FERMENTATION STUDIES OF BIOACTIVE FUNGAL STRAIN *ASPERGILLUS* SP. SS 7 ISOLATED FROM *ZINGIBER CASSUMUNAR* ROXB

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Abstract

Endophytic fungal strain *Aspergillus* sp. SS 7 isolated from the rhizome of *Zingiber cassumunar* Roxb. was used for the investigation of optimal fermentation conditions such as various carbon and nitrogen sources, different culture media, age of inoculum, size of inloculum and pH utilization. In utilization of carbon sources, starch and glycerol were the best whereas yeast extract and soybean were the best nitrogen sources. In antimicrobial activity of various carbon sources, glucose medium showed very high activity against *Candida albicans* whereas various nitrogen sources, oat meal medium indicated very high activity against *Malassezia furfur*. The investigation of the morphological characters on different media, medium 1, 3, 7, 9 and 10 were good media. As a result of antimicrobial activity on different media, medium 7 and medium 9 were the best for fermentation medium. 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. In the study of pH utilization, pH 6 was the best for extraction of the bioactive compounds.

Key words: antimicrobial activity, *Aspergillus* sp. SS 7, *Zingiber cassumunar* Roxb.

Introduction

Fermentation is a metabolic process that converts sugar to acids, gases or alcohol. From thousands of year's mankind has used natural product, chemicals produced by plants, fungi, bacteria and other living organism in a variety of application: drugs and food. During the last two decades endophytes have been targeted as valuable sources of new bioactive compounds (Tadych and White, 2009). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal and anticancer activities have been successfully discovered from the endophytic fungi. Methods to obtain

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bioactive compounds include the extraction from a natural source and the microbial production via fermentation (Parton and Willis, 1989). In the present study, optimal fermentation conditions and different media of endophytic fungal strain *Aspergillus* sp. SS 7 were conducted for extraction of the bioactive compounds.

Materials and Methods

Collection and outstanding characters of plant sample

The plant sample was collected from the Mhawe-Bi Township. Outstanding characters of plant sample was identified by Backer and Bakhuizen, 1968.

Morphological and microscopical characters of isolated fungal strain SS 7

Isolated fungal strain SS 7 grown on slant culture was transferred onto the plate containing sucrose, yeast extract medium. Then this plate was incubated at room temperature for 3-7 days. Colony forms, surface and reverse pigments of isolated strain and microscopical characters were studied according to Barnett, 1998.

Utilization of carbon and nitrogen sources

In this study the morphological characters of strain SS 7 were studied by using various carbon and nitrogen sources. Carbon sources are sucrose, glucose, starch, mannitol and glycerol whereas nitrogen sources are yeast extract, meat extract, malt extract, oat meal and soybean. Basal media for finding out suitable 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 finding out suitable nitrogen sources are glycerol 1.0%, K_2HPO_4 0.01%, $MgSO_4$ 0.01% and $CaCO_3$ 0.01% (Monaghan *et al*., 1999).

Antimicrobial activity of strain SS 7 by using various carbon and nitrogen sources

Fungal strain SS 7 grown on slant culture was transferred into 50 ml flasks containing 25 ml of various carbon and nitrogen sources and incubated for ten days. The fermented broth was used to check antimicrobial activity by paper disc diffusion assay (Phay, 1997).

Morphological characters of strain SS 7 on different media

In this study, different media were used for media optimization. A piece of fungus from plate culture of strain SS 7 was inoculated on each of different media plates and incubated for 3-5 days (Monaghan *et al*., 1999). 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) and **medium 11** (Sucrose, Malt extract, Soluble starch medium)

Antimicrobial activity of strain SS 7 by using different media

A piece of fungus from plate culture of strain SS 7 was inoculated into each of eleven (50 ml) conical flasks containing 20 ml of different fermentation medium. These flasks were incubated 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) as shown in Figure 1.

