EFFECTS OF CARBON AND NITROGEN SOURCES FOR THE GROWTH OF SOIL FUNGUS YY-13 AND ITS FERMENTATION OPTIMIZATION

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Abstract

In this study, fungal strain (YY-13) isolated from soil sample in Minhla Area, Magway Region were intended to produce antibacterial metabolites. In addition, it was focused on the growth and fermentation conditions of isolated soil fungus (YY-13) against *Escherichia coli* by using agar well diffusion method. Moderate growth and the best antibacterial activity of YY-13 were observed both in carbon and nitrogen sources. The addition of potato powder as a carbon sources resulted better growth and the inhibition zone reached (32.78 mm) in glucose. In the nitrogen sources, the maximum growth of YY-13 was found in peptone and the highest antibacterial activity was obtained by using the sodium nitrate (28.39 mm). The fermentation conditions of YY-13 were studied by the effect of age, size of inoculum, temperature, pH, static and shaking culture. In this investigation, YY-13 was found that 25% of size of inoculums and 84 hrs of age of old culture were suitable conditions. And then, the effects of different temperature and pH range were also determined. According to these studies, temperature 30 ˚C and pH-6 were found to be the best conditions of antibacterial activity against *E.coli*. In the comparison of static culture and shaking culture of YY-13, the static culture exhibited the higher antibacterial activity (26.91 mm) than that of shaking culture (20.38 mm).

Keywords: YY-13, Antibacterial activity, *Escherichia coli*, carbon and nitrogen sources, fermentation conditions

Introduction

Natural products are important sources in the drug discovery process. Accordingly, new ecological niches should be explored for natural bioactive agents in pharmaceutical, agricultural, and industrial fields. These products should be renewable, ecofriendly and easily obtainable. The most importance of natural products are plants, animals, marine microorganisms (sponge, corals and algae), and microorganisms (bacteria, actinomycetes, and fungi) (Liu, *et al*., 2004).

The presence of more than 200,000 natural metabolites presenting various bioactive properties (Berdy, 1974) demonstrates the important of natural product in new drugs discovery. The use of microorganisms has created a huge revolution in many aspects of human's life as witnessed by numerous studies conducted by scientists in different fields. Microorganisms are used in production of pharmaceutical products, especially antibiotics (Philippe, *et al*., 2009).

Antibiotics have an important role in human health. Their necessity emerged from the spread of various diseases. As a result, scientists are trying to produce and discover more antibiotics (Rolain, *et al.*, 2000). Soil fungi have been the most studied of fungi, and typical soil genera such as *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium* have shown ability to synthesize a diverse range of bioactive compounds. About one third of metabolites derived from fungi belong to *Aspergillus* and *Penicillium* (Berdy, 1974).

Modifying fermentation parameters such as time, temperature, pH, and nutrients can help expanding the range of those secondary metabolites (Pfefferle and Gurtler, 2000). The presence of antimicrobial properties may indicate a larger activity spectrum, including antitumor and antiparasitic characteristics (Demain, 1999).

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The purpose of the present study was to screen the ability of YY-13 to produce antibacterial compounds using different growth parameters namely, pH, temperature, static and shaking culture.

Materials and Methods

Source of Soil Fungi

The fungal strain (YY-13) isolated from soil sample in Minhla Area, Magway Region, were intended to produce antibacterial metabolites.

Study on Antimicrobial Activity

The isolated fungus YY-13 was cultured on BMEA medium for 5 days. The isolated fungus were inoculated into 25 mL seed medium and incubated at room temperature for 3 days (Ando, 2004). After 3 days, 25 mL seed culture was transferred into the 75 mL of fermentation medium and incubated at room temperature. Fermentation was carried out for 3-10 days.

Screening of Antimicrobial Activity by Agar Well Diffusion Method (Collins, 1965)

Two days old culture test broth (0.1 mL) was added to 25 mL of assay medium and thoroughly mixed and poured into plate. After solidification, cork borer was to make the wells (wells-8 mm). The fermentation broth (20 μL) was carefully added into the wells and incubated at room temperature for 24 to 48 hours. The diameter of the zones of inhibition around each well was measured and recorded after 24 to 48 hours incubation.

