources, honey and sucrose were good whereas yeast extract, meat extract and malt extract were the best nitrogen sources. In antimicrobial activity of various carbon sources, sucrose medium showed very high activity whereas various nitrogen sources, yeast extract medium indicated very high activity against eight test organisms. In the investigation of the morphological characters on various media, the seven cultural media were good media. According to the result of antimicrobial activity on various media, sucrose/yeast extract medium was the best for fermentation. In the study of inoculum optimization, two days old (age of inoculum) and 1.5% of seed culture at fifth day fermentation were suitable for the production of bioactive metabolites from this strain. In the study of pH utilization, pH 7 was the best for extraction of the bioactive compounds.

**Keywords:** *Cephalosporium* sp., Fermentation studies, *Hesperethusa crenulata*

## Introduction

Fermentation procedures have to be developed for the cultivation of microorganisms under optimal conditions and the production of desired metabolites or enzymes by microorganisms. A clear understanding of microbial growth kinetics is necessary if a large-scale process is to be properly managed. The physiological condition of the inoculum is crucial to the length of the lag phase. The proper cultivation and transfer of inoculums are essential for the production of both primary and secondary metabolites. The pure culture (seed culture) media and culture conditions often have to be designed for optimal yields. However, the kinetics of product formation is not necessarily correlated with the length of the lag phase (Yamane, 1984).

Seed culture must be made in order to have enough inoculum for a large fermenter. If a production fermenter is starter with too little inoculum, growth is delayed and the product formation rate can be unsatisfactory. The optimal inoculum concentration for the production determines the number of stages of seed culture that are required (Gaden, 1959).Optimal fermentation conditions such as proper kinetic growth (ages and sizes of inoculum) are very important for the production of metabolites (Omura, 1985).

Several media which must be optimized are composition of ingredients, quality, carbon and nitrogen relationship, impurities, variability from batch to batch, order of solution or suspension of ingredients, pH value before and after sterilization on the entire nutrient solution or on individual components, and changes in the sterilized nutrient solution before inoculation due to increase in temperature and aeration (Malek, 1984).

The objectives of this study are to study the utilization of carbon and nitrogen sources, to evaluate antimicrobial activity of *Cephalosporium* sp. on eight test organisms by using various carbon and nitrogen sources, to conduct the morphological characters and antimicrobial activity

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of *Cephalosporium* sp. on various media, to investigate the optimal fermentation studies such as age of inoculum, size of inoculum and pH utilization of *Cephalosporium* sp..

## Materials and Methods

### Utilization of carbon and nitrogen sources

In this study the morphological characters of *Cephalosporium* sp. were studied by using various carbon and nitrogen sources. Carbon sources are glycerol, glucose, sucrose, mannitol, honey and starch, whereas nitrogen sources are peptone, yeast extract, meat extract, malt extract, oat meal and soy bean. Basal media for carbon sources are yeast extract 0.3%, $K_2HPO_4$ 0.01%, $MgSO_4$ 0.01% and $CaCO_3$ 0.01% while basal media for nitrogen sources are glycerol 1.0%, $K_2HPO_4$ 0.01%, $MgSO_4$ 0.01% and $CaCO_3$ 0.01% (Monaghan, 1999).

### Antimicrobial activity of *Cephalosporium* sp. by using various carbon and nitrogen sources

Twelve 50mL flasks containing 25mL of various carbon and nitrogen sources were utilized. A small piece of fungal strain grown on slant culture was transferred into each flask. Twelve flasks were incubated on shaker at 180rpm for fourteen days. The fermented broth in each flask was used to check antimicrobial activity by paper disc diffusion assay (Monaghan, 1999; Phay, 1997).

### Morphological characters of *Cephalosporium* sp. on various media

In this study various media were employed for media optimization. A piece of fungus from plate culture of strain (*Cephalosporium* sp.) was inoculated in each of various media plates and incubated for 5-7 days. Various media were medium 1 **(**Polypeptone,Yeast medium), medium 2 (Meat, Polypeptone, NaCl medium), medium 3 (Yeast, Malt, Glucose medium), medium 4 (Glycerol, $K_2HPO_4$,$MgSO_4$, NaCl medium), medium 5 (Oat meal medium), medium 6 (Glycerol, $K_2HPO_4$ medium), medium 7 (Soybean, Mannitol medium), medium 8 ($K_2HPO_4$,$MgSO_4$, NaCl medium), medium 9 (Sucrose, Yeast extract medium), medium 10 (Malt, Meat extract medium), medium 11 (Sucrose, Malt extract, Soluble starch medium) and medium 12 (Honey medium) (Dubey & Maheshwari, 2009).

### Antimicrobial activity of *Cephalosporium* sp. by using various media

A fungal piece from plate culture of strain (*Cephalosporium* sp.) was inoculated into each of twelve (50mL) conical flasks containing 25mL of various fermentation media. These flasks were incubated at 180rpm at room temperature for two days. After two days, these fermented broths were checked for their inhibitory activity by paper disc diffusion assay (Phay, 1997).

### Age of inoculum for *Cephalosporium* sp.

One day old, two days old and three days old of seed culture were transferred into 250ml fermentation flasks containing 100mL of sucrose/yeast extract medium respectively. They were incubated for eight days. Then, these fermented broths were checked for their inhibitory activity by paper disc diffusion assay (Monaghan, 1999).