Figure 1. Different media for media optimization

Age of inoculum for strain SS 7

Two days old and three days old of seed cultures were transferred into 50 ml fermentation flasks containing 25 ml of medium 9 (SY: sucrose, yeast extract medium). They were incubated for seven days at room temperature. Then, these fermented broths were checked for their inhibitory activity by paper disc diffusion assay (Strobel and Sullivan, 1999).

Size of inoculum of strain SS 7

The proper cultivation and amount of inoculums are essential for the optimal production of bioactive metabolites. A piece from fungal plate culture of strain SS 7 was inoculated into 300 ml conical flasks containing 100 ml of medium 9 (SY; sucrose, yeast extract medium) seed medium. The flasks were incubated at room temperature 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 seven conical flasks containing 100 ml of fermentation medium as shown in Figure 2. The fermentation was carried out for ten days.

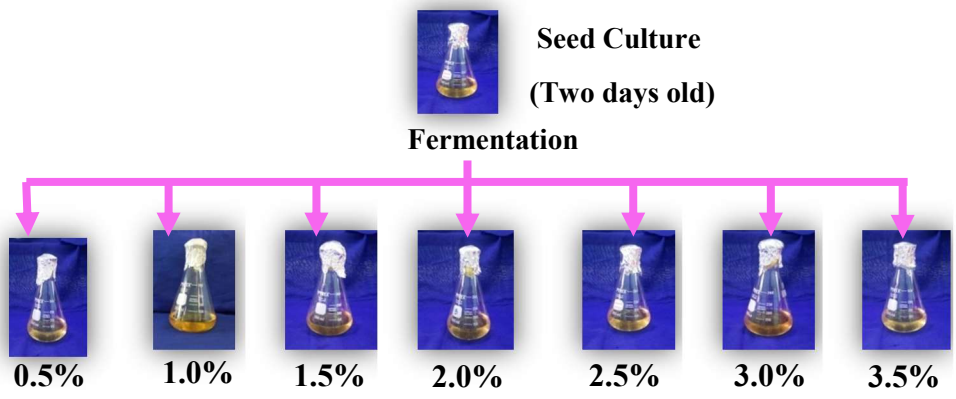


Figure 2. Seed culture and fermentation for size of inoculum

pH utilization of strain SS 7 (Monaghan *et al*., 1999)

For the seed culture, a piece from fungal plate culture of strain SS 7 was inoculated into 300 ml of conical flask containing 100 ml of medium 9 (SY; sucrose, yeast extract medium) and then flasks were incubated at room temperature for two days. Seven 300 ml conical flasks containing 100 ml fermentation medium 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 3 days. After three days, seven fermentation flasks were checked their antimicrobial activity.

Ferm

Result

Outstanding characters of plant sample

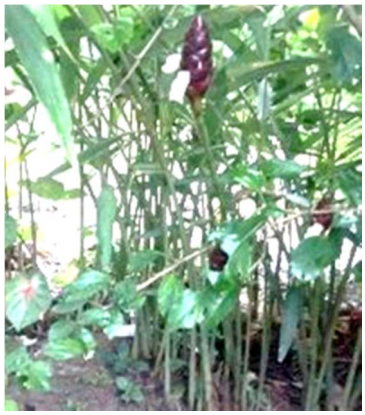


Figure 3. Habit of *Zingiber cassumunar* Roxb.

Scientific name - *Zingiber cassumunar* Roxb.
English Name - Bengal ginger
Myanmar name - Meik-Tha-Lin
Family - Zingiberaceae

Outstanding characters

Herbs with aromatic rhizome, rhizomes bright yellow; Leaves opposite and distichous, simple; Inflorescence borne separately from the leaves, peduncle, ovate spike, bracteolate; Flowers pale yellow, complete, bisexual, irregular, zygomorphic, epigynous; Sepals (3), synpetalous; Petals (3), synpetalous; Stamens $1+(2)^{st} + 2^{st}$, epipetalous; Filaments exserted, anthers dithecous, dorsifixed, longitudinal dehiscence; Pistil 1, tricarpellary, syncarpous, axile placentation, style long and slender, stigma capitates inferior; Fruits and seeds not seen.

