Optimization of growth and production on an antifungal metabolite from *Bacillus* species SK 23

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled **"Optimization of growth and production of an antifungal metabolite from *Bacillus* species SK 23"** is based on the results of investigations carried out by me in the Discipline Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Prof. Savita Kerkar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **"Optimization of growth and production of an antifungal metabolite from *Bacillus* species SK 23"** is a bonafide work carried out by Ms. Saishree Pratap Kamat under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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1. Introduction

Bacillus sp. are microorganisms which are Gram positive, rod-shaped, facultative aerobic, spore forming bacteria which are spread widely in nature. The genus *Bacillus* have been isolated from many surroundings with probable applications in the field of biotechnology. *Bacillus* species are a source of many secondary metabolites which have a number of applications as anti-cancer agents, antibiotics, biosurfactants, biopesticides, enzyme inhibitors, etc. *Bacillus* sp. shows a wide range of physiological abilities; hence it can survive in most of the environmental conditions and compete with other favourable organisms within that environment. It produces stable metabolites which have an inhibitory effect on other microorganisms (Faruk Adamu Kuta, 2009). *Bacillus* sp. synthesize different extracellular metabolites having broad-spectrum activity against various microbes and hence it is important to investigate it for antimicrobic activity (M. Awais, 2010).

Some of the antibiotics produced by *Bacillus* sp. are: Zwittermicin, Cerexin and Biocerin produced by *Bacillus cereus,* Tyrothricin, Brevin, Gramicidin, Brevistin by *Bacillus brevis*, Lateropsorin by *Bacillus laterosporus,* Esperin by *Bacillus mesentericus,* Xylostatin, Polypeptin, Ciculin and Butirocin by *Bacillus circulans,* Colistin and Polymyxin by *Bacillus polymyxa,* Micrococcin, Pumulin and Tetain by *Bacillus pumilus,* Licheniformin, Proticin, Bacitracin by *Bacillus licheniformis*, Baciphelacin and Octopytin by *Bacillus thiaminolyticus*, and Subtilin, Polymyxin, Difficidin, Bacitracin, Mycobcillin, Bacillomycin and Iturin by *Bacillus subtilis.*

However, for the reason of bioprospecting of *Bacillus* sp., terrestrial resources have been overexploited. Hence, focus is needed on the unexplored or under-explored habitats where *Bacillus* is found, such as environments with extreme conditions of heat, salinity, pressure, etc. There have been fewer studies on marine microorganisms as compared to terrestrial microorganisms. As marine microorganisms live in aquatic environment with high levels of dissolved salt as compared to terrestrial microorganisms, they have diverse metabolic pathways to produce metabolites, having exceptional structure and activity.

Fungi are responsible worldwide, for the contamination of crops and food raw materials and even for some human and animal diseases. Hence, prevention of fungal contamination is very important for the agri-food industry as well as in the human health sector. Synthetic fungicides based on different chemicals are usually used to control the growth and reproduction of fungi. Synthetic fungicides not only have possible toxicological risks, but also undesirable side-effects leading to fungicide-resistant populations. *Fusarium* is a complex genus of ascomycete fungi that consists of phytopathogens of agricultural relevance. It is a challenge to control *Fusarium* infection in crops which leads to substantial yield losses.

Post-harvest losses were reduced mainly by treatment with chemical pesticides. As some of the pathogens have developed pesticide resistance, there is an increase in the presence of undesirable chemical residues in the food chain. This has led to the search for safer methods to effectively control microbial infections which cause post-harvest decay. The use of natural antagonistic microorganisms has been extensively studied and biological control has been used as an alternative (Y Toure, 2002).

Microbes which are antagonistic to phytopathogens could make a substantial contribution to the prevention of plant diseases. They provide an alternative to the use of chemical pesticides in agriculture. *Bacillus* spp., *Pseudomonas* spp., and *Streptomycetes* spp. are among the 20 genera of bacteria that are widely used as biocontrol agents and *Bacillus* spp. is most desired of them (Adnan Amin, 2012) (Saira Ali, 2014). Numerous species of *Bacillus* have been identified as plant growth promoting bacteria and/or biocontrol agents. The most commonly studied ones are *B. amyloliquefaciens, B. subtilis* and *B. licheniformis.* Majority of commercial biocontrol products are *Bacillus*-based. These agents are ideal in the agri-food industry due to their chemical and physical diversity (Lili Chen, 2010).