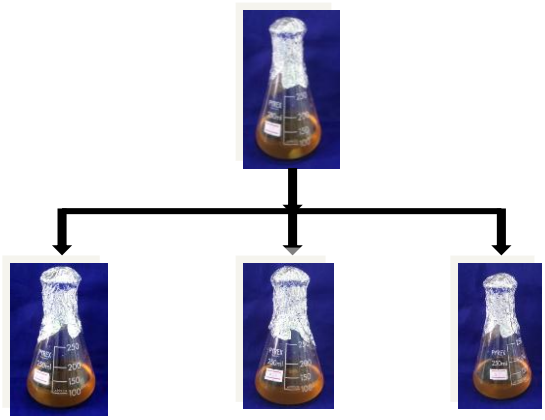


**Figure 1** Fermentation flasks for age of inoculum

**Size of inoculum for *Cephalosporium* sp.**

The proper cultivation and transfer (size of inoculum) are essential for the production of bioactive metabolites. A piece from fungal plate culture of strain *Cephalosporium* sp. was inoculated into 250mL of conical flasks containing 100mL of SY seed medium. The flasks were incubated at 30$^{o}$C for two days. After two days, the seed cultures (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) were transferred into the seven conical flasks (250mL) containing 100mL of fermentation medium. The fermentation was carried out for ten days (Monaghan, 1999).

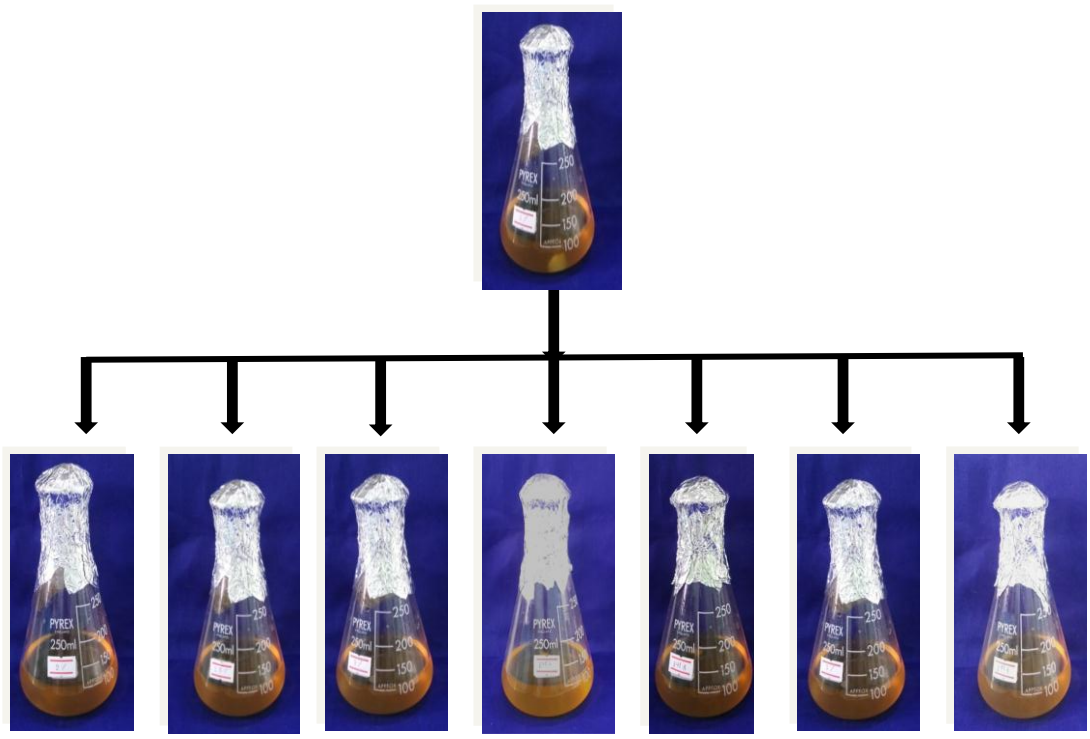


**Figure 2** Fermentation flasks for size of inoculum

**pH utilization for *Cephalosporium* sp.**

For the seed culture, a piece from fungal plate culture of strain (*Cephalosporium* sp.) was inoculated into 250mL of conical flask containing 100mL of SY medium and then the flasks were incubated at room temperature for two days. The seven 300mL conical flasks containing 100mL fermentation medium in each were adjusted at pH 4, 5, 6, 7, 8, 9, 10 and autoclaved. After two days, the seed culture (1.5%) was transferred to each fermentation flask with pH 4 to 10 and fermentation was carried out for 2 days. After 2 days, these flasks were checked antimicrobial activity (Monaghan, 1999).

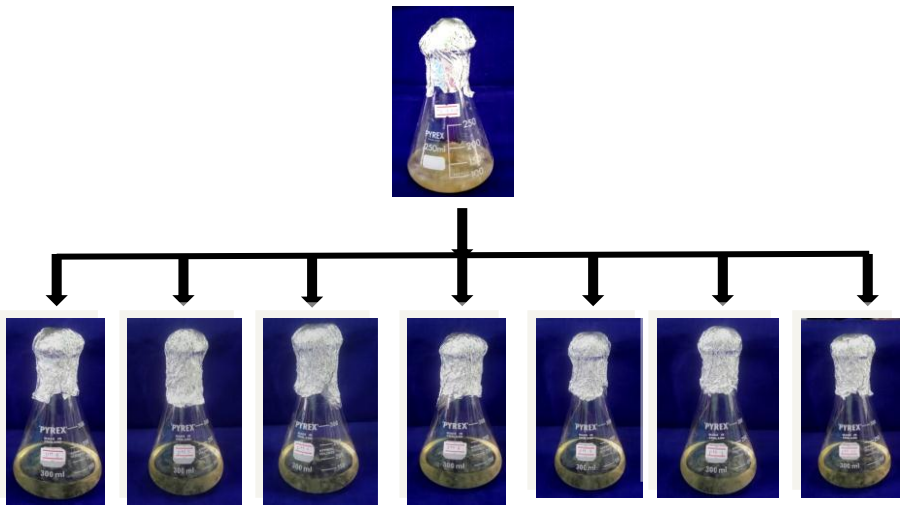
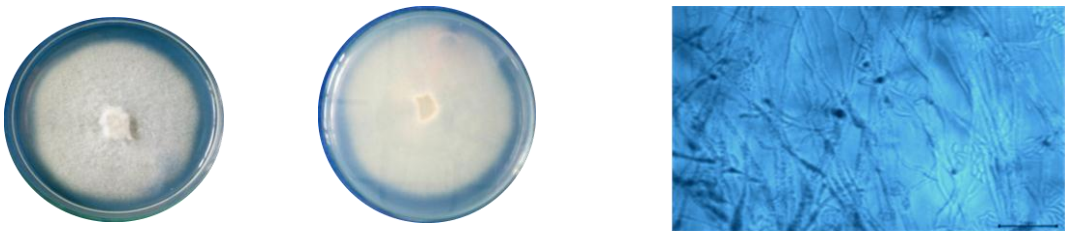


