# ISOLATION AND OPTIMIZATION OF ANTIBACTERIAL METABOLITE PRODUCTION OF ENDOPHYTIC FUNGI FROM THE LEAF OF *ANNONA SQUAMOSA* L.

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**Abstract**

In the course of the investigation of endophytic fungi were isolated from the leaf of *Annona squamosa* L. used PGA (Potato Glucose Agar) and LCA (Low Carbon Agar) media. After isolation, morphology of 7 days old culture were studied. According to this study, 3 different fungi (NA-01, NA-02 and NA-03) were isolated. In the study of antibacterial activities, endophytic fungus (NA-01) showed the highest antibacterial activities against *Micrococcus luteus* than other fungi. In the present investigation, endophytic fungi was isolated for antibacterial metabolite production, and effect of fermentation medium, pH and temperature variation were optimized for maximum antibacterial metabolite against *Micrococcus luteus.* The maximum production of antibacterial metabolite was observed in FM-1 medium (glucose, glycerol, yeast extract, peptone, $K_2HPO_4$, $CaCO_3$) at pH 5.0 and incubation temperature of 25ºC with shaking condition.

**Keywords:** *Micrococcus luteus*, *Annona squamosa* L., endophytic fungi

## Introduction

Endophytic fungi, at the beginning were applied for any organism found within plant (Petrini, 1986).

Plants may serve as a reservoir of large number of microorganisms known as endophytes (Bacon and White, 2000). Endophytes are microorganisms (mostly fungi and bacteria) that inhabit plant hosts for all or part of their life cycle. They colonize the internal plant tissue beneath the epidermal cell layers without causing any apparent harm or symptomatic infection to their host, living within the intercellular spaces of the tissue and its seems that they may penetrate the living cells (Strobel and Daisy, 2003).

Microorganisms are capable of amazing array of different types of fermentation. Various media types were tested to provide growth of the particular microorganisms. These media also could be modified in order toestablish the optimal conditions for production of the active secondary metabolite (Casida, 1968).

Medium optimization is still one of the most critically investigated phenomenon that is carried out before any large scale metabolite production, and possess many challenges too. Before 1970s, media optimization was carried out by using classical methods, which were expensive, time consuming, involving plenty of experiments with compromised accuracy. Nevertheless, with the advent of modern mathematical/statistical techniques, media optimization has become more vibrant, effective, efficient, economical and robust in giving the result for designing a production medium, the most suitable fermentation conditions (eg., pH, temperature, agitation speed, etc.) and the appropriate medium components (eg., carbon, nitrogen etc.) must be identified and optimized accordingly. Further, by optimizing the above said parameters, maximum product concentration could be achieved (Gupte and Kulkarni, 2003; Franco-Lara *et al.*, 2006; Wang *et al.*, 2011).

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In the present paper, the aim and objectives are (i) to isolate endophytic fungi from the leaf of *Annona squamosa* L., (ii) to study antibacterial activity of endophytic fungi (NA-01, NA-02 and NA-03) and (iii) to optimize the fermentation media, pH and temperature for the maximum production of antibacterial metabolite of endophytic fungus NA-01.

## Materials and Methods

### Medium used for isolation of fungi

**LCA medium**
**(Low Carbon Agar Medium)**
**(Ando and Inaba, 2004)**

| | |
|---|---|
| Glucose | 0.2 g |
| Sucrose | 0.2 g |
| $K_2HPO_4$ | 0.1 g |
| $KNO_3$ | 0.1 g |
| KCl | 0.05 g |
| Agar | 1.8 g |
| DW | 100 mL |
| pH | 6.5 |

**PGA medium**
**(Potato Glucose Agar Medium)**

| | |
|---|---|
| Potato | 20 g |
| Peptone | 0.3 g |
| Glucose | 2.0 g |
| Agar | 1.8 g |
| DW | 100 mL |
| pH | 6.5 |