Table 1 Test Organisms Utilized in the Antimicrobial Activity of Isolated Soil Fungi

No.	Test Organisms	Diseases
1	*Escherichia coli* AHU5436	Diarrhoea
2	*Bacillus subtilis* IFO 90571	DNA topoisomerase I
3	*Bacillus pumilus*IFO 12092	Wound and burn infection, fever
4	*Candida albicans* NITE 09542	Candidasis
5	*Staphylococcus aureus* AHU8465	Food poisoning, Methicillin Resistance
6	*Malassezia furfur* AUV 0255	Dandruff, Seborrhoeic dermatitis

The Effects of Carbon and Nitrogen Sources for the growth of Fungus YY-13

In this study, carbon sources such as glucose, xylose, sucrose, mannitol, lactose, fructose, maltose, glycerol, tapioca powder, molasses, soluble starch, potato powder, wheat powder and arabinose were used in each of 1.0 g. Nitrogen sources such as asparagine, malt extract, peptone, gelatin, casein, yeast extract, sodium nitrate, urea, ammonium nitrate, potassium nitrate, ammonium chloride and ammonium sulphate were utilized (each 1.0 g).

The Effect of Age and Size of Inoculum for the Fermentation (Omura, 1985)

In this study, the selected fungus YY-13 was cultured on BMEA medium at room temperature for 5 days and then was transferred into seed medium. Inoculation period 3 to 10 days were used for the production of antimicrobial metabolite and the procedure of seed culture medium was also used as the previous method. And then, seed culture was transferred to 100 mL conical flask containing of fermentation medium and incubated at room temperature. The inoculum age of

fermentation were studied by 48 hrs, 60 hrs, 72 hrs, 84 hrs, 96 hrs and 108 hrs. In the study of sizes of inoculum, (5%, 10%, 15%, 20%, 25%, and 30%) were utilized with 84 hr age of culture. Fermentation was done and antimicrobial activity was tested by agar well diffusion method.

Study on Different Carbon Sources Utilization for the Fermentation

Carbon sources (each 1.0 g or 1.0 mL) such as glucose, xylose, sucrose, mannitol, lactose, fructose, maltose, glycerol, tapioca powder, molasses, soluble starch, potato powder and arabinose were used. Fermentation were incubated at 30˚C for 6 days.

Basal fermentation medium used in carbon source

Yeast extract 0.3 g, K_2HPO_4 0.001 g, $MgSO_4$ 0.001 g, $CaCO_3$ 0.1 g, DW 100 mL and pH 6.5

Study on Different Nitrogen Sources Utilization for the Fermentation

Nitrogen sources (each 1.0 g or 1.0 mL) such as malt extract, peptone, asparagine, yeast extract, gelatin, casein, sodium nitrate, potassium nitrate, urea, ammonium nitrate, ammonium chloride and ammonium sulphate were used. Fermentation were incubated at 30˚C for 6 days.

Basal fermentation medium used in nitrogen source

Glucose 0.3 g, K_2HPO_4 0.001 g, $MgSO_4$ 0.001 g, $CaCO_3$ 0.1 g, DW 100 mL and pH 6.5

The Effect of Shaking and Static Culture for the Fermentation

100 mL conical flask containing 25 mL of the best fermentation medium was incubated on the shaker for 6 days. At the same time, another those fermentation medium was incubated under static condition without shaking. These shaking culture and static culture were compared by using agar well diffusion assay method.

The Effect of pH and Temperature on Culture of Fungus YY-13

In this study, optimum pH was studied by varying the medium pH as 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The different pH of fermentation medium was adjusted by using 0.1 M HCl and 1 M NaOH. In the effect of temperature, the selected fungus YY-13 was inoculated and incubated at five different temperatures by using 20 ˚C, 25 ˚C, 30 ˚C, 35 ˚C and 40 ˚C. The fermentation medium was assayed for antimicrobial activity.

Results and Discussion

The Effects of Carbon and Nitrogen Sources Utilization for the growth of Fungus YY-13

In the growth morphology on various carbon sources, good growth of YY-13 was found on potato powder, moderate growth soluble starch, xylose, glucose and lactose.