Morphological and microscopical characters of isolated fungal strain SS 7

Surface and reverse colour of strain SS 7 was dark green and yellow. Conidiophores upright, simple, terminating in a globose swelling, bearing phialides at the apex, conidia 1 celled, globose, often variously colored in mass. Therefore, strain SS 7 was identified as *Aspergillus* sp. (Figure 4)

Figure 4. Morphological and Microscopical character of strain SS 7 (X 400)

Carbon utilization

Among carbon sources, starch and glycerol media were the best carbon sources whereas sucrose, glucose and mannitol media were also suitable for fermentation as shown in and Figure 5.

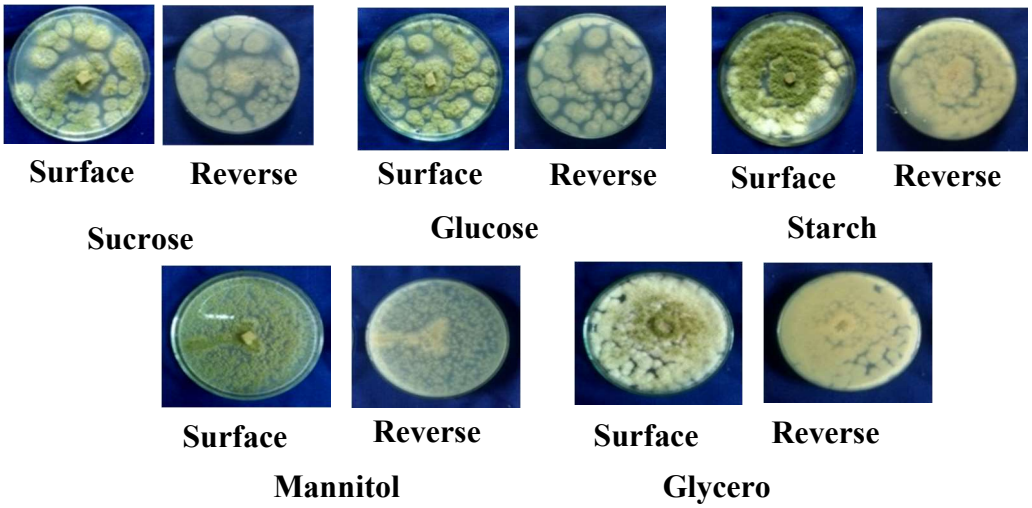


Figure 5. Strain SS 7 grown on the plates of various carbon source

Nitrogen utilization

In nitrogen sources, yeast extract and soybean media were the best nitrogen sources. Meat extract, malt extract and oat meal media were suitable for fermentation as shown in Figure 6.

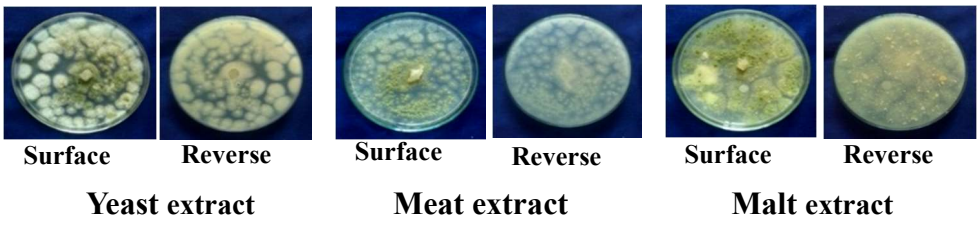


Figure 6. Strain SS 7 grown on the plates of various nitrogen sources

Antimicrobial activity of strain SS 7 by using various carbon sources

Antimicrobial activity of strain SS 7 in glucose medium showed very high activity against *Candida albicans*, starch medium indicated high activity against *Candida albicans*, *Salmonella typhi* and *Staphylococcus aureus* as shown in Figure 7.