### Isolation Procedure of Endophytes (Ando and Inaba, 2004)

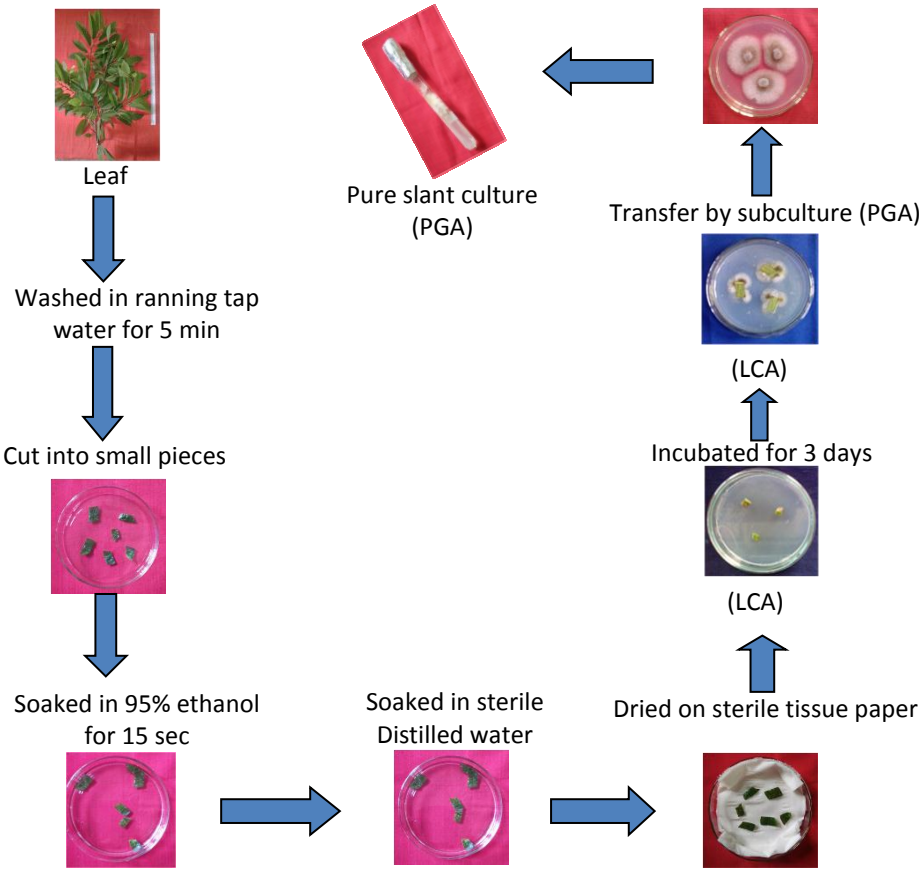



**Figure 1** Isolation Procedure of Endophytes from Plant Leaves

**Medium used antibacterial test (Ando and Inaba 2004)**

| **Seed medium** | | **Fermentation medium** | | **Assay medium used for test organism** | |
|---|---|---|---|---|---|
| Glucose | 2.0 g | Glucose | 2.0 g | Glucose | 1.0 g |
| Peptone | 0.3 g | Peptone | 0.3 g | Peptone | 0.3 g |
| $KNO_3$ | 0.1 g | $K_2HPO_4$ | 0.01 g | $KNO_3$ | 0.01 g |
| $K_2HPO_4$ | 0.01 g | $MgSO_4$ | 0.01 g | Agar | 1.8 g |
| DW | 100 mL | $CaCO_3$ | 0.1 g | DW | 100 mL |
| pH | 6.5 | DW | 100 mL | pH | 6.5 |
| | | pH | 6.5 | | |

**Screening for antibacterial activities by agar well diffusion assay (Mohanta, *et al.*, 2008)**

1. The isolated fungi were grown at room temperature for 7 days on PGA medium for sporulation
2. The isolated fungi were inoculated on seed medium and incubated at room temperature for 3 days
3. Five mL of seed culture was transferred into the fermented medium and incubated at room temperature for 7 days
4. 0.2 mL of fermented broth was filled into the holes on assay plate containing test organisms incubated for 24-36 hours.