Among the various antimicrobial metabolites produced by *Bacillus* sp, lipopeptide antibiotics which have been identified till date are divided into 3 main groups: surfactin, iturin and fengycin groups according to their structure. These lipopeptides occur as families of closely related isoforms differing in branching and length of the fatty acid side chains as well as in the amino acid substitutions in the peptide rings. These are all natural compounds with high potential for pharmaceutical and biotechnological applications. Their excellent surface and membrane-active properties and super emulsifying and foaming properties distinguish them from the other groups. These characteristics can be used in the food biotechnology and agricultural sector. Bioactive lipopeptides also act as antifungal agents, antiviral agents, antiamoebocytic agents and antimycoplasma agents (Muaaz Alajlani, 2016).

Antimicrobial metabolite production is always associated with growth and is influenced strongly by many factors such as temperature, pH, incubation period, NaCl

concentration, nutrient sources and cell density. It is very important for optimizing growth conditions for the improvement of antimicrobial metabolite production. To increase the production of the antimicrobial metabolites, many studies have been carried out on the optimization of the composition of the growth medium and culture conditions. An important role is played by the media composition in the economics and productivity of the process. Process parameter optimization is an attempt to improve the quality and to minimize the quality variation of the metabolite.

In order to ensure the maximum biosynthesis of the desired antimicrobial compounds, it is necessary to identify the best medium composition and process conditions for the cultivation of the selected producing strain. This can be done by traditional methods such as the One Factor At A Time (OFAT) approach or by statistical methods such as the Response Surface Methodology (RSM). OFAT is the technique used for optimization of the production media wherein only one factor of the production media composition or the growth conditions is changed and the rest are kept constant. The objectives to carry out optimization are to increase the productivity, lower the consumption of substrate, obtain a high-quality product, control metabolic activity, unit operations, downstream processing and process scheduling efficiently, lower the overall cost of the entire process and to increase the reproducibility and the reliability of the process. Optimization of antibiotic production for the enhancement of the yield is very important for scaling up the industrial process.

The purpose of this study is to optimize the growth of *Bacillus* sp. SK 23 in a defined production medium, maximize the production of the antifungal compound and extract the bioactive metabolite.

1.1 Aim and Objectives

Aim: To optimize the growth and production of antifungal compound by *Bacillus* sp. SK 23 and to extract it.

Objectives:

a) To optimize the media components for maximizing the growth of *Bacillus* sp. SK 23.

b) To design a defined production media for the optimum production of antifungal compound by *Bacillus* sp. SK 23.

c) To extract the antifungal compound from *Bacillus* sp. SK 23 using solvent extraction technique in the optimized media.

2. Review of Literature

The use of *Bacillus* species as antagonistic microorganisms which produce inhibitory substances for fungal pathogens is shown to be one of the most promising solutions to the problems of fungal crop, human and animal health diseases. Members of the *Bacillus* genera produce a high proportion of agriculturally important compounds and enzymes that degrade fungal structure (Zorana Trivunovic, 2022).

Various studies have been carried out for the exploration of antimicrobial metabolites from *Bacillus* species. The following table summarizes the international and national scenario of the reports.

The genus *Bacillus* is well studied for the production of various types of lipopeptides such as lichenysins, bacillomycin, fengycins and surfactins. Alajlani et. al. (2016) used *Bacillus subtilis* strain BIA for the production of bioactive lipopeptides. They observed that the compounds produced by the *Bacillus subtilis* strain BIA were surfactin and iturin-like.

Jiao et. al. (2021) used the *Bacillus amyloliquefaciens* strain YN201732 which produces lipopeptides having biocontrol activity (bacillomycin D) against the fungal pathogens *Fusarium solani* and *Erysiphe cichoracearum*. After extraction by acid precipitation and organic solvent extraction, the compound was identified as fengycin using HPLC and LCMS-IT-TOF.