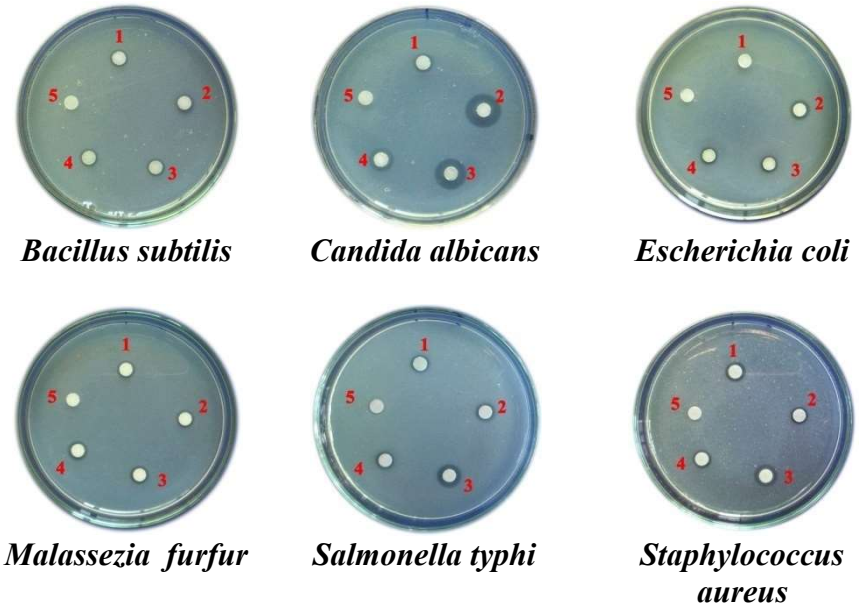


Figure7. Inhibitory zones of strain SS 7 on various carbon sources against six test organisms

Antimicrobial activity of strain SS 7 by using various nitrogen sources

Antimicrobial activity of strain SS 7 in yeast extract medium showed high activity against *Bacillus subtilis*, *Candida albicans*, *Salmonella typhi* and *Staphylococcus aureus*. Meat extract medium indicated high activity against *Candida albicans*. Malt extract medium indicated high activity against *Candida albicans* and *Staphylococcus aureus.* Oat meal medium indicated very high and high activity against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli* and *Malassezia*. Soybean medium indicated high activity against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur* and *Staphylococcus aureus* respectively as shown in Figure 8.

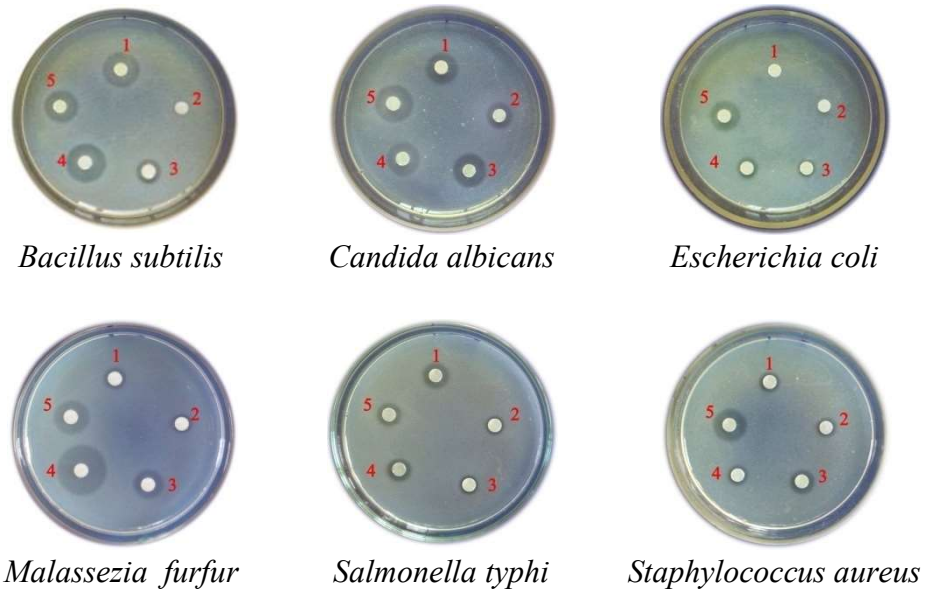


Figure 8. Inhibitory zones of strain SS-7 on various nitrogen sources against six test organisms