7 days old culture

100 mL conical flask containing 50 mL seed medium

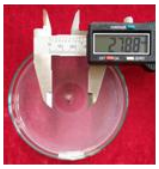


**Figure 2** Procedure of antimicrobial activity test

**Medium optimization for fermentation and production of antibacterial metabolite**

| **FM-1** | | **FM-2** | |
|---|---|---|---|
| Glucose | 2.0 g | Glucose | 1.5 g |
| Glycerol | 0.2 g | Yeast Extract | 0.3 g |
| Yeast extract | 0.3 g | Polypeptone | 0.3 g |
| Peptone | 0.3 g | $K_2HPO_4$ | 0.01 g |
| $K_2HPO_4$ | 0.01 g | $CaCO_3$ | 0.1 g |
| $CaCO_3$ | 0.1 g | DW | 100 mL |
| DW | 100 mL | pH | 6.5 |
| pH | 6.5 | | |

| **FM-3** | | **FM-4** | |
|---|---|---|---|
| Glycerol | 1.8 g | Glycerol | 0.2 g |
| Yeast extract | 0.8 g | Glucose | 1.0 g |
| Polypeptone | 0.3 g | Yeast extract | 0.3 g |
| $K_2HPO_4$ | 0.01 g | Peptone | 0.3 g |
| $CaCO_3$ | 0.1 g | $K_2HPO_4$ | 0.01 g |
| pH | 6.5 | $CaCO_3$ | 0.1 g |
| | | pH | 6.5 |

**Study on effect of pH**

The optimization of pH of the fermentation broth for antibacterial metabolite production was done by carrying out the fermentation at six different pH values viz 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. The medium was adjusted to the desired pH by adding 0.1 N NaOH or 0.1 N HCl (Naik *et al.*, 1988).

**Figure 3** Effect of pH on the fermentation

**Study on effect of incubation temperature on fermentation**

The optimization temperature for antibacterial metabolite production on fermentation of fungus NA-01 was carried out at five different incubation temperatures viz. 20ºC, 25ºC, 30ºC, 35ºC, and 40ºC. The fermentation was carried out 7 days and antibacterial activity was studied by agar well diffusion assay method.


**Figure 4** Different incubation temperature of (NA-01) for the fermentation

**Comparison of activity of static and shaking culture**

Two different flask containing 100 mL of (FM-1) fermentation medium with pH 5.0 at 25ºC were prepared. One fermented flask was incubated on the shaken (100 rpm) and another fermented flask was incubated under static condition (without shaking). These two culture were compared the antibacterial activity by using agar well diffusion method.


**Figure 5** Flask with shaking and static culture medium on the fermentation

## Results

Botanical name *Annona squamosa* L.

Myanmar name Awzar

Family Annonaceae

**Outstanding characters**

The plant is small tree. The leaves are simple, alternate, exstipulate, the margin entire, the tip acute. Inflorescence is solitary cymes. The flowers are greenish yellow colour, ebracteolate, pedicellate, bisexual. Fruit an aggregate of barriers, globose, fleshy. The seeds are large, brownish black.


**Figure 6** *Annona squamosa* L.

**Morphology of isolated endophytic fungi**

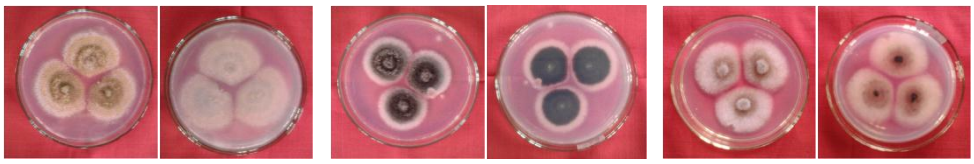
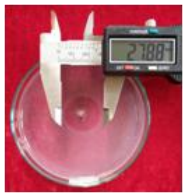



**Figure 7** Morphology of isolated endophytic fungi isolated from *Annona squamosa* L.

**Table 1 Antibacterial activities of isolate fungi from the leaves of *Annona squamosa* L.**

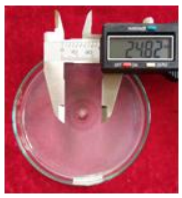
| | *Micrococcus luteus* | *Agrobacterium tumefaciens* | *Staphylococcus aureus* | *Salmonella typhi* | *Escherichia coli* |
|---|---|---|---|---|---|
| NA-01 | **27.88** | 17.10 | 25.44 | 19.94 | 17.02 |
| NA-02 | 16.70 | - | **24.82** | - | - |
| NA-03 | **20.28** | - | - | - | 16.53 |

(5 days fermentation)

