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EFFECT OF DIFFERENT NITROGEN SOURCES, INOCULUM SIZE AND FERMENTATION VESSEL SIZE ON ANTIMICROBIAL ACTIVITY OF *ASPERGILLUS IBERICUS*

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ABSTRACT

The present study highlights the effect of different nitrogen sources and their concentration, inoculum size and size of vessel on bioactivity of rhizosphere soil fungi *Aspergillus ibericus* from *Ficus religiosa* against opportunistic pathogens, through the production of secondary metabolite exhibiting antimicrobial activity. The various organic (peptone) and inorganic (ammonium nitrate, ammonium chloride and sodium nitrate) nitrogen sources were used in the fermentation medium. The optimization of inoculum size for production of antimicrobial metabolite was carried out by using fungal disks of various sizes viz. 10mm, 12mm, 14mm, 16mm and 18mm cut from four days old colony of *A. ibericus* added as inoculum in each flask. Antimicrobial metabolite obtained from *A. ibericus* was found to be effective against test microbes when dextrose was used as a carbon source. The optimization of size of fermentation vessel on antimicrobial metabolite production was carried out by taking Erlenmeyer's flasks of different sizes viz. 100, 250, 500 and 1000ml, respectively. The fungus exhibited maximum antimicrobial activity in the presence of unmodified potato dextrose broth without any nitrogen source. Fungal disk size of 10mm was found to be optimum for maximum yield of antimicrobial metabolite. Whereas, 250ml vessel size was

found to be the optimized size for fermentation on small scale in laboratory for production of fungal bioactive metabolite by strain *A. ibericus*.

Keywords: Rhizosphere Soil, Oppurtunistic Pathogens, *Aspergillus ibericus*, Nitrogen sources, inoculum size, fermentation vessel size

INTRODUCTION

Antibiotics are the most important pillar of current medications **[1]**. The antimicrobial compounds have been found to cure various kind of bacterial and fungal infections, but the discovery of these agents has been tempered by appearance of resistant microbial pathogens. Emergence of drug resistance in them has emphasized the need of research for new compounds. Antibiotics nowadays are taken for granted to treat bacterial infections. The antibiotics are successfully used for prophylactic or therapeutic purposes regularly in clinical, veterinary and agricultural purposes. However, overconsumption of antibiotics caused an enormous selective pressure on the bacteria to gain resistance or die **[2]**. The increasing frequency of multi-resistant pathogenic bacteria has compromised the clinical treatment of an emerging infectious diseases. There is an urgent demand for new antimicrobial compounds active against current resistant pathogens and emerging pathogens **[3]**.

Nature and concentration of some components in fermentation medium have a significant effect on antimicrobial metabolite production. Influence of particular nutrients on the antimicrobial compound biosynthesis is determined by the chemical structures of antimicrobial substances. When carbon or nitrogen source is a limiting factor, growth is rapidly reduced and the antimicrobial metabolite biosynthesis takes place in the stationary phase. In other cases, antimicrobial substance production is associated with the growth phase. Thus, some nitrogen sources can be incorporated in antimicrobial molecules as precursors or their amino groups transfer to specific intermediate products **[4]**. The culture medium should provide energy, carbon and nitrogen sources, and minerals for cellular growth and natural product biosynthesis. As a nitrogen source, some fungal species can use amino acids like aspartate, arginine, and histidine **[5]** or phenylalanine, isoleucine, methionine, and tyrosine **[6]**. Fungi and actinomycetes can also use some organic and inorganic nitrogen sources for growth and production of antimicrobial components. Ripa *et al*. **[7]** has suggested that the highest activity of *Streptomyces* sp. was obtained with yeast extract as a nitrogen source. Adinarayana *et al*. **[8]** in their research discovered that among the nitrogen sources, corn steep was best followed by soybean meal and sodium glutamate, while sodium nitrate and soya peptone showed similar titers. In general, cultivation medium should contain nutrients easily available in the market and, if possible, relatively inexpensive **[9]**. In accordance with the previous facts, there is diversity in nitrogen sources that can be metabolized by different fungal species. Thus, each research should start with defining the culture medium composition, especially carbon and nitrogen sources as the most important macronutrients for each bioprocess. The present study highlights the effect of nitrogen sources and their concentration, inoculum size and fermentation vessel size on antimicrobial metabolite production by rhizospheric soil fungus *Aspergillus ibericus* obtained from *Ficus religiosa* against opportunistic pathogens.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from rhizosphere of medicinal plant Peepal (*Ficus religiosa*) from Botanical garden, Kurukshetra University, Kurukshetra, by removing 1-1.5 inch of top soil with sterilized spatula.