Morphological characters of strain SS 7 on different media

In the investigation of morphological characters of strain SS 7 on different media, medium 1, 3, 7, 9 and 10 were good. In these media, the surface and reverse colour of medium 1 was white to light green and cream colour, another media such as medium 3, 7, 9 and 10 were green and cream colour. Medium 2 and 5 were moderate for fermentation. These surface and reverse colour were white to light green and white for medium 2 and green and white colour for medium 5. However, medium 4, 6, 8 and 11 were not suitable for fermentation to produce antimicrobial metabolites from strain SS 7. The surface and reverse colour of medium 4, 6 and 8 were white colour respectively whereas medium 11 was light green and white colour as shown in Figure 9.

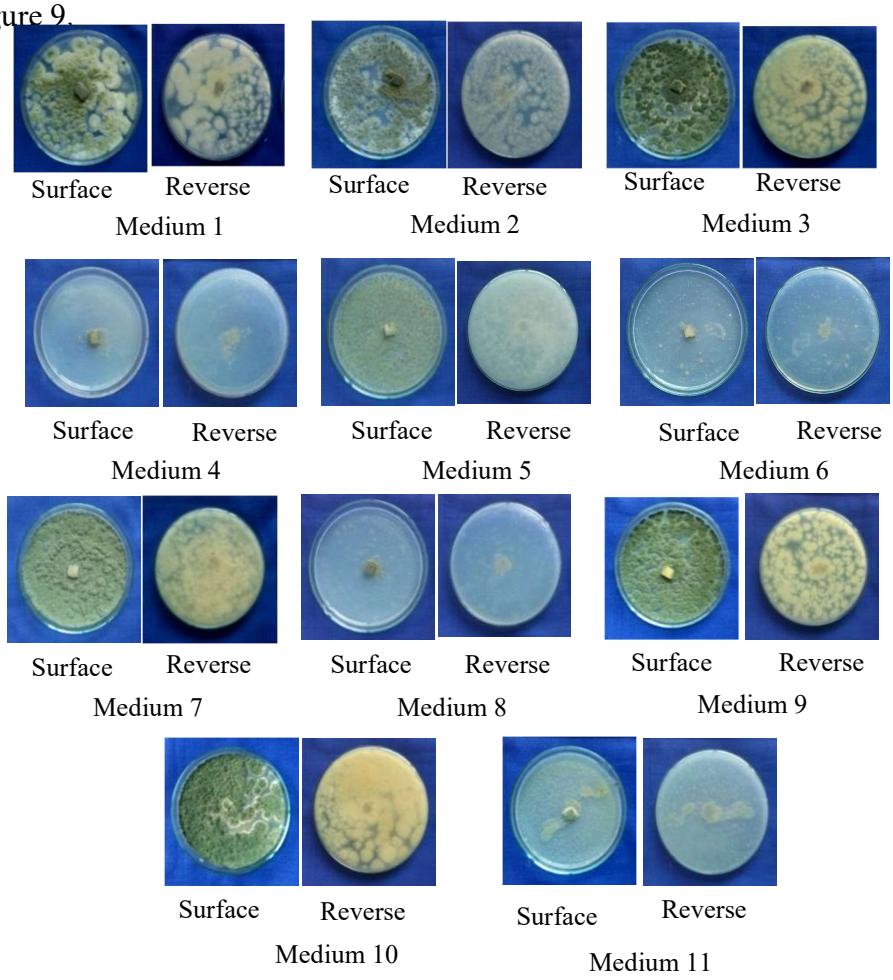
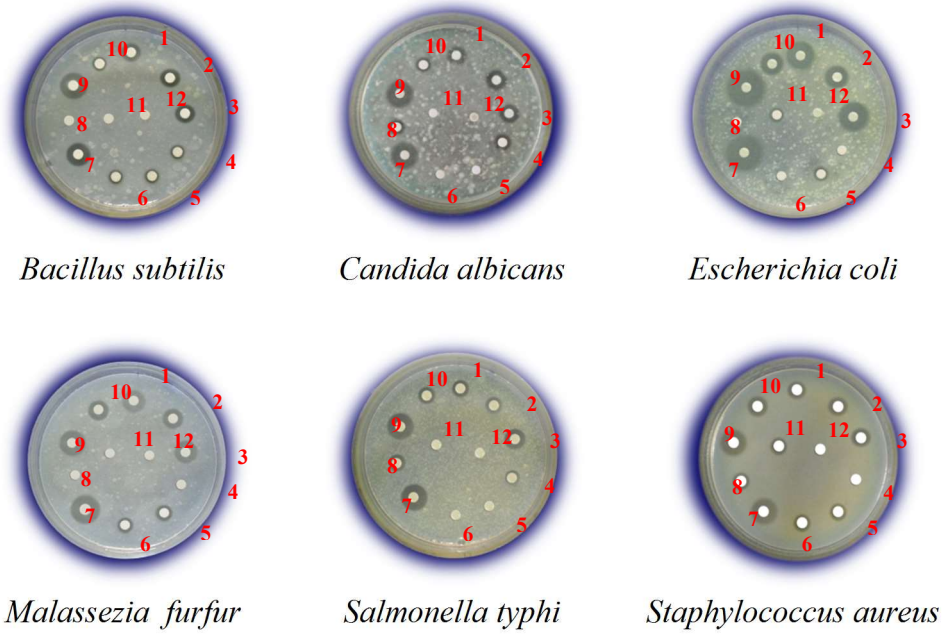