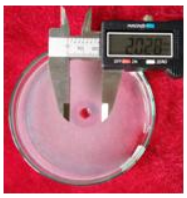


NA-01

**Figure 8** Antibacterial activity of isolated fungus (NA-01) against *Micrococcus luteus*



NA-02



NA-03

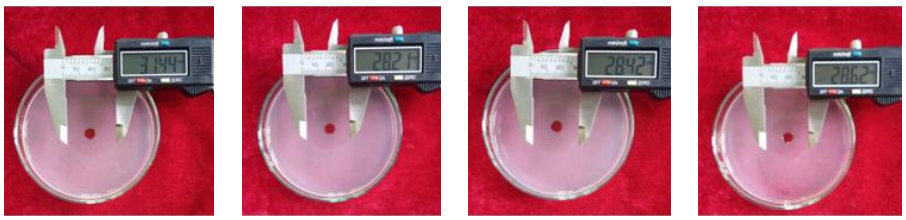
**Figure 9** Antibacterial activity of isolated fungus (NA-02) against *Staphylococcus aureus* and (NA-03) against *Micrococcus luteus* (5 days fermentation)

**The effect on media on the fermentation (size of agar well 8 mm)**

**Table 2 The effect of different media on the antibacterial activity of isolated endophyte NA-01**

| Medium | Activity (Clear zone, mm) |
|---|---|
| **FM-1** | **31.44** |
| FM-2 | 28.21 |
| FM-3 | 28.42 |
| FM-4 | 28.62 |

**Test organism was *Micrococcu luteus***



FM-1 FM-2 FM-3 FM-4

**Figure 10** The effects of fermentation medium for the production of antibacterial metabolite of (NA-01) against *Micrococcus luteus*

**Effect of pH on fermentation**

**Table 3 The effect on pH on the fermentation (size of agar well 8 mm)**

| pH | Activity (Clear zone, mm) |
|---|---|
| 4.0 | 26.28 |
| 4.5 | 27.28 |
| **5.0** | **33.09** |
| 5.5 | 25.59 |
| 6.0 | 23.68 |
| 6.5 | 22.83 |

**Test organism was *Micrococcu luteus***

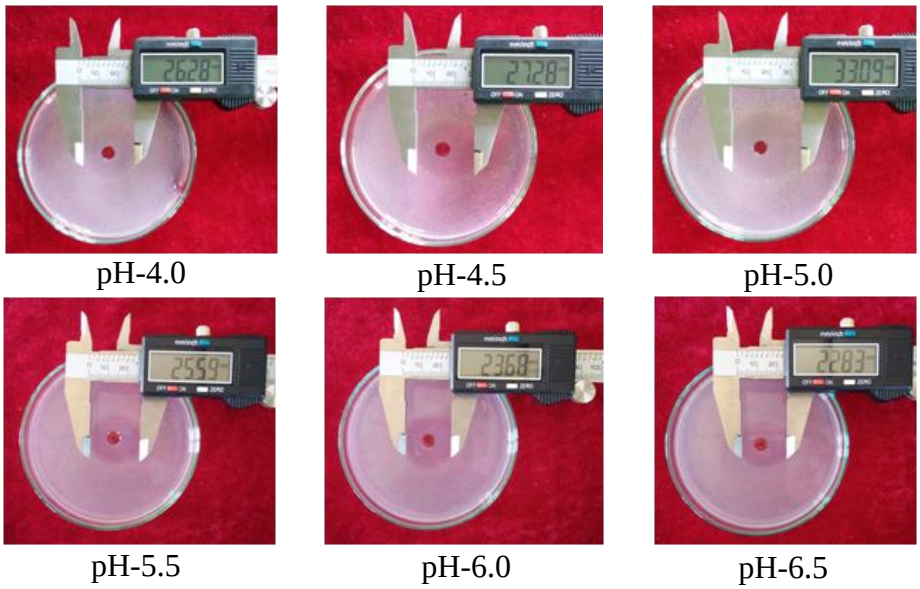



**Figure 11** Effect of pH on the fermentation for the antibacterial metabolite of (NA-01) against *Micrococcus luteus*

**Effect of temperature**

**Table 4 Effect of incubation temperatures on the fermentation for (NAO-01) against *Micrococcus luteus***

| Temperature (°C) | Inhibitory zone (mm) |
|---|---|
| 20°C | - |
| **25°C** | **31.24** |
| 30°C | 22.35 |
| 35°C | 18.02 |
| 40°C | 15.46 |