Isolation of *Aspergillus ibericus* from soil

The serial dilution agar plate method was used for isolation of *Aspergillus ibericus* from soil sample **[10, 11]**. Potato dextrose agar (PDA) (CDH) for fungi was used as isolation medium. One gram of soil (finely pulverized and air dried) was suspended in 9ml sterilized distilled water blank no. 1 and shaken vigorously to obtain uniform suspension. 1ml of suspension was transferred, while in motion, from stock suspension (No. 1) to sterile water blank no. 2 with sterile pipette under aseptic conditions to make 1:100 (10^{-2}) dilution. For fungi, 10^{-2} to 10^{-5} dilutions were used. Approximately 20-25ml cooled (45°C) molten nutrient agar and potato dextrose agar was added to each petri plate. After solidification of agar media, 100µl aliquots of suspension of dilutions were added to labeled and sterilized petriplates and spread with spreader. Inoculated plates were incubated at 30°C for 4-5 days for fungi.

Purification and maintenance of isolates

Fungal colonies appearing on their respective media were transferred to potato dextrose agar plates (one colony on each plate) at 30°C for 4-5 days. The colonies were then transferred on potato dextrose agar slants and incubated at 30°C for 4-5 days for fungi and were maintained at 4°C in a refrigerator for further studies.

Effect of nitrogen sources on antimicrobial metabolite production

For evaluating the most suitable nitrogen source for antimicrobial metabolite production, potato dextrose broth was supplemented with various organic (peptone) and inorganic (ammonium nitrate, ammonium chloride and sodium nitrate) nitrogen sources. Each flask with 100ml of potato dextrose broth supplemented with different nitrogen sources was autoclaved at 121°C for 15 minutes. For each source, three replicates were used. Unmodified broth was used as control. Three disks cut from four days old colony of selected fungus were added as inoculum in each flask. The inoculated flasks were incubated at 30°C for 12-14 days under stationary condition. The extracts after filtration with filter paper were assayed for antimicrobial activity against test microbes by using agar well diffusion method **[12]**.

Effect of inoculum size on antimicrobial metabolite production

The optimization of inoculum size for production of antimicrobial metabolite was carried out by using disks of various sizes viz. 10mm, 12mm, 14mm, 16mm and 18mm cut from four days old colony of selected fungus added as inoculum in each flask. The inoculated flasks were incubated at 30°C for 12-14 days under stationary condition. The extracts after filtration with filter paper were assayed for antimicrobial activity against test microbes by using agar well diffusion method **[13]**.

Effect of fermentation vessel size on antimicrobial metabolite production

The optimization of size of fermentation vessel on antimicrobial metabolite production was carried out by taking Erlenmeyer's flasks of different sizes viz. 100, 250, 500 and 1000ml. 50, 100, 200 and 400ml of potato dextrose broth was taken in Erlenmeyer's flasks of various sizes viz. 100, 250, 500 and 1000ml respectively and autoclaved at 121°C for 15 minutes. For each vessel size, three replicates were used. Three disks of cut from four days old colony of selected fungus were added as inoculum in each flask. The inoculated flasks were incubated at 30°C for 12-14 days under stationary condition. The extracts after filtration with filter paper were evaluated for antimicrobial activity against test microbes by using agar well diffusion method **[13]**.

Statistical analysis

The data obtained from experiments of nitrogen source and their concentration, inoculum size and fermentation vessel size were subjected to analysis of variance (One Way ANOVA) to evaluate the significance of each parameter by estimating p-value and f-value **[14]**.