**Figure 3** Fermentation flasks for pH utilization

## Results

**Morphological and microscopic characters of isolated strain *Cephalosporium* sp.**

The surface color and reverse color of *Cephalosporium* sp. was the same color white. It shows conidia (phialospores) hyaline, 1 celled, septate hyphae which are typically very fine and narrow. They usually appear in clusters, in balls or rarely as fragile chains. It is identified as *Cephalosporium* sp. as shown in Figure 4.



Surface view Reverse view

**Figure 4** Morphological and microscopic characters of *Cephalosporium* sp.

**Carbon utilization**

Among carbon sources, honey medium was the best carbon sources whereas soluble starch medium was also suitable for fermentation as shown in Table 1 and Figures 5.

**Table 1 Morphological characters of *Cephalosporium* sp. on various carbon sources**

| No. | Carbon sources | Growth | Surface color | Reverse color |
|---|---|---|---|---|
| C1 | Glucose | Poor | White | White |
| C2 | Sucrose | Poor | White | White |
| C3 | Soluble starch | Moderate | White | White |
| C4 | Glycerol | Poor | White | White |
| C5 | Mannitol | Poor | White | White |
| C6 | Honey | Good | White | White |


**Figure 5** Morphological characters of *Cephalosporium* sp. on various carbon sources

**Nitrogen utilization**

In nitrogen sources, meat extract, malt extract and yeast extract media were the best nitrogen sources as shown in Table 2 and Figure 6.

**Table 2** Morphological characters of *Cephalosporium* sp. on various nitrogen sources

| No. | Nitrogen sources | Growth | Surface color | Reverse color |
|---|---|---|---|---|
| N1 | Peptone | Poor | White | White |
| N2 | Oat meal | Poor | White | White |
| N3 | Meat extract | Good | White | White |
| N4 | Malt extract | Good | White | White |
| N5 | Yeast extract | Good | White | White |
| N6 | Soybean | Poor | White | White |

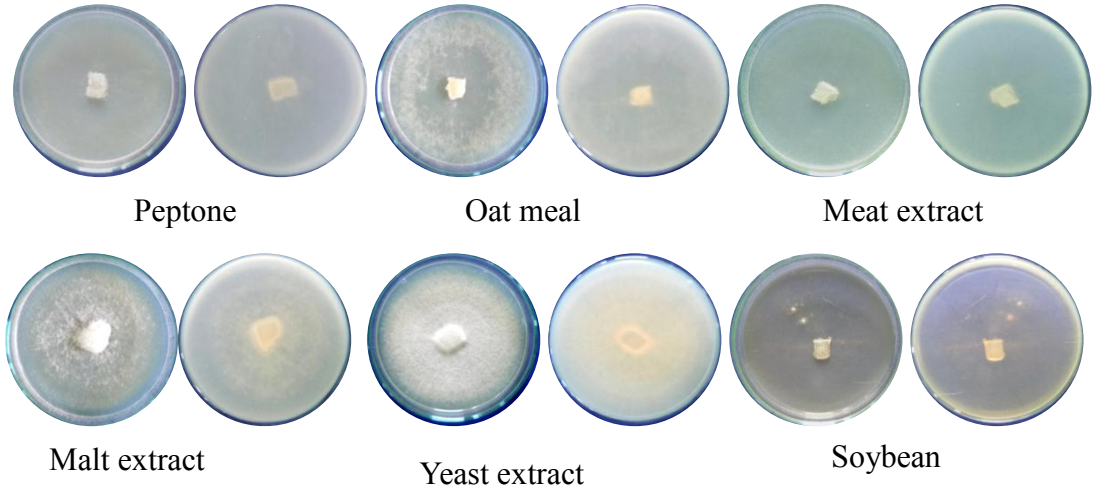



**Figure 6** Morphological characters of *Cephalosporium* sp. on various nitrogen sources

**Antimicrobial activity of *Cephalosporium* sp. on carbon sources and nitrogen sources**

Fermented broths of *Cephalosporium* sp. in four carbon media C1, 2, 3 and 6 showed very high antimicrobial activity on eight test organisms from 2 to 7 days. Fermented broth of *Cephalosporium* sp. in mannitol medium exhibited weak activity on some test organisms from 2 to 5 days while fermented broth of *Cephalosporium* sp. in glycerol medium was also indicated weak activity on *Agrobacterium tumefaciens* and *Aspergillus flavus* on 4$^{th}$ and 5$^{th}$ days. Fermented broths of *Cephalosporium* sp. in four nitrogen media N1, 2, 4 and 5 exhibited very high activity on eight test organisms from 2 to 8 days. Fermented broth of *Cephalosporium* sp. in nitrogen medium (meat extract) indicated weak activity on eight test organisms from 2 to 6 days, but soybean medium did not show any activity as shown in Table 4 & 5 and Figure 7.