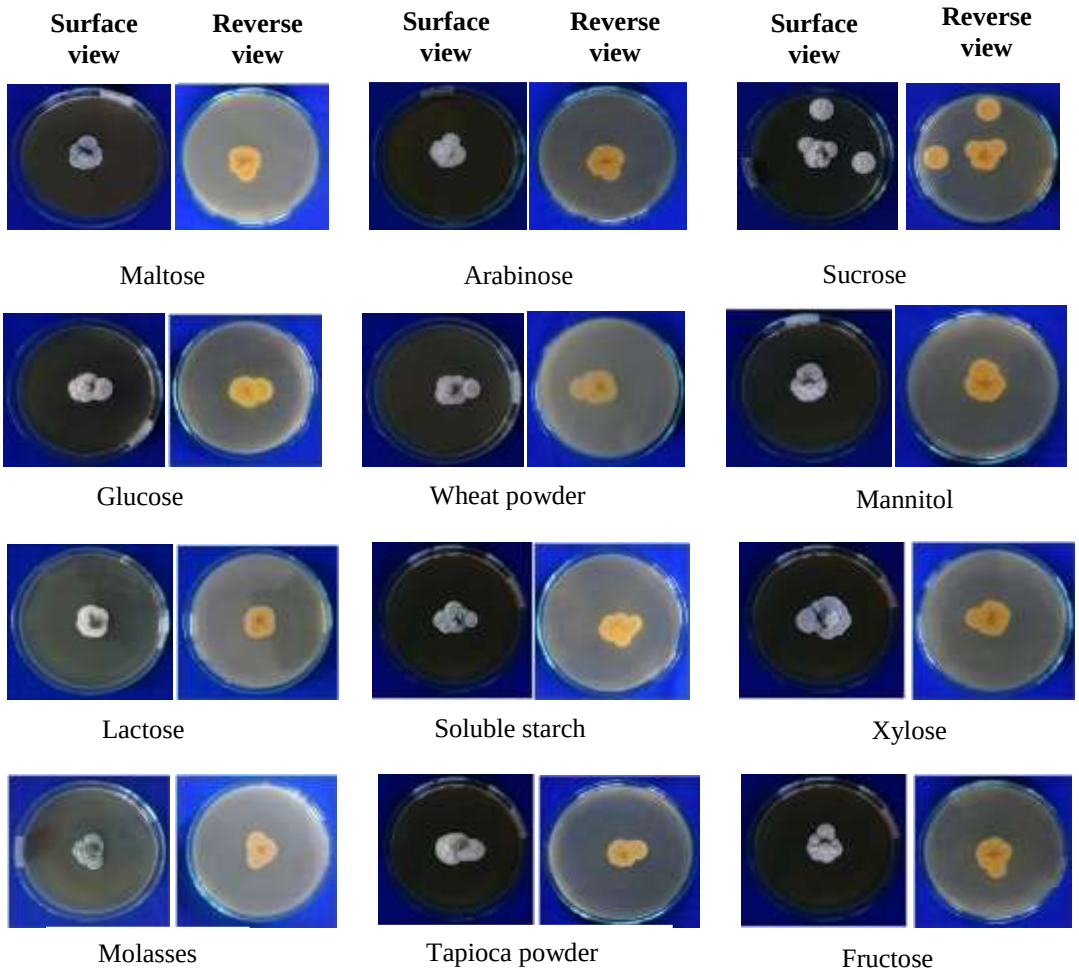
In the nitrogen sources, YY-13 were good growth on peptone, potassium nitrate, sodium nitrate, yeast extract and casein, moderate growth of ammonium chloride, asparagine, malt extract, gelatin, ammonium sulphate, ammonium nitrate, and while urea were poor growth.

As many issued (Buchanan *et al.,* 1984) and (Calvo *et al.,* 2002) support, simple sugar such as glucose, fructose, sucrose enhanced growth as well as secondary metabolite production by microorganisms slightly than complex carbon sources like starch, galactose, xylose, mannitol, etc.

