Punjab University Journal of Zoology

OPEN ACCESS

37(2): 175-182 (2022)
https://dx.doi.org/10.17582/journal.pujz/2022.37.2.175.182

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Research Article

Isolation and Identification of Antifungal Bacteria from Rhizosphere of Citrus Field

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Article History
Received: October 08, 2022
Revised: 25 November 2022
Accepted: December 18, 2022
Published: December 28, 2022

Authors' Contributions
AAK conducted the experiments and wrote the manuscript. MI conceived the study design, supervised the study and did final editing of manuscript.

Keywords
Molecular identification, *Bacillus subtilis* 2i, *Schizophyllum* sp. B2A, Antifungal activity, Fermentation

Abstract | Fruit rotting is major problem in the world and the fungus mostly causes it. Citrus is susceptible to postharvest decay caused by fungus. The use of synthetic fungicides, which have negative effects on human health and the environment, reduces the widespread economic losses in citriculture caused by these pathogens. In the current study, 68 bacterial strains were isolated from citrus field soil and tested for antifungal activity using the streak plate method. Among them, one bacterium was found to be antagonist against the pathogen. This was identified as *Bacillus subtilis* 2i through 16SrRNA. The disease causing fungus was also isolated and identified as *Schizophyllum* sp. B2A through 18SrRNA. The strain *Bacillus subtilis* 2i was cultivated in sub-merged fermentation for the production of bioactive compounds. Various parameters were optimized to obtain maximum bioactive compounds production. Maximum antifungal activity was observed at temperature 37°C, pH 8, inoculum size 2.5% and 24h fermentation period. These results recommended the potential utilization of *Bacillus subtilis* 2i for intention to control the disease causing fungus *Schizophyllum* sp. B2A in citrus.

Novelty Statement | It is the first antifungal bacteria reported in this region. It is helpful in the control of fungal diseases in citrus.

To cite this article: Khan, A.A., and Irfan, M., 2022. Isolation and identification of antifungal bacteria from rhizosphere of citrus field. *Punjab Univ. J. Zool.*, **37**(2): 175-182. https://dx.doi.org/10.17582/journal.pujz/2022.37.2.175.182

Introduction

The rhizospheric soil contains numerous species of fungi and bacteria living innocently. *Bacillus* belongs to gram-positive bacteria. It is found primarily in soil, water, dust and air. They belong to bacterial domain and its phylum, class, order, family and genus are *Birmicutes*, *Bacilli*, *Bacillales*, *Bacillaceae* and *Bacillus,* respectively. The *Bacillus* genus contains 377 species of rod shaped gram positive bacteria (Alina *et al*., 2015). *Bacillus* can lessen them to oval endospore and retain in this condition for years, under stressful condition, they form spores. It consists of free-living parasitic and non-parasitic pathogenic species. The peptide bioactive compounds are produced by *Bacillus* through non-ribosomal and ribosomal mechanism (Dobbelaere *et al*., 2003). The plants root system is mainly affecting the rhizosphere zone with comparison with bulk soil, enrich with nutrients, it is due to the reason because it contains amino acid and sugars coming from decaying exudates of plants, providing the nutrients and energy to the plants by the bacteria (Gray and Smith, 2005).

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Bacteria are the main component of the soil. The various biotic activities are carried out by these bacterial strains and make the soil fruitful for the yield of nutrient and enhancement of crops production (Ahemad and Kibret, 2014). The nutrients in soils is recycled by bacteria which is effective in plants growth stimulation, various plant growth regulators are also produced by bacteria some rhizobacteria are acting as biological control for various pathogen and this method is very effective in controlling the soil and environmental pollution (Ahemad and Kibret, 2014). Rhizobacteria can increase the growth of plants under non favorable conditions. The plant growth is promoted by these microbes through hormones and nutritional balance, nutrients solubilizing regulation and plants pathogens resistance (Braud *et al*., 2009). The rhizobacteria strains belong to *Azospirillum, Bacillus*, *Enterobacter, Burkhobacter*, *Acinetobacter, Arthrobacter, Flavobacterium, Serratia* and *Beijerinckia* enhance the production of crops (Bharti *et al*., 2013). The great interest is present for the use of useful microorganism in agriculture due to beneficial nature of microbes as growth promoter of shoots and roots and resistance against disease. Many of the crop species such as tomatoes, tobacco, cucumbers, wheat, bell peppers, barleys and *Brassica junicea* are benefitted by the practice of rhizobacteria (Kang *et al*., 2014).