**Table 3** Inhibitory zones of fermented broths of *Cephalosporium* sp. on various carbon sources at 7$^{th}$ day fermentation

| Carbon sources<br>T.O | C 1 | C 2 | C 3 | C 6 |
|---|---|---|---|---|
| *Agrobacterium tumefaciens* | 18 | **18** | 12 | 17 |
| *Aspergillus flavus* | 16 | **20** | 12 | 19 |
| *Bacillus subtilis* | 17 | **13** | 13 | 18 |
| *Candida albicans* | 22 | **25** | 14 | 20 |
| *Malassezia furfur* | 18 | **23** | 13 | 23 |
| *Micrococcus luteus* | 17 | **23** | 13 | 18 |
| *Salmonella typhi* | 14 | **22** | - | 17 |
| *Xanthomonas oryzae* | 19 | **23** | 13 | 25 |

**Table 4 Inhibitory zones of fermented broths of *Cephalosporium* sp. on various nitrogen sources at 7th day fermentation**

| Nitrogen sources T.O | N 1 | N 2 | N 4 | N 5 |
|---|---|---|---|---|
| *Agrobacterium tumefaciens* | 13 | 20 | 17 | **21** |
| *Aspergillus flavus* | 11 | 19 | 19 | **21** |
| *Bacillus subtilis* | 15 | 19 | 20 | **21** |
| *Candida albicans* | 11 | 19 | 22 | **25** |
| *Malassezia furfur* | 11 | 21 | 15 | **21** |
| *Micrococcus luteus* | 10 | 17 | 17 | **21** |
| *Salmonella typhi* | 12 | 21 | 16 | **25** |
| *Xanthomonas oryzae* | - | 20 | 21 | **23** |

10-12 mm = weak activity, 13-17 mm = high activity, >18 mm = very high activity, Disc size = 6 mm, T.O = Test organism

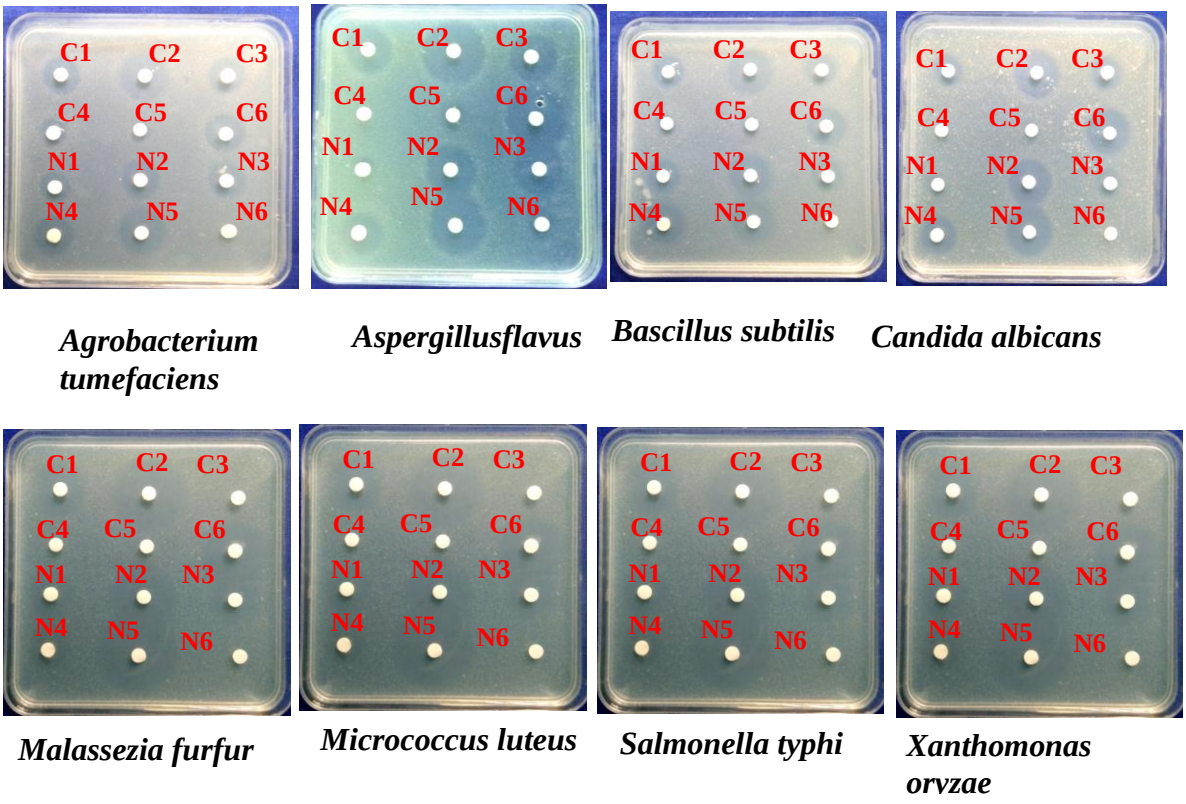



**Figure7** Inhibitory zones of *Cephalosporium* sp. on various carbon and nitrogen sources against eight test organisms

**Morphological characters of *Cephalosporium* sp. on various media**

In the investigation of morphological characters of *Cephalosporium* sp. on various media, seven media (1, 3, 5, 7, 9, 10 and 11) were good whereas *Cephalosporium* sp. showed moderate growth on medium 2 and also its showed poor growth on media 4, 6, 8 and 12. Surface and reverse colors of *Cephalosporium* sp. on various media were white, but reverse color of medium 3 was pale yellow and that of medium 10 was yellow as shown in Table 5 and Figure 8.