Figure 9. Surface and reverse color of strain SS 7 grown on different media

Antimicrobial activity of strain SS 7 by using different media

Antimicrobial activity of strain SS 7 on different media, soybean, mannitol medium (M-7) showed highest activity against *Malassezia furfur* and *Escherichia coli*. Sucrose, Yeast extract medium (M-9) also showed highest activity against *Escherichia coli* and *Salmonella typhi* as shown in Figure 10.



1. Medium 1	2. Medium 2	3. Medium 3	4. Medium 4
5. Medium 5	6. Medium 6	7. Medium 7	8. Medium 8
9. Medium 9	10. Medium 10	11. Medium 11	12. Control

Figure 10. Inhibitory zones of strain SS 7 on different media against six test organisms

Fermentation studies of strains SS 7

Age of inoculum

In the study of age of inoculum, fermentation 1 (Two days old) showed the highest activity against *Bacillus subtilis*, *Candida albicans, Malassezia furfur* and *Escherichia coli* respectively as shown in Table 1 and Figure 11. Fermentation 2 (Three days old) showed weak activity against four test organisms as shown in Table 2 and Figure 11.

Table 1. Inhibitory zones (mm) of strain SS 7 for Fermentation 1 (Two days old)

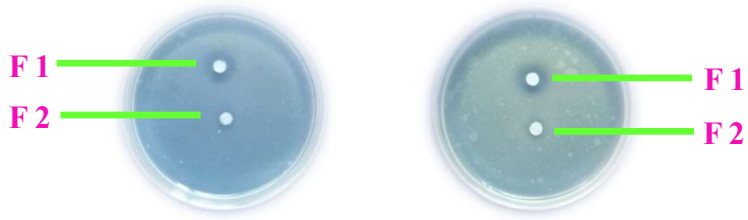
Day / Test organism	1	2	3	4	5	6	7
Bacillus subtilis	-	12	14	**16**	14	12	11
Candida albicans	-	15	15	**17**	12	11	10
Escherichia coli	-	12	13	15	**17**	14	12
Malassezia furfur	-	12	14	17	14	12	10

10-12 mm = weak activity, 13-17 = high activity, >18 mm =very high activity (Disc size =6mm