The rhizobacteria, which are present around the soil of plants are more effective and versatile in solubilization, mobilization and the nutrients transformation compare to bulk soil (Ahemad and Kibret, 2014). The soil bacteria have great contribution in soil fertility and nutrient recycling (Glick, 2012). This approach of using rhizobacteria is very effective and strongly recommended and liked by environmentalists and agronomists for improving the soil fertility. In this context scientist are mainly focused on such types of rhizobacteria strains which are having novel characters like pest degradation, metal detoxification, production of bio control substances, phosphate solubilization, nitrogenase activity, ammonia production and cellulose production (Ahemad and Khan, 2012; Jahanian *et al*., 2012; Glick, 2012). The various stresses like herbicides, insecticides, fungicides and salinity are greatly effecting the plants growth and development, which is eliminated by using the rhizobacteria as bioinoculants (Mayak *et al*., 2004). The method for growth promotion in plants by rhizobacteria is not completely identified, the above mentions properties are reported in the development and growth of plant (Khan *et al*., 2009; Zaidi *et al*., 2009). *Bacillus* is used as biological control among all bacteria (Choudhary and Johri, 2009). The biocontrol and excellent colonizing capacity of *Bacillus subtilis* strains are significant and safe for the surrounding environment (Zhao *et al*., 2014). The *Bacillus* species are having significant ability to take over the roots of plants (Turner and Backman, 1991). The rhizobacteria have the great ability to establish plants and microbes interaction due to the efficient in plants roots colonization, formation of microcolonies and production of biofilm. The *Bacillus* species are facilitating the host plant by formation of biofilm which is helpful in protecting the plants from external danger, eliminating the competition of microbes, which is indirectly supportive for growth, crop quality and yield. The spore formation ability of *Bacillus* species makes it dominant in the soil environment (Nihorimbere *et al*., 2013). Great amount of peptides, antibiotics, volatile compounds, low molecular weight and several lipopeptide are produced by *Bacillus* species with particular activity against pathogenic fungi (Shafi *et al*., 2017).

The antimicrobial activity is strongly related to variant structure of lipopeptide (Matsui *et al*., 2020). The fengycin and iturin have the capacity to oppose the growth of fungus (Cao *et al*., 2018). Lipopeptide is compound cluster and exist in different form and it is also call surfactin. The nutrients and cultural conditions of *Bacillus* strains are greatly effecting the production of various types of lipopeptide. The liquid and solid media providing physiological changes is influencing the microbial molecular behaviors having great effect on the yield formation. The use of chemical pesticides is replacing by the microorganism having antagonistic behavior against plant pathogen (Cawoy *et al*., 2011). The properties which make the biological control prominent are low cost, highly specific to their target, no pollutant and waste management problems for the environment.

It is reported that the mechanism through which microorganism have an effect on pathogens population are not at all time clear. It is normally credited to the following effects, the death of pathogen by direct parasitism, competition of food and space between pathogen and antagonist, poisonous effect on the pathogen by mean of substances that are antibiotic in nature (Whipps, 2001).

Many types of decaying and infection on fruit are quite persistent and commonly caused by fungi. Now it is very necessary to find proper and cheap remedies to control the pathogenic fungal diseases and infection (Chakrabarti and Shivarakash, 2005). The limited arsenal availability of antifungal compound is the major difficulty in treating the fungal diseases. The three active compounds which are antifungal by retarding the formation of cell wall, regulatory approval increase the search for further additional candidates which is showing inhibitory effect against the formation of cell wall (Richardson and Warneck, 2003; George and Selitrennikoff, 2006). The humid and warm conditions of climate of Pakistan throughout the year wide spread the fungal diseases in the flora. The current study was thus planned to characterize and isolate bacteria from the soil of an antifungal nature, then extract the compounds through submerged fermentation and checked their antifungal activity through bioassay.