Table 2 Morphological Characters of Fungus YY-13 on various Carbon Sources

No.	Carbon sources	Color		Growth size (mm)
		Surface colour	Reverse colour	
1	Glucose	White	Pale yellow	29.20-30.21
2	Tapioca powder	Pale green	Yellow	25.46-26.32
3	Potato powder	Pale green	Pale yellow	**34.03-35.06**
4	Glycerol	White	Pale yellow	24.99-26.22
5	Arabinose	Pale green	Pale yellow	24.39-25.13
6	Soluble starch	Pale green	Pale yellow	28.39-29.32
7	Maltose	Pale green	Pale yellow	23.70-24.76
8	Mannitol	White	Pale yellow	26.54-27.04
9	Molasses	Deep green	Yellow	24.80-25.68
10	Sucrose	Pale green	Pale yellow	20.50-22.37
11	Xylose	Pale green	Yellow	28.35-29.44
12	Wheat powder	Pale green	Pale yellow	23.33-24.62
13	Fructose	White	Pale yellow	26.64-27.92
14	Lactose	White	Pale yellow	27.21-28.68

20-30 mm = Moderate growth, 30-40 mm = Good, 40 to above = Excellent



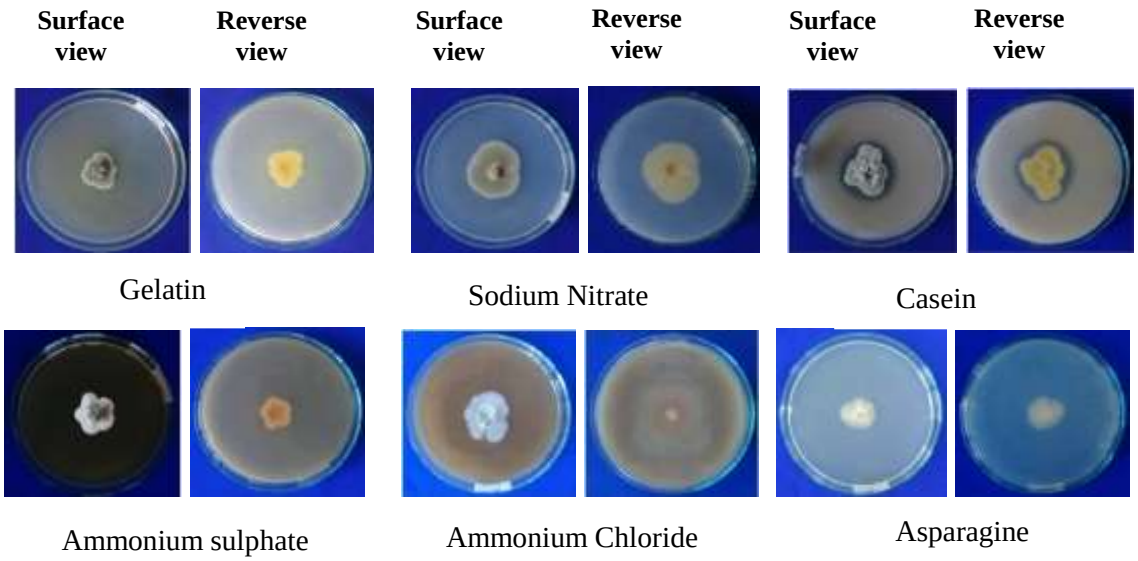
Potato powder Glycerol

Figure 1 Morphological characters of fungus YY-13 on various carbon sources

Table 3 Morphological Characters of Fungus YY-13 on various Nitrogen Sources

No.	Nitrogen sources	Color		Growth size (mm)
		Surface colour	Reverse colour	
1	Ammonium Nitrate	Grey	Pale orange	18.40-18.65
2	Potassium Nitrate	Grey	Pale orange	37.84-39.94
3	Sodium Nitrate	Grey	Pale orange	36.88-38.41
4	Malt Extract	White	Pale gray	29.20-30.06
5	Gelatin	White	Pale yellow	23.32-24.22
6	Yeast Extract	White	Gray white	24.39-25.08
7	Peptone	Brown	Pale yellow	38.65-40.79
8	Casein	White	Pale yellow	32.94-33.16
9	Ammonium Chloride	White	Pale gray	26.54-27.47
10	Asparagine	Pale yellow	White	22.91-20.64
11	Ammonium Sulphate	White	Pale grey	19.71-20.63
12	Urea	Whitish grey	Pale yellow	11.34-12.12