Table 2. Inhibitory zones (mm) of strain SS-7 for Fermentation 2 (Three days old)

Day / Test organism	1	2	3	4	5	6	7
Bacillus subtilis	-	11	10	10	10	10	-
Candida albicans	-	10	10	10	10	10	-
Escherichia coli	-	10	10	10	10	11	-
Malassezia furfur	-	10	11	10	10	10	-

10-12 mm = weak activity, 13-17 = high activity, >18 mm =very high activity (Disc size =6mm)



Bacillus subtilis *Candida albicans*

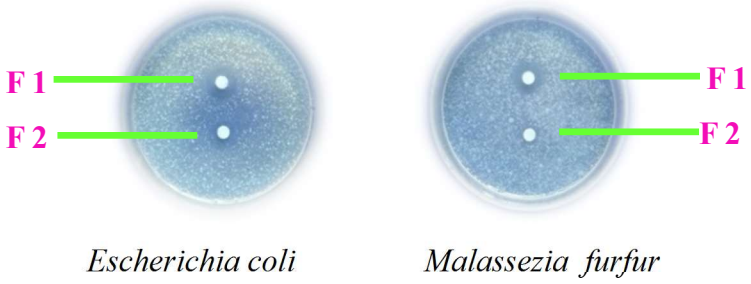


Figure 11. Inhibitory zones of strain SS 7 for Fermentation 1 and fermentation 2 against four test organisms

Size of inoculum

In the study of size of inoculum optimization, among the seed culture (0.5%, 1.0%, 1.5%, 2%, 2.5%, 3.0% and 3.5%) 1.5% of seed culture at fifth day fermentation was suitable for the production of the bioactive compound as shown in Figure 12.

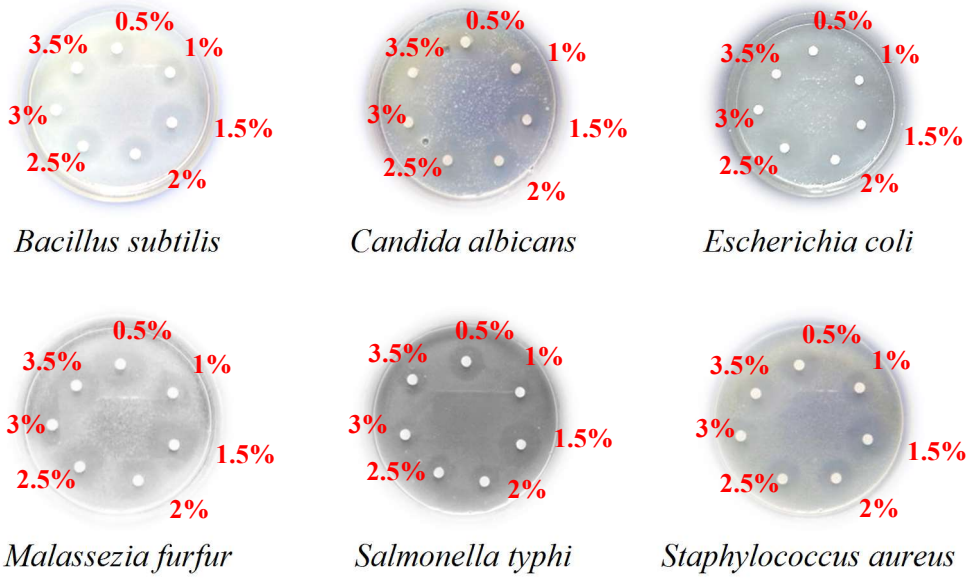


Figure 12. Inhibitory zones (mm) of size of inoculum for strain SS 7

Effect of different pH of strain SS 7

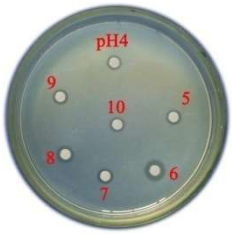
Among pH 4, 5, 6, 7, 8, 9 and 10 of fermented broth of strain SS 7, pH 6 was the best for extraction of the bioactive compounds from fermented broth according to the result of inhibitory zones against six test organisms as shown in Table 3 and Figure 13.