Materials and Methods

Isolation of bacteria

Soil samples collection were done from different tehsils of Sargodha Shahpur (DMS Lat: 32° 17' 11.8068" N, DMS Long: 72° 25' 48.9072" E), Sahiwal (DMS Lat: 30° 40' 39.7812" N, DMS Long: 73° 6' 24.5232" E), Sargodha (DMS Lat: 32° 4' 56.8776" N, DMS Long: 72° 40' 8.8608" E) Sillanwali, (DMS Lat: 31°49'29.46"N, DMS Long: 72°32'28.27"E), Bhalwal (DMS Lat: 32° 15' 55.4256" N, DMS Long: 72° 54' 19.3968" E) Bhera (DMS Lat: 32°28'54.03"N, (DMS Long: 72°54'27.6"E) and Kotmomin (DMS Lat: 32°11'24.1"N, DMS Long:73°1'32.77"E) Punjab, Pakistan (Figure 1). Soil samples were collected from 15 to 20 cm depth along with plant roots. The soil samples were processed for antifungal bacteria isolation. Soil sample of one gram was mixed with 10 ml sterilized distilled water in test tube (Youseif, 2018). All the test tubes were positioned on shaker at 130 rpm for time period of overnight. Then they were allowed to stand for 2 h at room temperature. After that, 200 µl extracts of soil were spread on plate of nutrient agar from each tube and incubated at 37°C for 24 h and isolated bacteria were checked by antifungal activity through streak plate method against already isolated fungal strain (Ahsan *et al*., 2017).

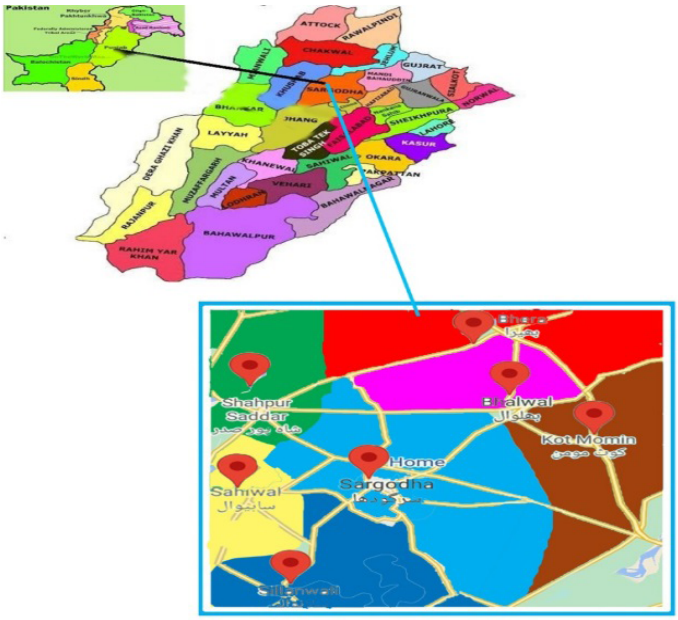

Figure 1: Map of Sargodha, Pakistan sampling sites.

Sample collection for fungal isolation

Citrus fruits samples were collected from local market of Sargodha. The samples were placed in sterile plastic bags and placed in refrigerator at 4°C till further processing.

Isolation of fungi

Using the techniques followed by Akhtar *et al*. (2007) fungi were isolated from each sample of fruit that was collected. A small piece of infected tissue was cut with a sterile scalpel, placed on Potato dextrose agar medium plates, and incubated at 30°C for 7 days. After growth, the tissue was processed for molecular identification (Ahsan *et al*., 2017).