20-30 mm = Moderate growth, 30-40 mm = Good, 40 to above = Excellent



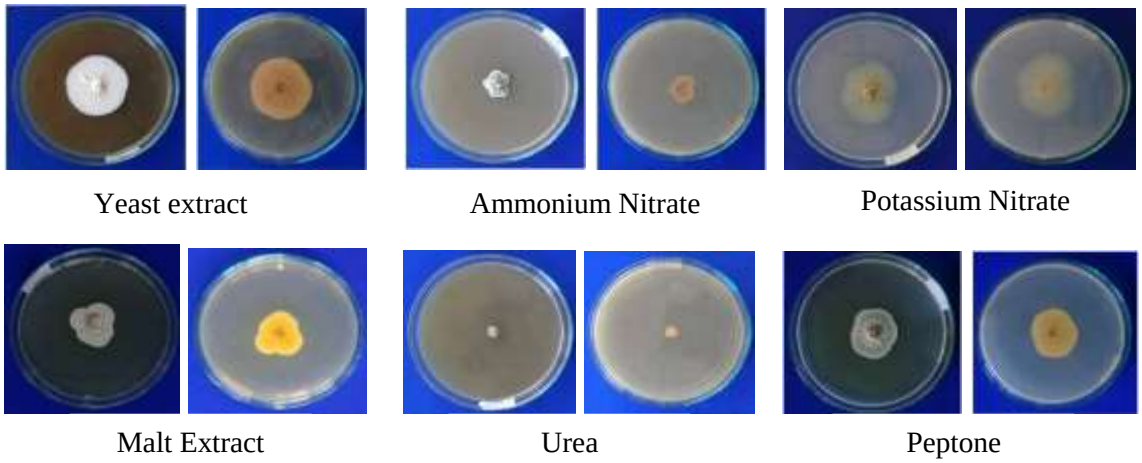


Figure 2 Morphological characters of fungus YY-13 on various nitrogen sources

Effects of Age of Inoculum on Fungus YY-13 against *E.coli*

In the effect of age of inoculum, the antimicrobial activity of fungus YY-13 was determined in different age of culture (48 hrs, 60 hrs, 72 hrs, 84 hrs, 96hrs and 108 hrs). In 84 hrs age, YY-13 showed the highest antibacterial activity (33.08 mm) followed by (32.28 mm) in 72 hrs on *E. coli*. Jain, 2010 reported that the variations in the fermentation environment often result in alteration in antibiotic production. (Table-4 and Figure-3)

Table 4 Effect of Age of Inoculum on Fungus YY-13

Ages of inoculum (hrs)	Inhibition Diameter Zone (mm)
48	22.33
60	23.81
72	32.28
84	**33.08**
96	31.37
108	30.85

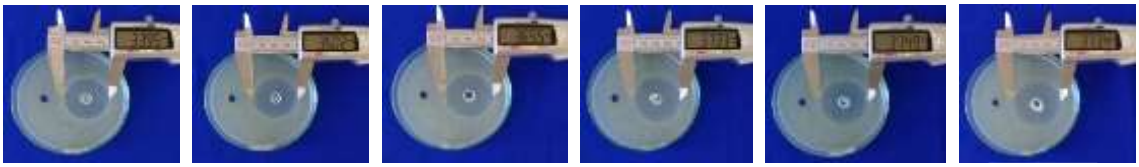
Figure 3 Inhibition zone of fungus YY-13 at different age of inoculum

Effect of Size of Inoculum on Fungus YY-13 against *E.coli*

In the study of size of inoculum, different size of inoculum (5%, 10%, 15%, 20%, 25% and 30%) 84 hrs seed culture were used. The best antibacterial activity of YY-13 was obtained by using 25% size of inoculum followed by 20% and 30% on *E. coli*, respectively. (Table-5 and Figure-4)

Table 5 Effect of the Size of Inoculum on Fungus YY-13

Size of inoculum (%)	Inhibition Diameter Zone (mm)
5	33.95
10	36.02
15	36.55
20	37.33
25	**37.49**
30	37.34



5% 10% 15% 20% 25% 30%

Figure 4 Inhibition zone of fungus YY-13 at different size of inoculum

Effect of Antibacterial Activity on Carbon Sources Utilization of YY-13 against *E.coli*

There are variation in the level of antibacterial activity, when the different carbon sources were tested in fermentation. The effects of carbon sources were different for the maximum antimicrobial metabolites productions. The highest antibacterial activity were observed by the addition of glucose (32.78 mm) and tapioca (31.23 mm) followed by potato powder (31.15 mm) and glycerol (30.78 mm) against *E. coli*. These results were shown in Table-6 and Figure-5. Bhavana, *et al.*, 2014 who observed glucose as favourable carbon source for maximum antimicrobial compound and mycelium growth of *Streptomyces carpaticus* MTCC 11062. So, it could be assigned that carbon sources was the best in the fermentation process used in this study.