RESULTS AND DISCUSSION

In the present study, the fungus *Aspergillus ibericus* was isolated from rhizosphere soil of medicinal plant *Ficus religiosa* and was studied for effect of nitrogen sources and their concentration, inoculum size and fermentation vessel size on antimicrobial potential against test microbes *B. subtilis, S. aureus, S. mutans, S. pyogenes, P. aeruginosa, E. coli, C. albicans* and *C. tropicalis* by agar well diffusion method, which could cause opportunistic infections in host.

Changes in the type and concentration of nitrogen source influence significantly the antibiotic production **[15]**. In the present study, various nitrogen sources such as ammonium nitrate, ammonium chloride, sodium nitrate, peptone were added in PDB to optimize the nitrogen source for antimicrobial compound production by strain *A. ibericus*. Unmodified PDB was kept as control. The zone of growth inhibition was found in range of 0mm to 17mm (ammonium chloride), 0mm to 13mm (sodium nitrate) and 0mm to 13mm (peptone). The broth amended with ammonium nitrate showed no activity against any of the test microbe. Whereas unmodified PDB (no nitrogen source) showed maximum antimicrobial activity (18mm to 26mm). Therefore, addition of nitrogen source supported the growth of fungus (*A. ibericus*), but inhibit the antimicrobial activity.One-way ANOVA analysis at 5% significance level shows calculated F value (20.13) greater than F critical value (2.64) and P value (1.11E-08) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. **Table 1** and **Figure 1** shows antimicrobial activity of fungus *A. ibericus* incubated in unmodified media and media supplemented with different nitrogen sources. **Figure 2** shows antimicrobial activity of fungus *A. ibericus* incubated in unmodified media and media supplemented with different nitrogen sources against test microbes *S. pyogenes* and *S. aureus*. Vahidi *et al*. **[16]** also reported lower antifungal activity of *Mycena leptocepahala* when NH_4Cl and $NaNO_3$ were used as nitrogen sources. Holkar *et al*. **[17]** optimized the nitrogen source for improved biomass and antibiotic production by *Streptomyces spectabilis* and found that peptone supported the maximum antibiotic production. In comparison with the inorganic nitrogen sources, organic nitrogen sources induced comparatively higher biomass as well as antimicrobial metabolite production. In contrary, peptone in present study inhibited the antimicrobial activity.

The inoculum size also affected the ability of the strain to produce antimicrobial compound. Lower inoculum size subjects any microbial culture to multiply at slower rate and hence insufficient utilization of substrate to produce metabolites. Presence of abundant spores in inoculum facilitates rapid proliferation and biomass synthesis. Beyond a certain inoculum load, enzyme production may decrease due to depletion of nutrients and developed oxygen tension in the medium resulting from enhanced biomass. These factors may individually or collectively result in decrease of metabolic activity **[18]**. Adequate inocula can initiate fast mycelium growth and product formation, thereby reducing other organism's contamination **[19]**.

Disk size of 10mm, 12mm, 14mm, 16mm and 18mm cut from seven days old colony of strain *A. ibericus* were inoculated in 250ml flask to visualize the effect of disk size on the production of antimicrobial metabolite. Disk size of 10mm was found to be optimum for maximum yield of antimicrobial metabolite as compared to 12, 14, 16 and 18mm. The diameter of zone of inhibition was 27mm against *C. albicans* and *S. pyogenes*, 26mm against *P. aeruginosa*, 25mm against *S. mutans*, 24mm against *B. subtilis*, *S. aureus* and *E. coli* and 22mm against *C. tropicalis*. When 12mm fungal disk was used, the antimicrobial activity measured by diameter of zone of inhibition was decreased (16 to 21mm), similar was the case with 14mm disk (18 to 24mm), 16mm disk (18 to 22mm) and 18mm disk (19 to 22mm). One-way ANOVA analysis at 5% significance level shows calculated F value (13.22) greater than F critical value (2.64) and P value (1.16E-06) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Antimicrobial activity of fungus *A. ibericus* incubated with different disk size is shown in **Table 2** and **Figure 3**. Antimicrobial activity of fungus *A. ibericus* incubated with different disk sizes against test microbes *S. mutans*, *P. aeruginosa* and *S. pyogenes* is shown in **Figure 4**.