Antagonist effect against fungus Schizophyllum *sp. B2A by streak plate method*

A fungus *Schizophyllum* sp. B2A was cultured in potato dextrose agar medium. The piece of that was placed on potato dextrose agar medium. The pure bacterial culture inoculated loop was inoculated on the potato dextrose agar medium plate surface already inoculated with *Schizophyllum* sp. B2A and incubated for one week at 37°C. *Bacillus subtilis* 2i was processed for optimization of growth conditions (Jayasinghe and Parkinson, 2008).

Identification of bacteria strain 2i

Among all the isolates, *Bacillus subtilis* 2i having antifungal activity was subjected for the genomic DNA extraction by using DNA extraction kit (Thermo scientific ®, USA). The experiment was performed as per manufacturer instructions. The 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' PCR primers were used. The 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' sequencing primers were used in this reaction. PCR reactions conditions were according to Khalid *et al*. (2017).

Identification of fungus Schizophyllum *sp. B2A*

For identification, 18SrRNA gene was used. The isolated fungus was subjected for the genomic DNA extraction using DNA extraction kit (Thermo scientific ®, USA). The experiment was performed as per manufacturer instructions. The NS1 5' (GTA GTC ATA TGC TTG TCT C) 3' and NS8 5' (TCC GCA GGT TCA CCT ACG GA) 3' PCR primers and sequencing primers were used in this reaction. PCR reactions were performed according to Panzer *et al*. (2015).

Phylogenetic analysis

Sequences of *Bacillus subtilis* 2i and *Schizophyllum* sp. B2A were subjected for Phylogenetic analysis. The Neighbor-Joining method MEGA X (version 11.0) was used to find the evolutionary history.

Production of antifungal compounds

In the 250 ml Erlenmeyer flask containing 30 ml of medium comprised of potato peel 2%, yeast extracts 0.583% and $MgSO_4$ 0.233% was taken and sterilized at 121°C for 15 min. After medium sterilization, it was cooled at room temperature and then inoculated with 1% vegetative cells of *Bacillus subtilis* 2i (San-Lang *et al*., 2002). The inoculated flask was placed an orbital shaker (JSR model SA12038CA2HT) at 120 rpm and 37°C for 24h. After completion of fermentation, broth was taken and centrifuge (DLAB model DMO4125) at 10,000

rpm for 10 min. The supernatant obtained was used for antifungal activity (Ahsan *et al*., 2017).

Antimycotic activity against Schizophyllum *sp. isolate B2A*

The *Bacillus subtilis* 2i was selected showing antifungal activity againt *Schizophyllum* sp. B2A. The antimycotic ability of *Bacillus subtilis* 2i was further analyzed by confirmatory experiments (Quiroga *et al*., 2009). The six test tubes having 5ml of nutrient broth were labelled and the fresh bacterial strain culture was inoculated in each and kept for growth at optimized conditions for 24 h. Two mililiter sample was taken and centrifuged at 10000 rpm for the time period of 10 min. After centrifugation 200 µl of cell free liquid was poured in the well of PDA plate inoculated with *Schizophyllum* and incubated at temperature of 37˚C for time period of one week. Zone of inhibitons was monitered with regular intervals (Ahsan *et al*., 2017).

Optimization of process parameters for antifungal compounds productions

Various process parameters such as incubation temperature (25˚C 30˚C 35˚C, 37˚C, 40˚C), pH (5.5, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0), inoculum size (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%) and fermentation period (24, 48, 72h) was optimized for antifungal compounds production by *Bacillus subtilis* 2i in submerged fermentation (Ahsan *et al*., 2017).

Stability test of the fermentation broth

To check the stability of fermentations broth thermal stability, pH stability and exposure to UV light for different time period stability were performed according to Ahsan *et al*. (2017). All the experiment repeated three times and antifungal activity was determined by well diffusion method as discussed above.

Statistical analysis

All the data presented was mean values of triplicates.

Results and Discussion

Isolation and selection of antagonistic bacteria

From the soil, *Bacillus subtilis* 2i was isolated and evaluated against the *Schizophyllum* sp. B2A which is pathogen of citrus (Figure 2c). This is one of the main key criteria considered by scientists, when bio prospecting for potent biological control agent (Khalili *et al*., 2016).