**Table 5** Morphological characters of *Cephalosporium* sp. on various media

| Media | Various media | Growth | Surface color | Reverse color |
|---|---|---|---|---|
| M 1 | Peptone/Yeast | Good | White | White |
| M 2 | Meat/Polypeptone/NaCl | Moderate | White | White |
| M 3 | Yeast/Malt/Glucose | Good | White | Pale yellow |
| M 4 | Glycerol/$K_2HPO_4$/$MgSO_4$/NaCl | Poor | White | White |
| M 5 | Oat Meal | Good | White | White |
| M 6 | Glycerol/$K_2HPO_4$ | Poor | White | White |
| M 7 | Soybean/Mannitol | Good | White | White |
| M 8 | $K_2HPO_4$/$MgSO_4$/NaCl | Poor | White | White |
| M 9 | Sucrose/Yeast/NaCl/$CaCO_3$ | Good | White | White |
| M 10 | Malt/Meat | Good | White | Yellow |
| M 11 | Sucrose/Malt/Starch | Good | White | White |
| M 12 | Honey | Poor | White | White |

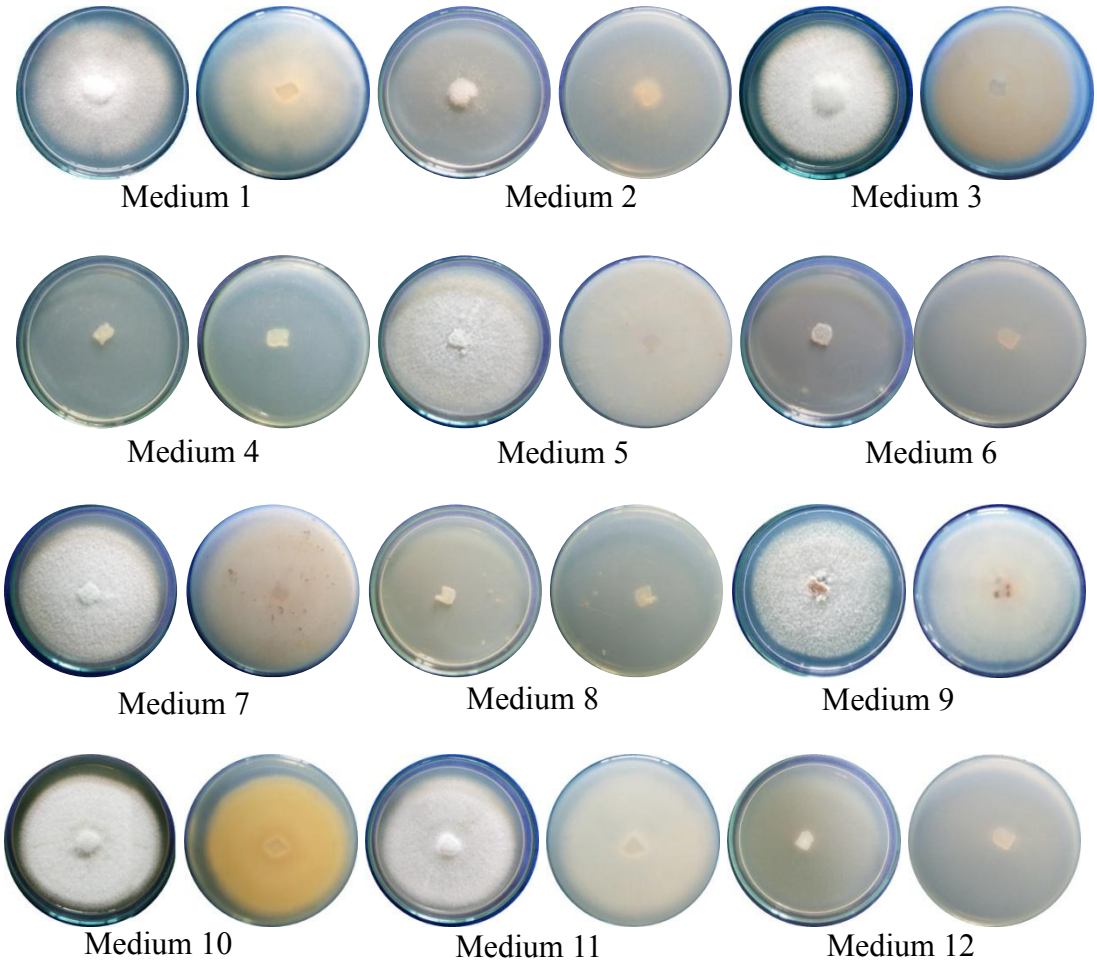



**Figure 8** Morphological characters of *Cephalosporium* sp. on various media

## Antimicrobial activity of *Cephalosporium* sp. on various media

Strain (*Cephalosporium* sp.) in medium 11 (sucrose/malt/soluble starch) and medium 9 (sucrose/yeast extract medium) showed highest antimicrobial activity on eight test organisms. The fermented broths of *Cephalosporium* sp. in media 2, 10 and 12 indicated high activity on eight test organisms while the fermented broths on media 3, 5, 7 and 8 were also exhibited weak activity on eight test organisms. The fermented broths of *Cephalosporium* sp. in medium 4 showed antimicrobial activity on some test organisms, but medium 6 did not show any activity as shown in Table 6 and Figure 9.