The fermentation vessel size is also an important factor for growth and metabolic activity of microorganisms and subsequently for production of bioactive metabolites. The selection of fermentation vessel for the fermentation depends upon the ease of oxygen transport, adequate liquid mixing, convenience in handling and familiarity with the type of microbe used for fermentation. The experimental fermentation conditions as regulated in flasks at small scale in laboratory, are employed for the operation of the large production fermenters **[13]**.

The present study revealed that different vessel sizes of 100ml, 250ml, 500ml and

1000ml has significant effect on yield of metabolite produced by strain *A. ibericus*. As in 250ml vessel size, bioactive metabolite showed maximum activity (28mm against *B. subtilis*, 27mm against *S. aureus*, 23mm against *S. mutans*, 26mm against *S. pyogenes*, 27mm against *P. aeruginosa*, 28mm against *E. coli*, 24mm against *C. albicans*, 25mm against *C. tropicalis*) followed by 100ml vessel size (27mm against *B. subtilis*, 26mm against *P. aeruginosa* and *E. coli*, 25mm against *S. aureus*, *S. pyogenes,* 23mm against *C.tropicalis*, 22mm against *C. albicans* and 20mm against *S. mutans*). In 500ml vessel size, metabolite showed activity 25mm against *P. aeruginosa*, 24mm against *E. coli*, 23mm against *B.subtilis*, *S. aureus* and *S. pyogenes*, 21mm, 19mm and 18mm against *C. albicans*, *C. tropicalis* and *S. mutans* respectively. Antimicrobial activity of metabolite decreased in vessel size of 1000ml in range of 17mm to 24mm. One-way ANOVA analysis at 5% significance level shows calculated F value (13.97) greater than F critical value (2.94) and P value (9.34E-06) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Antimicrobial activity of fungus *A. ibericus* incubated in different vessel size is shown in **Table 3** and **Figure 5**. **Figure 6** shows antimicrobial activity of fungus *A. ibericus* inoculated in fermentation vessels of different sizes against test microbes *S. mutans*, *S. pyogenes* and *S. aureus*. 250ml vessel size was found to be the optimized size for fermentation on small scale in laboratory for production of fungal bioactive metabolite by strain *A. ibericus*. However, Jain and Pundir **[13]** found 500ml vessel size as optimum for fermentation of *Aspergillus terreus* for antimicrobial metabolite production.

Table 1: Optimization of nitrogen source and antimicrobial activity

Nitrogen source	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
Ammonium nitrate	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
Ammonium chloride	0±0.00	16.66±0.57	14.66±0.57	15.66±0.57	0±0.00	12.66±0.57	15.66±0.57	14.00±0.00
Sodium nitrate	0±0.00	12.33±0.57	0±0.00	12.00±0.00	12.66±0.57	0±0.00	0±0.00	11.33±0.57
Peptone	0±0.00	12.66±0.57	11.66±0.57	13.00±1.00	0±0.00	0±0.00	0±0.00	0±0.00
Unmodified PDB	22.00±0.00	23.33±0.57	20.00±0.00	24.33±0.57	21.33±0.57	20.00±0.00	25.66±0.57	18.33±0.57

Values are mean inhibition zone ± Standard deviation of three replicates
Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcusmutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