Molecular identification of isolates Bacillus subtilis *2i*

Based on16SrRNA gene sequencing the strain was identified as *Bacillus subtilis* having 99% homology in the NCBI gene bank database. *Bacillus subtilis* 2i isolates phylogenetic tree is shown in Figure 3. The 16SrRNA gene sequence was submitted to NCBI with accession number of MW590677.1.

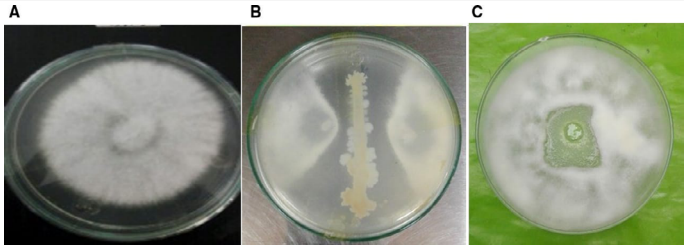

Figure 2: (A) Control *Schizophyllum* sp. B2A. (B) Streak plate method showing antifungal activity of *Bacillus subtilis* 2i against *Schizophyllum* sp. B2A. (C) Well diffusion method, showing antifungal activity against *Schizophyllum* sp. B2A.

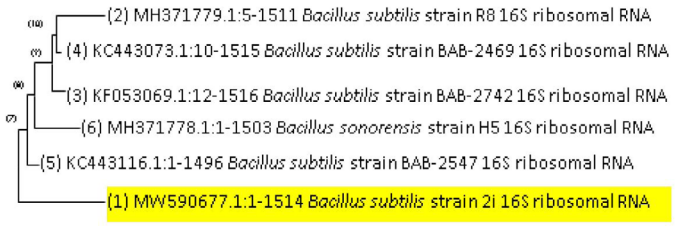

Figure 3: Relationship between isolates *Bacillus subtilis* 2i and several other strains based on their 16SrRNA gene sequences through neighbor- joining Phylogenetic tree.

Evolutionary relationships of taxa

The evolutionary history was finding by using the Neighbor-Joining method. The branch length sum of the optimal tree was 0.00935843. The replicate tree percentage was shown after the branches containing associated clustered taxa with the 500 replicates of bootstrap. The evolutionary distance unit was used for scaling of tree drawing. Evolutionary distances were calculated using the maximum composite likelihood method and expressed as the number of base substitutions per location. Six nucleotide sequences are involved in these sequences. The pair wise deletion option was used for the removal of ambiguous position for each pair sequence. MEGA X was used for the analyses conduction of evolutionary dataset. The final dataset contains total 1516 positions (Kumar *et al*., 2018).

Molecular identification of Schizophyllum *sp. B2A*

Based on the 18SrRNA sequencing; B2A strain was identified as *Schizophyllum* sp. (99% homology) in the NCBI gene bank database. B2A isolates phylogenetic tree is shown in (Figure 4). The 18SrRNA gene sequence was submitted to NCBI under the name *Schizophyllum* sp. B2A and accession number (MW603607.1) was obtained.

Evolutionary relationships of taxa

The Neighbor-Joining method was used to find the evolutionary history. The branch length sum of the optimal tree was 0.08665617. The replicate tree percentage was shown after the branches containing associated clustered taxa with the 500 replicates of bootstrap. The

evolutionary distance unit was used for scaling of tree drawing. Maximum composite likelihood was used for the computation of evolutionary distances are in the units of the per site number of base substitutions. Six nucleotide sequences are involved in these sequences. The pair wise deletion option was used for the removal of ambiguous position for each pair sequence. MEGA X was used for the analyses conduction of evolutionary dataset. The final data set contain total 2125 positions (Kumar *et al.*, 2018).