**agar well size = 8 mm**


**Figure 12** The effects of incubation temperatures (25 ºC, 30 ºC, 35ºC and 40ºC) of NAO-01 against *Micrococcus luteus*

**Comparison of activity of static culture and shaking culture**


**Figure 13** The effect of shaking and static culture of (NAO-01) against *Micrococcus luteus*

**Maximum antibacterial activity of endophytic fungus NA-01**

Maximum antibacterial activity was observed in fermentation medium-1 (FM-1) (glucose, glycerol, yeast extract, peptone, $K_2HPO_4$, $CaCO_3$), pH-5.0 at 25ºC with shaking condition. The maximum activity reached at 5 days fermentation as 33.86 mm clear zone on *Micrococcus luteus.*

## Discussion and Conclusion

Endophytes are microorganisms that includes bacteria and fungi within plant tissue without causing and immediately negative effects, and has been found in every plant species examined to date and recognized as the potential source of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms (Strobel and Daisy, 2003).

In the present study, isolation of endophytic fungi from the leaf of *Annona squamosa* L. According to result, 3 different endophytic fungi (NA-01, NA-02 and NA-03) were isolated. In the present, antibacterial activities of isolated endophytic fungi NA-01, NA-02 and NA-03 were studied. It was observed that endophytic fungus NA-01 showed the antibacterial activity on *Micrococcus luteus* (27.88 mm), *Agrobacterium tumefaciens* (17.10 mm), *Staphylococcus aureus* (25.44 mm), *Salmonella typhi* (19.94 mm) and *Escherichia coli* (17.02 mm). Endophytic fungus NA-02 showed the activity on *Micrococcus luteus* (16.70 mm) and *Staphylococcus aureus* (24. 82 mm) and endophytic fungus NA-03 showed the activity on *Micrococcus luteus* (20.28 mm) and *Escherichia coli* (16.53 mm) clear zone respectively.

According to this result, endophytic fungus (NA-01) showed the highest antibacterial activities against on *Micrococcus luteus* (27.8 mm) than other fungi. Therefore, NA-01 was selected for further investigation.

The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production (Stanbury *et al.,* 1997). The yield of bioactive compound can sometimes be substantially increased by the optimization of physical (temperature, salinity, pH and light) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes (Thakur *et al.,* 2009).

In the present study, medium optimization for fermentation and production of antibacterial metabolite by using four different fermentation media (FM-1, FM-2, FM-3 and FM-4). It was observed that fermentation medium (FM-1) is the best medium for the production of antibacterial metabolite.

The pH level of the growth medium has a marked effect on secondary metabolite production (Rizk *et al.,* 2007).

In the present study, pH 5.0 were best for the production of antibacterial metabolite.

Physical factors such as incubation temperature, can exert different on the growth and production phase of secondary metabolite (Rizk *et al.,* 2007).

In the present study the effect of incubation temperature values, it was observed that optimum temperature 25ºC (31.24 mm clear zone) was suitable for the production of antibacterial metabolite.

Aeration is critical factor for cell growth and metabolite production by aerobic microbial culture. The previous investigation showed that oxygen supply plays an important role in the cell

growth and production of bio-metabolite by the fungus (Shih, *et al.*, 2007; Fang and Zhong, 2002).

In the present study effect of different fermentation condition such as shake culture and stationary culture were studied. The results revealed that metabolite production was higher in shaking culture (31.43 mm clear zone) than static culture (27.10 mm clear zone).

The maximum production of antibacterial metabolite of endophytic fungus NA-01 could be achieved in fermentation medium (FM-1) (glucose, glycerol, yeast extract, peptone, $K_2HPO_4$, $CaCO_3$). Further process parameters like incubation temperature at 25ºC, pH 5.0 are found to be optimum for the maximum production of antibacterial metabolite. According to above conditions maximum activity was found to be (33.86 mm) clear zone on *Micrococcus luteus.*

The present study concluded that the optimum conditions require for the production of antibacterial metabolite by endophytic fungus NA-01 were determined and metabolites production showed the highest antibacterial activity against *Micrococcus luteus.* Hence further studies will be carried out on extraction, purification and identification of antibacterial metabolites of endophytic fungus NA-01.

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