Table 6 Effect of Different Carbon Sources Utilization of YY-13 against *E. coli*

No.	Carbon sources	Inhibition Diameter Zone (mm)
1.	Lactose	22.69
2.	Glycerol	30.78
3.	Glucose	**32.78**
4.	Mannitol	29.72
5.	Fructose	27.23
6.	Sucrose	29.04
7.	Maltose	30.04
8.	Arabinose	30.62
9.	Soluble starch	30.37
10.	Molasses	29.41
11.	Xylose	28.28
12.	Tapioca powder	31.23
13.	Potato powder	31.15
14	Wheat powder	27.98

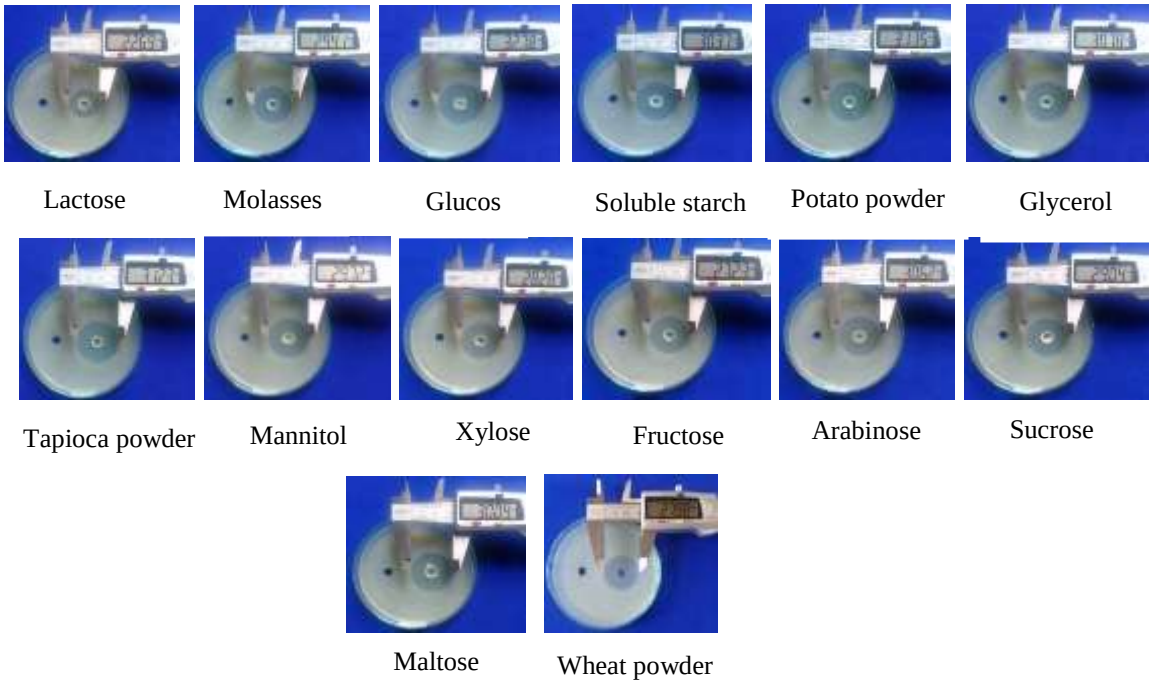


Figure 5 Inhibition zone of fungus YY-13 on various carbon sources

Effect of Antibacterial Activity on Nitrogen Sources Utilization of YY-13 against *E.coli*

In this study, the addition of malt extract, potassium nitrate, sodium nitrate, gelatin, casein displayed the greatest activity. These results were shown in Table-7 and Figure-6. Ismaiel, *et al.*, 2010 reported that sodium nitrate as best nitrogen sources for the production of metabolite by fungus *Fusarium roseum*. According to the observation, it could be denoted that nitrogen sources was the best in the fermentation medium.