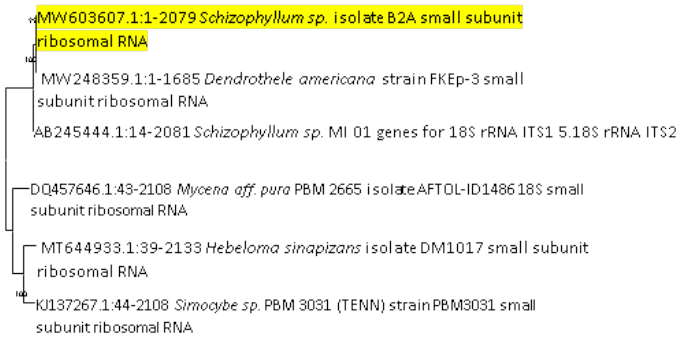

Figure 4: Relationship between *Schizophyllum* sp. B2A and several other strains based on their 18SrRNA gene sequences through neighbor-joining Phylogenetic tree.

Optimization of process parameters

The antifungal compounds were produced by *Bacillus subtilis* 2i and found antimycotic activity against *Schizophyllum* sp. B2A. It was processed for optimization of growth conditions. The inoculated culture of *Bacillus subtilis* 2i was incubated at various temperature levels from 25 to 40°C, the zones of growth inhibition were measured to be increased from 8 to 15 mm, at 40°C activity was decreased from 15 to 12 mm (Figure 5). It was demonstrated by Ahsan *et al.* (2017) that antibiotic activity was increasing from 24 to 30°C but beyond this range when we further increase the temperature it shows inverse effect. Lai *et al.* (2005) stated that temperature had countless influence on the production of secondary metabolites; low temperature affected the secondary metabolites production. We observed that antibiotic production was greatly affected by low and high temperature. Growth was also affected by temperature variations. Favorable growth temperature was 23–37 °C (Chen *et al.*, 1996).

The inoculated culture of *Bacillus subtilis* 2i was incubated at various pH. The pH 8 was giving maximum results. Extreme conditions (pH 5.5, 9.0) resulted in decreased antifungal activity (Figure 6). Ahsan *et al.* (2017) reported that temperature, pH and inoculum volume (%) has great influence on antifungal compounds productions. When pH was increased from 3 to 7, antibiotic activities increase but beyond 7 the activities decrease and show high peak activity near pH 7. High pH value and low pH values resulted in decreased antibiotic production. The pH plays central role in secondary metabolites production (Jain *et al.*, 2019).

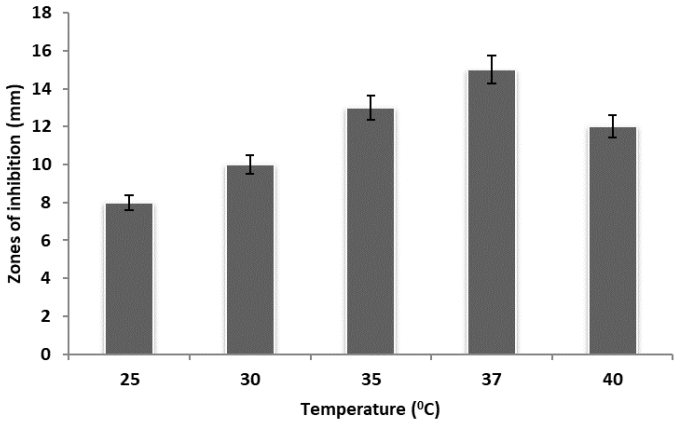

Figure 5: Effect of temperature on antifungal substance production.

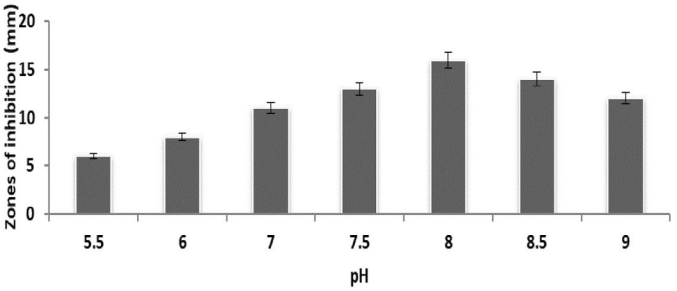

Figure 6: Effect of pH on antifungal substance production.