Table 2: Antimicrobial metabolites from *Bacillus* species

Sr. No.	Isolate	Source	Antimicrobial Compound	Active against	References
International scenario					
1.	*Bacillus subtilis* strain GA1	Strawberry fruits, Belgium	Fengycin	Fungus *Botrytis cinerea*	Toure et. al., 2004
2.	*Bacillus* sp. strain PP19-H3	Macroalga *Schizymenia dubyi*, Omaezaki coast, Japan	Macrolactins (A, F, G, H, I, J, K, L, M)	Bacteria *Staphylococcus aureus*	Nagao et. al., 2001
3.	*Bacillus amyloliquefaciens* strain B94	Field soil, Urbana, USA	Iturin A	Fungus *Rhizoctonia solani*	Yu et. al., 2002

4.	*Bacillus amyloliquefaciens* strain RC-2	Mulberry leaves, Tsukuba, Japan	Iturins	Fungus *Colletotrichum dematium*	Hiradate et. al., 2002
5.	*Bacillus* sp. strain ICBB 1582	Farmyard soil, South Sulawesi Province, Indonesia	7-O-Malonyl Macrolactin A	Bacteria Methicillin-Resistant *Staphylococcus aureus*, Vancomycin-Resistant *Enterococci* & *Burkholderia cepacia*	Romero-Tabarez et. al., 2006
6.	*Bacillus amyloliquefaciens* strain SH-B10	South China Sea, China	Fengycin	Fungi *Fusarium oxysporum* & *Fusarium graminearum*	Chen et. al., 2010
7.	*Bacillus* sp. strain Sc026	East coast, Gulf of Thailand	Macrolactin (A and F)	Bacteria *Bacillus subtilis* and *Staphylococcus aureus*	Jaruchoktaweechai et. al., 2000

8.	Marine *Bacillus* sp. strain Sc026	Sediments around Sichang Island, East coast, Gulf of Thailand	Macrolactin F, 7-O-succinyl macrolactin F and 7-O-succinyl macrolactin A	Bacteria *Bacillus subtilis* and *Staphylococcus aureus*	Jaruchoktaweechai et. al., 1999
9.	*Bacillus mojavensis* strain CWBI-B1568 and *Bacillus subtilis* strain CWBI-B1567	Soil of different palm trees of arid regions, South-East Algeria	Antifungal compound	Fungus *Candida albicans*	Youcef-Ali et. al., 2014
10.	*Bacillus* sp. strain AT28	Soil near Daejeon City, Chungcheongnam-Do, Korea	Macrolactin S	Bacteria *Staphylococcus aureus* FabG, *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli*	Sohn et. al., 2008
11.	*Bacillus subtilis* strain CMB32	Soil in Gwangju, Korea	Iturin A, Fengycin and Surfactin A	Fungus *Colletotrichum gloeosporioides*	Kim et. al., 2009
12.	*Bacillus subtilis*	Source not known	Iturin A	Yeast *Saccharomyces cerevisiae*	Latoud et. al., 1987

13.	*Bacillus cereus* strain 8A	Source not known	Bacteriocin	Bacteria *Listeria monocytogenes*, *Clostridium perfringens* and several *Bacillus* sp.	Bizani and Brandelli, 2002
14.	*Bacillus subtilis* and *Bacillus amyloliquefaciens*	Source not known	Iturin	Bacteria *Xanthomonas oryzae*	Beric et. al., 2011
15.	*Bacillus* strain GU057	District D.I. Khan and Kohat (KPK), Pakistan	Bacitracin	Bacteria *Micrococcus luteus* and *Staphylococcus aureus*	Amin et. al., 2012
16.	*Bacillus amyloliquefaciens* ESB-2	Seawater in Yuhuan, Zhejiang, China	Macrolactin A	B16-F10 murine melanoma cell, *Herpes simplex* type I and II, HIV virus	He et. al., 2012
17.	*Bacillus amyloliquefaciens* strain PPCB004	Surface of fruits	Iturin A	Post-harvest fungal phytopathogens	Arrebola et. al., 2009
Indian scenario					

1.	Halotolerant *Bacillus subtilis* strain SK.DU.4	Rhizosphere soil sample	2 antimicrobial peptides	Gram-positive bacteria	Baindara et. al., 2013
2.	*Bacillus amyloliquefaciens* strain MBL27	Dairy wastes	Bacteriocin & Antimicrobial protein	Bacteria	Vijayalakshmi and Rajkumar, 2010
3.	*Bacillus subtilis* strain MTCC-8114	Garden soil sample of DEI, (Dayalbagh, Agra, India)	Peptide antifungal antibiotic	Fungi *Microsporum fulvum* & *Trychophyton* sp.	Kumar et. al., 2008

Bacillus subtilis R1 isolated from an oil contaminated desert site in India was used as a biocontrol agent and biosurfactant in microbial enhanced oil recovery. Extraction and characterization of the biosurfactant revealed it to be similar to the lipopeptide group surfactins and fengycin. Various plant pathogenic fungi were also inhibited by the strain on potato dextrose agar (Sujata S. Jha, 2016).