Table 7 Effect of Different Nitrogen Sources Utilization of YY-13 against *E. coli*

No.	Carbon sources	Inhibition Diameter Zone (mm)
1.	Ammonium Nitrate	16.38
2.	Potassium Nitrate	26.02
3.	Sodium Nitrate	**28.39**
4.	Malt Extract	25.61
5.	Gelatin	21.42
6.	Yeast Extract	17.05
7.	Peptone	17.93
8.	Casein	20.00
9.	Ammonium Chloride	14.04
10.	Asparagine	16.38
11.	Ammonium Sulphate	12.10
12.	Urea	+

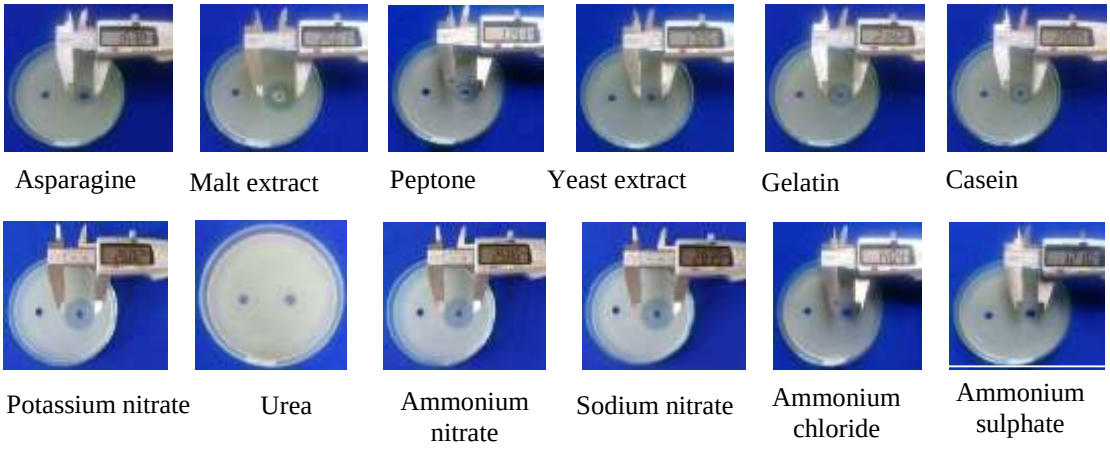


Figure 6 Inhibition zone of fungus YY-13 on various nitrogen sources

Comparison between Shaking Culture and Static Culture of Fermentation Optimization

In this investigation, the comparison between shaking culture and static culture were carried out. The maximum production of antimicrobial metabolite of shaking culture showed the inhibitory zone 20.38 mm against *E. coli.* The static culture of fermentation broth showed activity 26.91 mm and 25.76 mm against *E. coli* (Figure-7). Stinson *et al.*, 2003 observed that maximum growth and production of antimicrobial agent was recorded after the fungus reached its stationary phase. From the observation, it could be remarked that the finding results were agreeable with literature.

(A) Static (B) Shaking

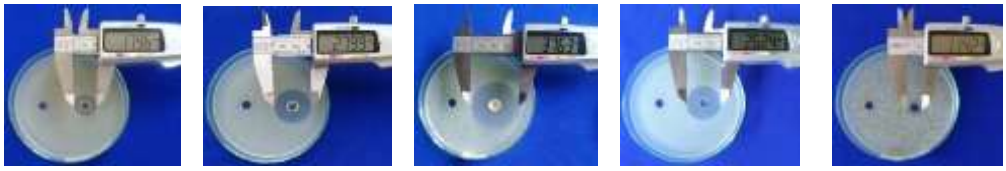
Figure 7 Inhibition zone of YY-13 in shaking and static culture

Effects of Incubation Temperature on Culture of YY-13

In this study, the effect of incubation temperature on antibacterial activity of YY-13 was determined by changing the temperature at 20 ˚C, 25 ˚C, 30 ˚C, 35 ˚C and 40 ˚C. The optimum temperature was found at 30 ˚C (37.63 mm) against *E. coli.* Incubation temperature is known to influence directly the overall growth and development of any organism. It affects the physiology and subsequently the synthesis of various metabolites (Pandey, *et al.*, 2008). The finding of present study (Table-8 and Figure-8) could be assigned that it is agreeable with the literature preview on Gunasekaran and Poorniammal, 2008 have reported highest secondary metabolite production at a temperature of 30 ˚C in their study.