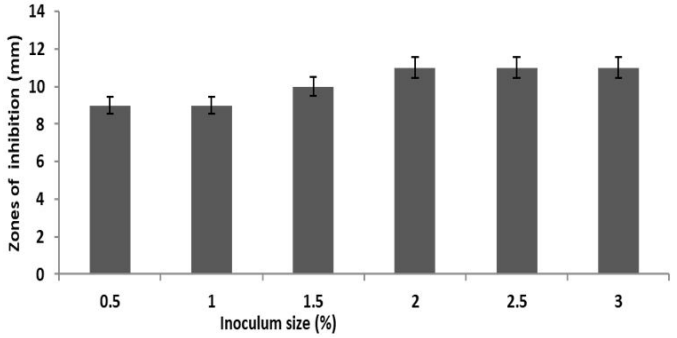

Figure 7: Effect of inoculum size on antifungal substance production.

Different concentration of inoculum was observed ranging from 0.5 to 3.0 % and the inhibition zone was found increased from 8 to 12 mm and become static by further increase in concentration (Figure 7). The inoculum volume had effect on the activity of antibiotic, with in optimum range, from 2% to 7% volume increase enhance antifungal activity and beyond the highest range increase in volume had adverse effect by decreasing the antibiotic activity (Ahsan *et al.*, 2017). Optimum volume of inoculum has good effect on yield and its effectiveness. The increased volume beyond the optimum range would lessen the space and oxygen supply, the toxin produced as a result of extra material and unfit the fermentation products (Lai *et al.*, 2005). The incubation period has great influence on the antimicrobial compounds productions (Oskay, 2009). Prapagdee *et al.* (2008) also reported that extension in the

incubation period has negative effect on the production of antifungal compounds and culture filtrate. In our findings incubation period were examined up to 72 h at regular interval and inhibition zone was found decreasing above 24 h (Figure 8).

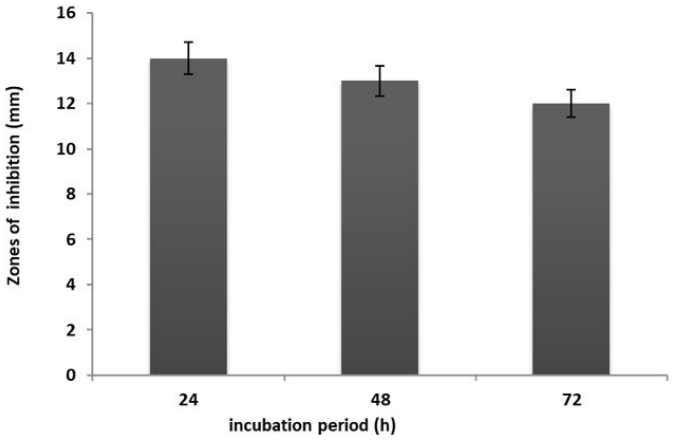

Figure 8: Effect of incubation period on antifungal substance production.

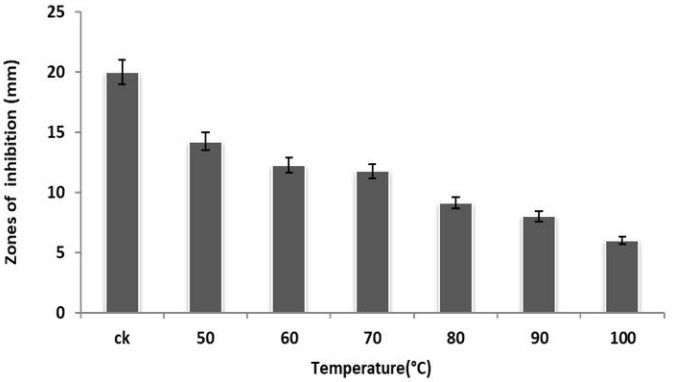

Figure 9: Effect of temperature on stability of fermented broth.