Table 3. Inhibitory zones (mm) of different pH of strain SS 7 on antimicrobial activity

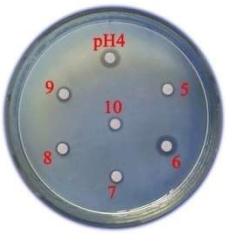
pH	***Bacillus subtilis***	***Candida albicans***	***Escherichia coli***	***Malassezia furfur***	***Salmonella typhi***	***Staphylococcus aureus***
pH 4	10	11	12	13	11	13
pH 5	10	10	10	10	10	11
pH 6	10	**13**	**14**	**13**	10	**15**
pH 7	10	10	14	11	10	10
pH 8	10	11	10	10	10	11
pH 9	10	10	10	10	10	12
pH 10	10	10	10	11	10	10

10 -12 mm = weak activity, 13 - 17 mm= high activity, >18 mm = very high activity. (disc size = 6 mm)

Bacillus subtilis



Candida albicans



Escherichia coli

Malassezia furfur *Salmonella typhi* *Staphylococcus aureus*

Figure 13. Inhibitory zones (mm) of pH utilization for strain SS 7

Discussion and Conclusion

Endophytic fungal strain *Aspergillus* sp. SS 7 isolated from the rhizome of *Zingiber cassumunar* Roxb. was used for the investigation of optimal fermentation conditions in order to produced its bioactive secondary metabolites. In utilization of carbon sources, starch and glycerol were the best fermentation whereas in nitrogen sources, yeast extract and soybean were the best fermentation for strain SS 7. Kyawt Kyawt Aung (2014) also reported that in utilization of carbon sources, starch was the best fermentation whereas in nitrogen sources, yeast extract and soybean were the best fermentation for endophytic fungal strain.

In antimicrobial activity of various carbon sources, glucose medium showed very high activity against *Candida albicans* whereas starch medium showed high activity against *Salmonella typhi* and *Staphylococcus aureus*. In various nitrogen sources oat meal medium indicated very high and high activity against *Malassezia furfur* and *Bacillus subtilis* whereas yeast extract media showed high activity against *Salmonella typhi* and soybean media indicated high activity against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. Yee Yee Thu (2006) reported that glucose and yeast extract media indicated high activity against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. Kyawt Kyawt Aung (2014) reported that starch and soybean medium showed high activity against *Escherichia coli*. In the study of morphological characters of different media, 1, 3, 7, 9 and 10 were good for fermentation to produce antimicrobial metabolites from strain SS 7. As a result of antimicrobial activity on different media, medium 7 and medium 9 were the best for fermentation medium.

In the study of size of inoculum optimization 1.5% of seed culture at fifth day fermentation was suitable for the production of bioactive metabolites. Yee Yee Soe (2014) observed the highest activity against *Bacillus subtilis* at 1.5% of seed culture for fermentation of bioactive strain. The large numbers of known bioactive compounds of microbial origin are currently produced by fermentation (Parkinsan, 1994). Optimal fermentation conditions such as proper age and size of inoculum are very important for the production of metabolites (Omura 1984). In the screening of optimal pH for fermentation, pH 6 was the best for extraction of the bioactive compounds from fermented broth of strain SS 7 according to the results of inhibitory zones on six test organisms. Yee Yee Thu (2006) has reported that endophytic fungus isolated from *Mimusops elengi* L. showed high activity at pH 6. Kyawt Kyawt Aung (2014) reported that endophytic fungus isolated from *Coccinia indica* Wight and ARN indicated high activity at pH 7. In conclusion, the best fermentation medium for strain SS-7 should consist of starch, mannitol and glycerol for carbon sources, yeast extract and soybean for nitrogen sources. The best fermentation condition was 1.5 % of two days old seed culture and pH 6 to produce bioactive metabolites from strain SS 7.

Acknowledgements

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