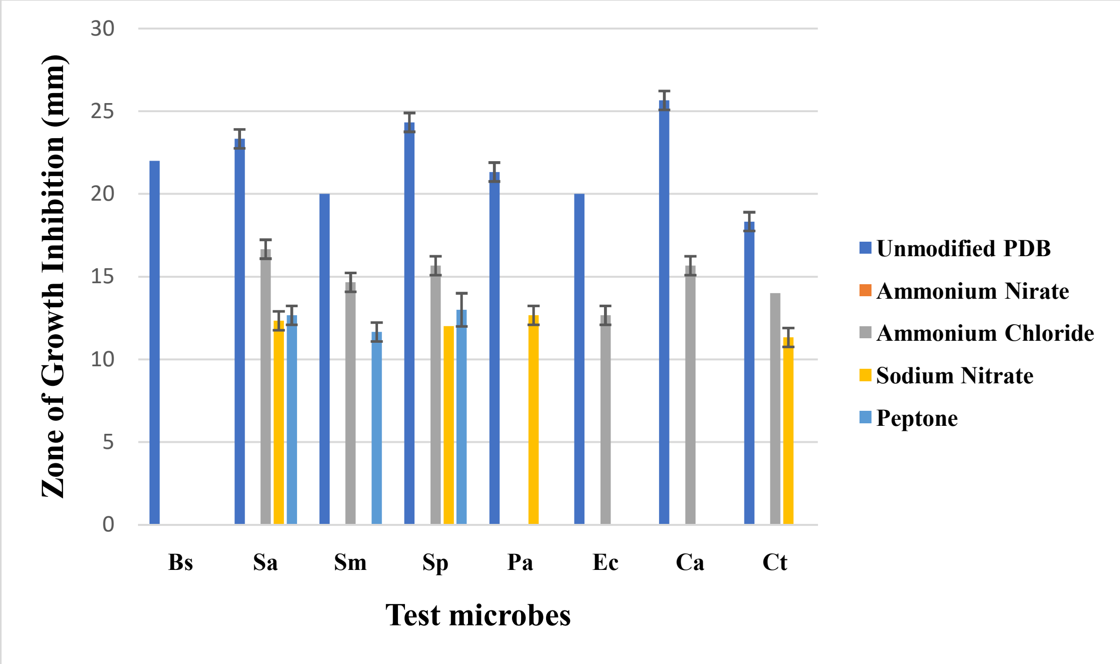

Figure 1: Optimization of nitrogen source and antimicrobial activity Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Sm: *Streptococcusmutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different

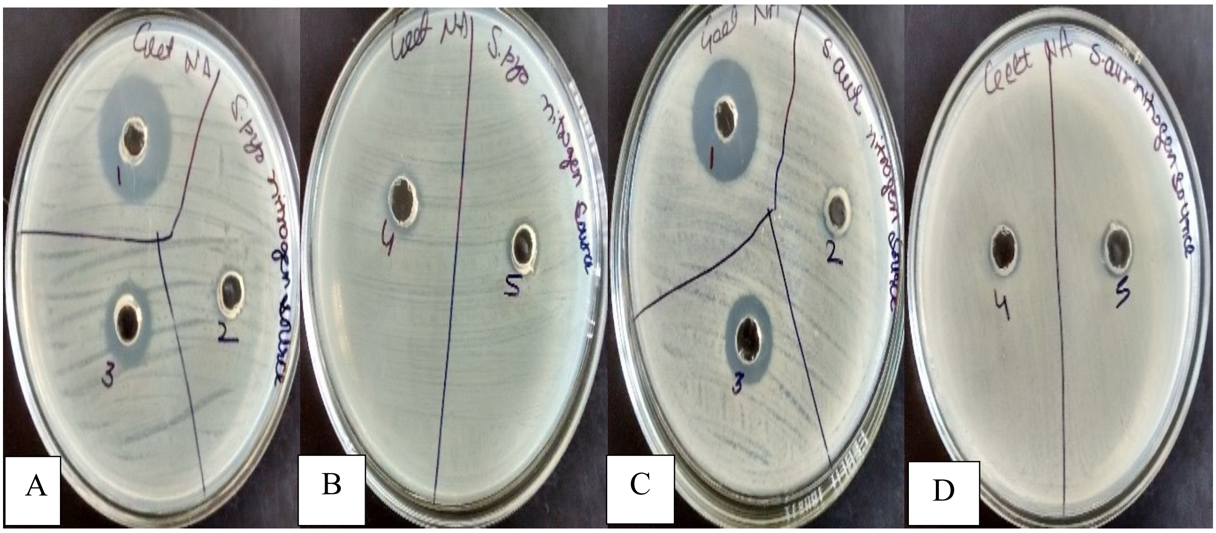

Figure 2: Optimization of nitrogen source and antimicrobial activity against test microbes A),B) *S. pyogenes* and C), D) *S. aureus*; 1: Unmodified PDB, 2: Ammonim nitrate, 3: Ammonium chloride, 4: Sodium Nitrate and 5: Peptone