Stability analysis of the fermentation broth

Fermentation broth was treated with temperature range between 50 to 100°C and observed their antifungal activity. By increasing the temperature, the antifungal activity of broth decreases (Figure 9). The fermentation broth antifungal activity was noted at various pH values from 2 to 9 and results revealed that antifungal activity was stable at pH 7 while further increased or decreased pH beyond this result decreased antifungal activity (Figure 10). To study the effect UV on the stability of antifungal activity, the fermented broth was treated with UV for various time period and results shown that UV treatment had adverse effect on the antifungal activity (Figure 11). Ahsan *et al.* (2017) demonstrated that KX852460 *Streptomyces* strain cultural filtrate having antifungal activity was not stable for long time, temperature and UV treatment and it was further explained that 5.0 and 8.0 pH range was stable and beyond that lower and upper range it was found unstable. The stability of filtrate of antimicrobial activity at various pH and temperature were also reported by Sharma *et al.* (2018). Illuminated light have no effect on the stability of cultural filtrate, treatment with UV light disturb the culture filtrate stability as reported by Nakatsuji *et al.* (2018).

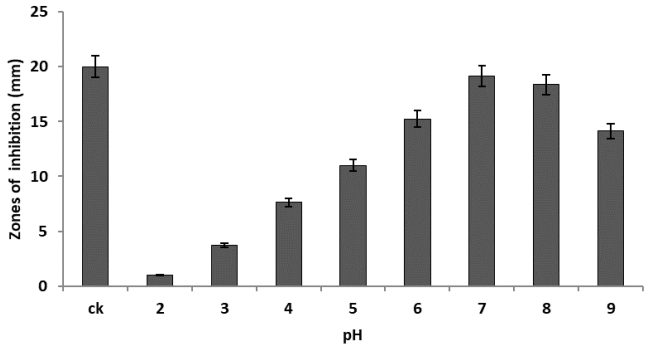

Figure 10: Effect of pH on stability of fermented broth.

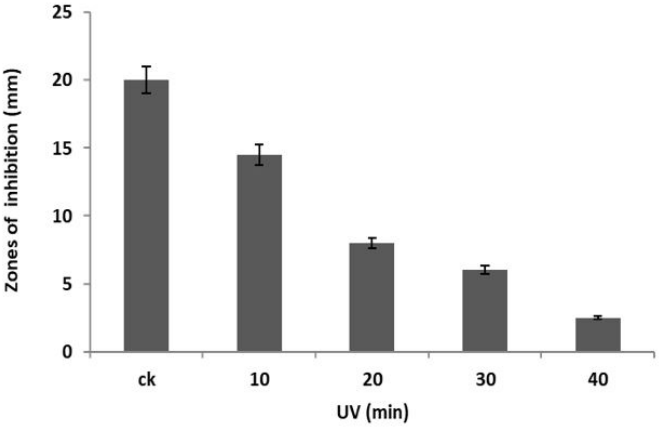

Figure 11: Effect of UV on stability of fermented broth.

Conclusions and Recommendations

In this study *Bacillus subtilis* 2i has great strength against *Schizophyllum* sp. B2A. Optimizations of the fermentation conditions are pre-requisite for large scale production of antifungal compounds. To get optimum antifungal activity, stability of the fermentation broth should be studied. Disease due to fungus is most common in Pakistan due to variant climatic change. The effective antifungal drugs and compound is the need of today alarming situation. It has not been worked out that how the antifungal compound shows their mode of action and what is the chemical nature of antifungal compound. Further investigation of these compounds may high lights their therapeutic usefulness. It is significant that the bacteria *Bacillus subtilis* 2i showing antifungal potential isolated from the local environment and their antimycotic and therapeutic ability may show more significant results as compared to the imported antibiotic and drugs. Based on these findings, we conclude that this strain will be useful for the biological control of citrus diseases caused by *Schizophyllum* sp. B2A.

Conflict of interest

The authors have declared no conflict of interest.

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