**Table 6** Inhibitory zones (mm) of fermented broths of *Cephalosporium* sp. on various media at 7th day

| Various media / T.O | M 1 | M 2 | M 3 | M 5 | M 7 | M 8 | M 9 | M 10 | M 11 | M 12 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Agrobacterium tumefaciens* | 22 | 17 | 13 | 12 | 15 | **27** | 16 | 19 | 14 | 22 |
| *Aspergillus flavus* | 25 | 21 | 11 | 10 | 17 | **26** | 18 | 22 | 17 | 25 |
| *Bacillus subtilis* | 24 | 21 | 14 | 10 | 15 | **26** | 17 | 21 | 13 | 24 |
| *Candida albicans* | 21 | 20 | 14 | 11 | 13 | **27** | 16 | 18 | 14 | 21 |
| *Malassezia furfur* | 20 | 20 | 11 | - | 14 | **28** | 13 | 20 | 16 | 20 |
| *Micrococcus luteus* | 23 | 21 | 13 | 10 | 16 | **23** | 14 | 20 | 15 | 23 |
| *Salmonella typhi* | 26 | 22 | 11 | - | 15 | **33** | 19 | 19 | 13 | 26 |
| *Xanthomonas oryzae* | 25 | 19 | 11 | - | 17 | **32** | 21 | 22 | 17 | 25 |

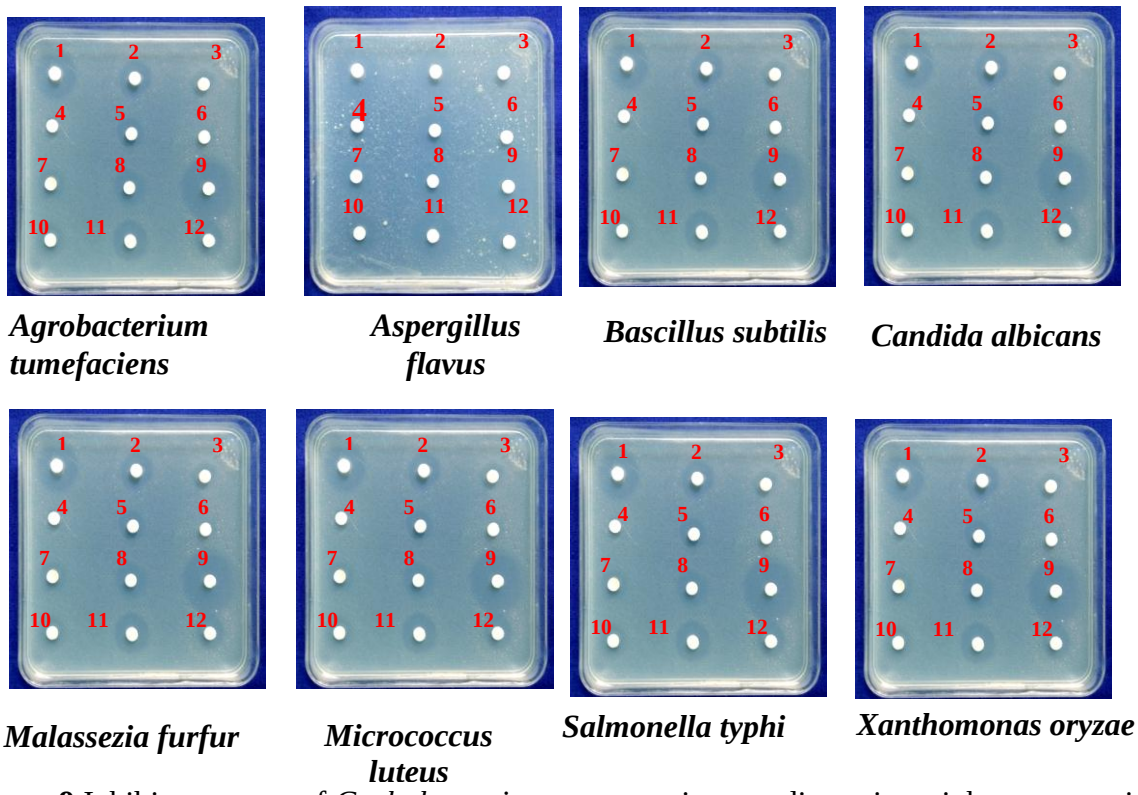



**Figure 9** Inhibitory zones of *Cephalosporium* sp. on various media against eight test organisms

**Fermentation studies of *Cephalosporium* sp.**

**Age of inoculum**

In the study of age of inoculum, fermented broth of two days old seed culture showed the highest activity against eight test organisms: (*Agrobacterium tumefaciens, Aspergillus flavus, Bacillus subtilis, Candida albicans, Malassezia furfur, Micrococcus luteus, Salmonella typhus* and *Xanthomonas oryzae*) as shown in Table 7 and Figure 10.

**Table 7. Age of inoculum (mm) for *Cephalosporium* sp. on *Salmonella typhi***

| *Cephalosporium* sp. | 2nd day | 3rd day | 4th day | 5th day | 6th day | 7th day |
|---|---|---|---|---|---|---|
| Fermentation 1 (one day old) | 25 | 26 | 25 | 25 | 24 | 16 |
| **Fermentation 2 (two days old)** | **35** | **36** | **36** | **35** | **27** | **25** |
| Fermentation 3 (three days old) | 21 | 24 | 24 | 19 | 19 | 18 |

10-12 mm = weak activity, 13-17 mm = high activity, >18 mm = very high activity, Disc size = 6 mm, T.O = Test organisms