Table 8 Effects of Incubation Temperature on Culture of YY-13

Temperature (˚C)	Inhibition Diameter Zone (mm)
20	17.96
25	27.99
30	**37.63**
35	26.24
40	11.42



20 ˚C **25 ˚C** **30 ˚C** **35 ˚C** **40 ˚C**

Figure 8 Inhibition zone of YY-13 at different temperature

Effect of pH Utilization for YY-13

In the present study, the antimicrobial activity of YY-13 was investigated by varying the pH, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The maximum antimicrobial activity of YY-13 (38.10 mm) against *E. coli* was found at pH 6.0. These results are shown in Table-9 and Figure 9. Similarly, the effect of culture medium on mycelial growth, metabolite profile and antimicrobial compound yield by a marine derived fungus *Arthrinium* c.f. *saccharia* was investigated by Maio *et al*., 2006.

Table 9 Effect of Different pH on culture of YY-13

pH	Inhibition Diameter Zone (mm)
4.0	35.10
4.5	36.46
5.0	37.68
5.5	37.78
6.0	**38.10**
6.5	37.89
7.0	37.26
7.5	36.81
8.0	35.92

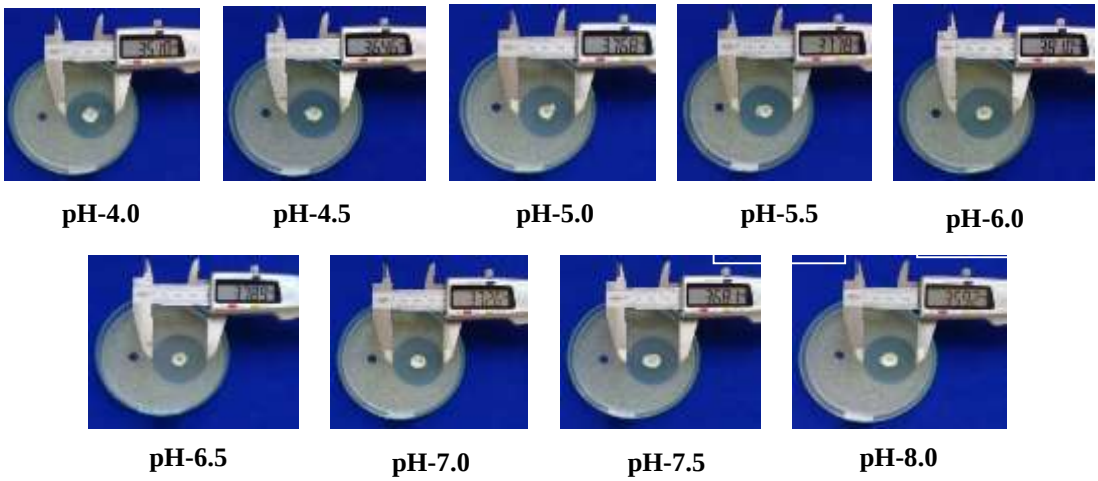


Figure 9 Inhibition zone of YY-13 at different pH

Conclusion

In this study, colony morphology and maximum metabolite production of YY-13 were investigated. The supplement of carbon and nitrogen sources effect the growth of colony morphology. In the fermentation studies, it was found that 84 hrs age of inoculum and 25% of size of inoculum were suitable for fermentation. The antibacterial substance production of YY-13 was influenced by addition of glucose and sodium nitrate.

In this study, the optimum temperature of incubation was found at 30 ˚C against *E. coli* (37.63 mm). In the present study, the highest antibacterial activity of YY-13 were found at pH 6.0 against *E. coli* (38.10 mm). The fermentation broth was studied at two conditions such as shaking culture (20.38 mm) and static culture (26.91 mm) on *E. coli*.

It can be concluded that this research may be supported for maximum production of antibacterial metabolite. In fact, this paper can contribute to the prevention and treatment of the bacterial diseases related to the *E. coli*.

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