Table 2: Optimization of inoculum size and antimicrobial activity

Inoculum size	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
10mm	24.33±0.57	24.00±0.00	25.33±0.57	27.33±0.57	22.66±0.57	24.00±0.00	27.00±1.00	22.66±0.57
12mm	21.00±1.00	18.66±0.57	18.66±0.57	21.66±0.57	19.66±0.57	19.66±0.57	20.00±1.00	16.33±0.57
14mm	21.00±0.00	18.33±0.57	19.66±0.57	23.66±0.57	23.00±1.00	22.66±0.57	24.66±0.57	19.66±0.57
16mm	22.66±0.57	21.00±1.00	21.66±0.57	21.66±0.57	20.66±0.57	21.33±0.57	19.33±0.57	18.66±0.57
18mm	22.66±0.57	19.66±0.57	20.66±0.57	21.00±0.00	19.66±0.57	22.33±0.57	21.66±0.57	19.33±0.57

Values are mean inhibition zone ± Standard deviation of three replicates

Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcusmutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

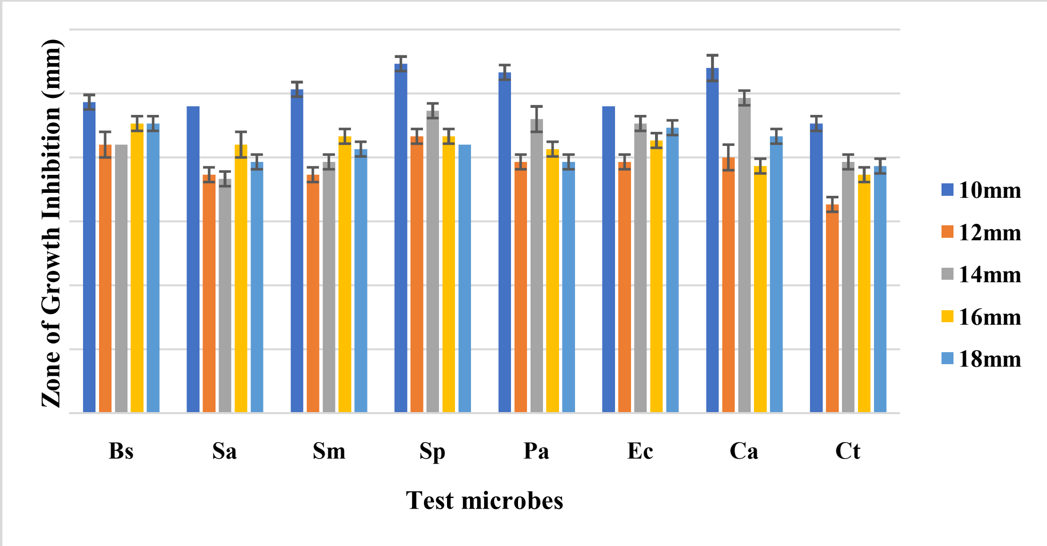

Figure 3: Optimization of inoculum size and antimicrobial activity Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichiacoli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different