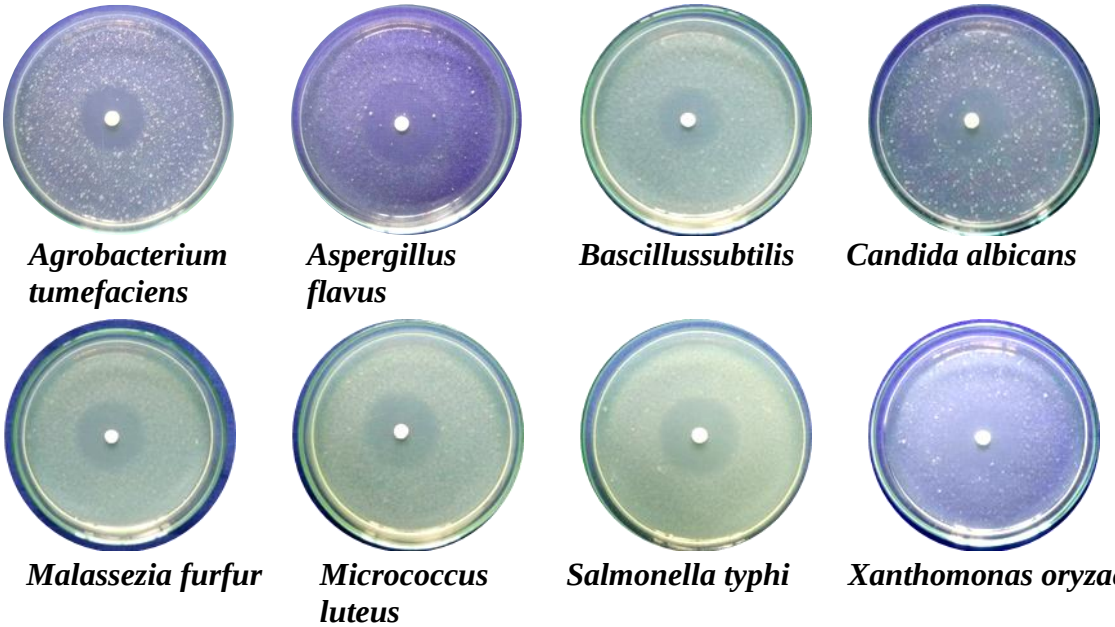



**Figure 10** Inhibitory zones of fermented broth (*Cephalosporium* sp.) of two days old against eight test organisms

**Size of inoculum**

In the study of size of inoculum optimization for *Cephalosporium* sp., among the seed cultures (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) 1.5% of seed culture at fifth day fermentation was suitable for the production of the bioactive compounds as shown in Table 8 and Figure 11.

**Table 8 Age of inoculum (mm) for *Cephalosporium* sp. on *Salmonella typhi***

| *Cephalosporium* sp. | 2nd day | 3rd day | 4th day | 5th day | 6th day | 7th day |
|---|---|---|---|---|---|---|
| Fermentation 1 (one day old) | 25 | 26 | 25 | 25 | 24 | 16 |
| **Fermentation 2 (two days old)** | **35** | **36** | **36** | **35** | **27** | **25** |
| Fermentation 3 (three days old) | 21 | 24 | 24 | 19 | 19 | 18 |

10-12 mm = weak activity, 13-17 mm = high activity, >18 mm = very high activity, Disc size = 6 mm, T.O = Test organism

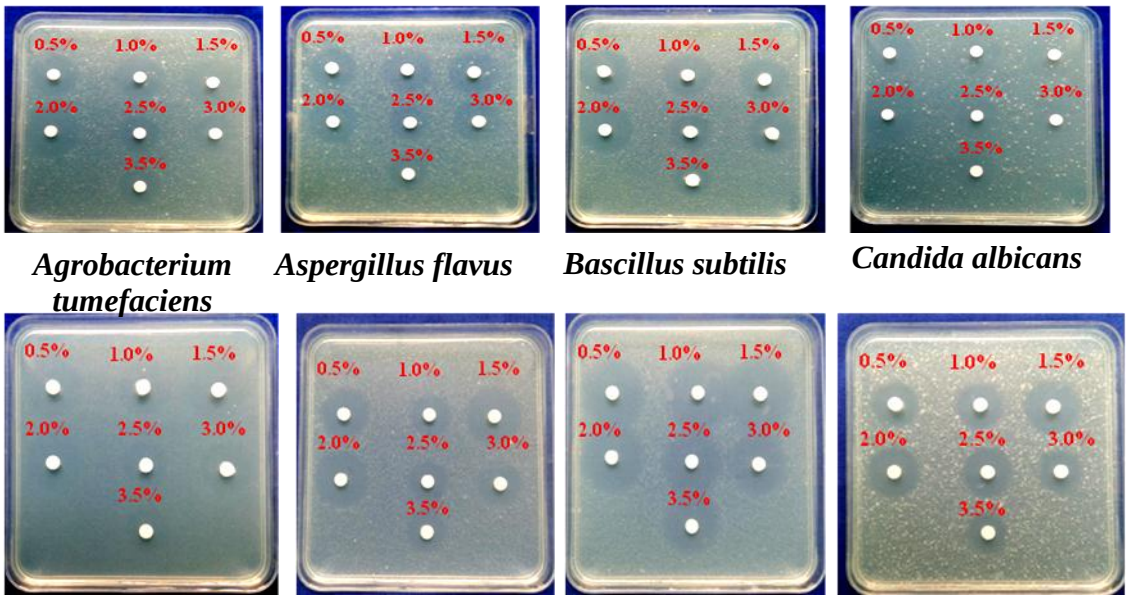



**Figure 11** Inhibitory zones (mm) for sizes of inoculum (*Cephalosporium* sp.)

**Effect of various pH for *Cephalosporium* sp.**

Among pH 4, 5, 6, 7, 8, 9 and 10 of fermented broths of strain *Cephalosporium* sp., pH 7 was the best for extraction of the bioactive compounds from fermented broth according to the result of inhibitory zones against eight test organisms as shown in Table 9 and Figure 12.

**Table 9** Inhibitory zones (mm) of *Cephalosporium* sp. with various pH on *Salmonella typhi*

| Day / pH | 2 days | 3 days | 4 days | 5 days | 6 days | 7 days |
|---|---|---|---|---|---|---|
| pH-4 | 18 | 20 | 25 | 16 | 22 | 23 |
| pH-5 | 17 | 14 | 19 | 18 | 23 | 22 |
| pH-6 | 17 | 16 | 22 | 19 | 21 | 19 |
| **pH-7** | **21** | **23** | **28** | **28** | **26** | **25** |
| pH-8 | 12 | 12 | 17 | 15 | 10 | 15 |
| pH-9 | 18 | 18 | 26 | 20 | 22 | 21 |
| pH-10 | 13 | 12 | 17 | 15 | 12 | 15 |

10-12 mm = weak activity, 13-17 mm = high activity, >18 mm = very high activity, Disc size = 6 mm, T.O = Test organism

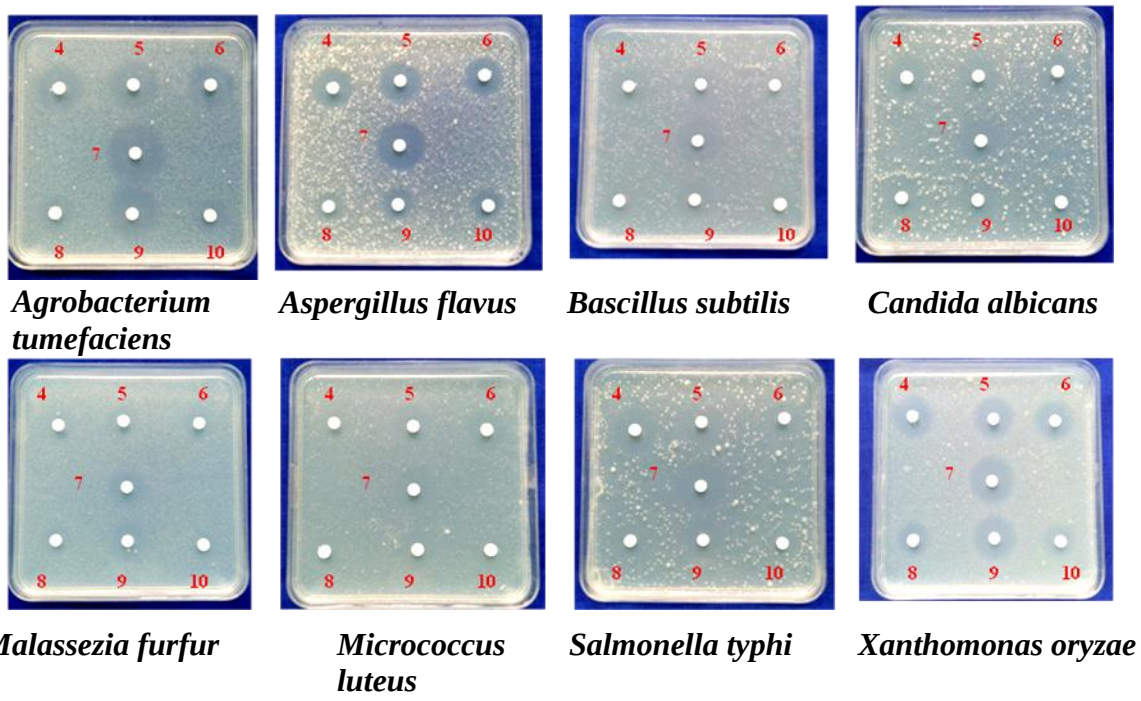



**Figure 12** Inhibitory zones (mm) of pH utilization for *Cephalosporium* sp.

## Discussion and Conclusion

Endophytic fungal strain *Cephalosporium* sp. isolated from the wood of *Hesperethusa crenulata* (Roxb.) Roem. was used to investigate the optimal fermentation conditions in order to produce its bioactive secondary metabolites. In the morphological character of carbon sources, honey was the best whereas in nitrogen sources, yeast extract, meat extract and malt extract were the best for *Cephalosporium* sp.. Kyawt Kyawt Aung (2014) also reported that starch was the best whereas in nitrogen sources, yeast extract and soybean were the best for endophytic fungal strain.

Klaic *et al.*, (2014) carried out optimization of various media, carbon and nitrogen sources for fungal growth and they also reported that peptone and yeast extract were the best nitrogen sources for the fungal growth. In 2015, Na Yu and Lu He investigated the optimal fermentation of endophytic fungus BS002 which was isolated from *Sophora flavescens*. They also reported that glucose, potato starch as carbon sources and peptone as nitrogen sources were the best for fermentation.

In antimicrobial activity of carbon and nitrogen sources, four carbon sources (glucose, sucrose, soluble starch and honey) and four nitrogen sources (peptone, yeast extract, malt extract and oat meal) showed very high activity on eight test organisms. Yee Yee Thu (2006) reported that glucose and yeast extract media indicated high activity against *Candida albicans.*

In the morphological characters of various media, seven media were good for fermentation to produce antimicrobial metabolites from *Cephalosporium* sp.. As a result of antimicrobial activity on various media, sucrose/yeast extract medium and sucrose/malt extract/soluble starch medium were the best for fermentation medium. For age of inoculum, two days old seed culture of this strain showed the highest activity against eight test organisms. Hnin

Wit Mhone (2018) and Soe Soe Yu Hnin (2018) also reported that two days old seed culture was the best for fermentation.

In the study for size of inoculum optimization, 1.5% of seed culture was suitable for the production of bioactive metabolites. Hnin Wit Mhone (2018) and Soe Soe Yu Hnin (2018) also stated that 1.5% of seed culture was suitable for the production of bioactive metabolites. In screening of optimal pH for fermentation of *Cephalosporium* sp., pH 7 was the best for extraction of the bioactive compounds from fermented broth. Kavish *et al.,* (2016) investigated the effects of pH, carbon sources and nitrogen sources on activity and they found the highest activity at pH 7. Shweta *et al.,* (2015) reported that the optimal pH of many endophytic fungi was pH 7 and they also carried out carbon and nitrogen sources for fermentation.

In conclusion, the best fermentation medium for strain *Cephalosporium* sp. consists of either sucrose/yeast extract medium or sucrose/malt/soluble starch medium. The best fermentation condition was 1.5 % of two days old seed culture at pH7 to produce bioactive metabolites from *Cephalosporium* sp..

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