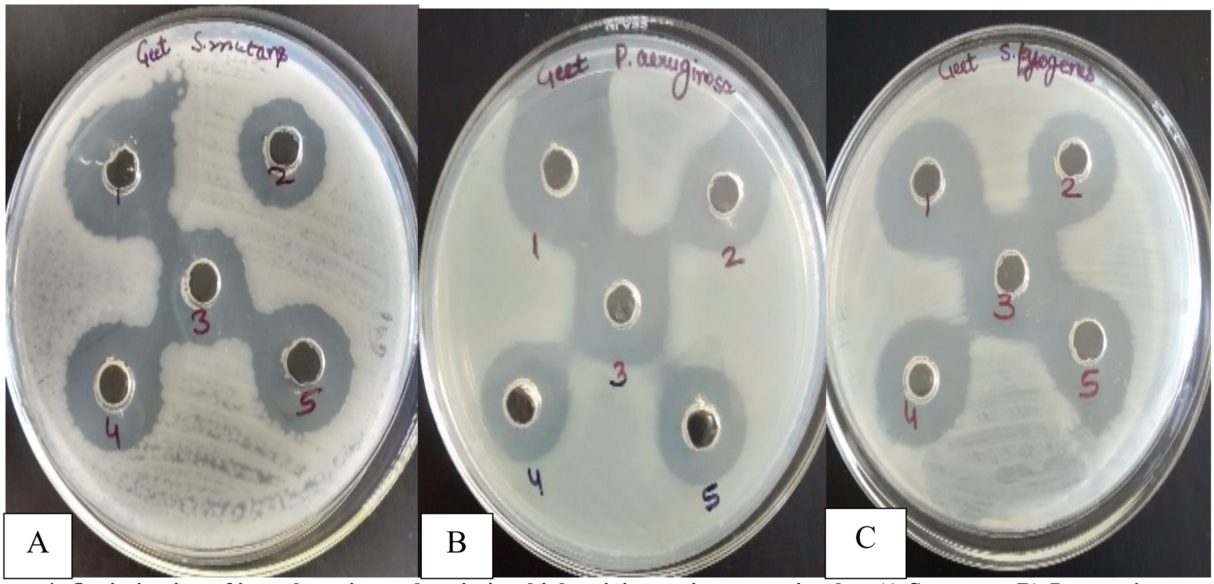

Figure 4: Optimization of inoculum size and antimicrobial activity against test microbes A) *S. mutans*, B) *P. aeruginosa* and C) *S. pyogenes*; 1: 10mm; 2: 12mm; 3: 14mm; 4: 16mm and 5: 18mm

Table 3: Optimization of fermentation vessel size and antimicrobial activity

Vessel size	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
100ml	26.66±0.57	25.00±0.00	20.33±0.57	25.00±0.00	25.33±0.57	25.33±0.57	22.66±0.57	23.33±0.57
250ml	27.33±0.57	26.33±0.57	23.33±0.57	26.66±0.57	26.33±0.57	28.00±0.00	25.00±1.00	25.00±0.00
500ml	23.33±0.57	22.66±0.57	17.33±0.57	22.33±0.57	24.66±0.57	23.66±0.57	21.66±0.57	18.66±0.57
1000ml	20.00±0.00	23.00±1.00	16.00±1.00	21.66±0.57	19.66±0.57	20.66±0.57	18.33±0.57	16.66±0.57

Values are mean inhibition zone ± Standard deviation of three replicates
Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcusmutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

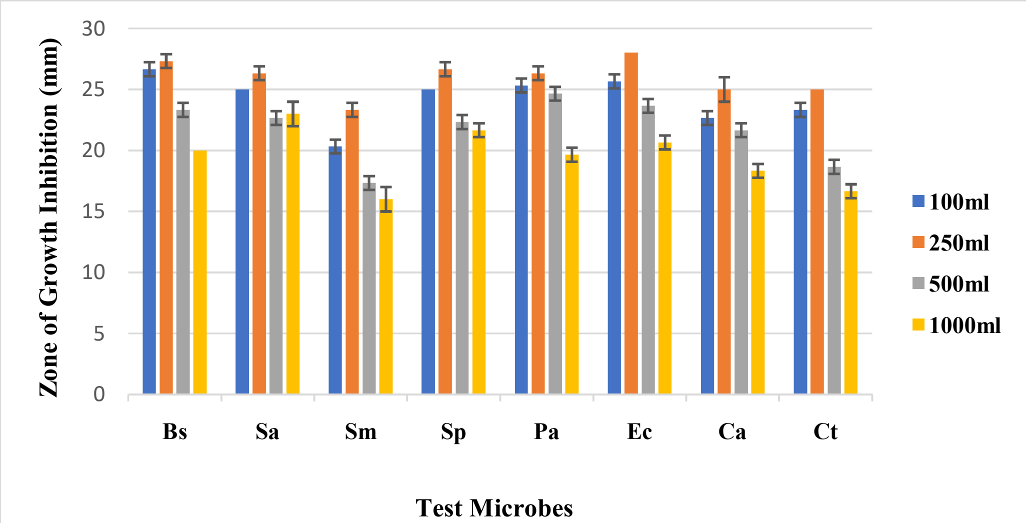

Figure 5: Optimization of fermentation vessel size and antimicrobial activity Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichiacoli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca:*Candida albicans;* Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different

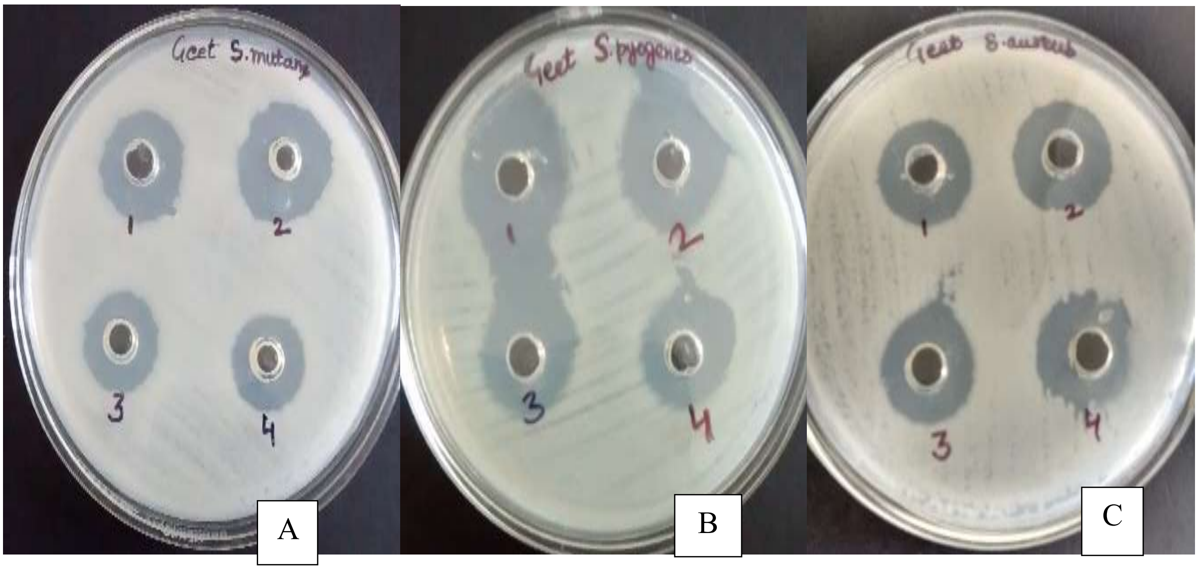

Figure 6: Optimization of fermentation vessel size and antimicrobial activity against test microbes A) *S. mutans*, B) *S. pyogenes* and C) *S. aureus*; 1: 100ml, 2: 250ml, 3: 500ml and 4: 1000ml

CONCLUSION

It may be concluded that fungus *Aspergillus ibericus* isolated from rhizosphere soil of medicinal plant *Ficus religiosa* is a promising source of antimicrobial metabolite and exhibits maximum antimicrobial activity in the presence of unmodified potato dextrose broth. Therefore, addition of nitrogen source supported the growth of fungus (*A. ibericus*), but inhibit the antimicrobial activity. Fungal disk size of 10mm was found to be optimum for maximum yield of antimicrobial metabolite. Whereas, 250ml vessel size was found to be the optimized size for fermentation on small scale in laboratory for production of fungal bioactive metabolite by strain *A. ibericus*. It may also be suggested that further research is needed to determine the cytotoxicity and in vivo efficacy against opportunistic pathogens before it is used for commercialization purpose.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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