Amin et. al. (2012) carried out the production of peptide antibiotics by *Bacillus* sp. GU 057 indigenously isolated from saline soil from different areas of district KPK, Pakistan, against the *Micrococcus luteus* and *Staphylococcus aureus.* After 48 hours of incubation at pH 8 and 4% concentration of glucose, maximum antibiotic activity i. e. 18 mm of inhibition was observed against *Staphylococcus aureus* and the antibiotic was identified as bacitracin by autoradiography.

Islam et. al. (2012) isolated antagonistic bacterial isolate C9 against *Rhizoctonia solani*. The bacterium was identified as *Bacillus subtilis* subsp. *subtilis*. The best carbon and nitrogen sources for antibiotic production were found to be Mannitol (1%) and Soytone (1%). An antibiotic compound – DG4 – was separated and purified from ethyl acetate extract from the culture broth of isolate C9 and its chemical structure was established.

Barale et. al. (2022) worked on purification and characterization of antibacterial surfactin isoforms produced by *Bacillus velezensis* strain SK. Good antimicrobial activity (1600 AU/mL) was exhibited by the isolated surfactin against *Bacillus cereus* and *Staphylococcus aureus.*

Meena et. al. (2018) used the two factors at a time (TFAT) approach by RSM to increase the synthesis of surfactin metabolite by the *Bacillus subtilis* KLP2015 and use it as an

antifungal agent. Optimization of the physico-chemical parameters such as nitrogen source, fermentation temperature, fermentation time and pH of the broth by 'One Factor at a Time' (OFAT) approach increased the lipopeptide production from 200.0 to 547.0 mg/L. The yield of lipopeptides increased from 547 mg/L to 985 mg/L after using the 'Two Factors at a Time' (TFAT) approach using Response Surface Methodology (RSM). The purified lipopeptide were found to be similar to an authentic Surfactin molecule. The purified lipopeptide showed potent 75.1% and 41.9% antifungal activity against *Mucor* sp. and *Aspergillus niger* respectively.

Awais et. al. (2010) studied the production of antimicrobial metabolites by *Bacillus subtilis*. They optimized the fermentation condition parameters for the maximum production of peptide antibiotics. The antimicrobial activity was checked against *Micrococcus luteus* through antibiotics diffusion assay. The maximum production of the peptide antibiotic was found to be optimized at pH 6 to 9, incubation time of 0 to 144 hours and glucose concentration 1 to 5%. The activity was found to be maximum at pH 8 after 4 hours of incubation.

This study is being carried out in order to optimize the growth and production of antifungal compound from *Bacillus* sp. SK 23 as well as the extraction of the antifungal compound. As the *Bacillus* sp. SK 23 was sourced from a saltpan in Goa, it survives and thrives in a marine environment. Many studies have been conducted on terrestrial *Bacillus* sp. but not many have been conducted on marine bacteria. Hence, this study focuses on optimization of the production media utilized by the *Bacillus* sp. SK 23 as well as the antifungal activity of the extracted antifungal compound.

3. Materials and Methods

3.1 Bacterial and test fungal culture

The bacterial culture of *Bacillus* sp. strain SK 23 and plant pathogen *Fusarium solani* were obtained from Dr. Savita Kerkar's culture collection, Programme Biotechnology, Goa University. *Bacillus* sp. was found to be a rod-shaped, Gram-positive bacterium and was maintained on Zobell Marine Agar (ZMA) media (25%) (Appendix I) at 4°C whereas the fungal culture was maintained on Potato Dextrose Agar (PDA) media (Appendix I) at 4°C. *Fusarium solani* showed white mycelia on upper side and orange coloration on reverse of PDA.

3.2 Growth of bacterial and test fungal culture

Bacillus sp. strain SK 23 was sub-cultured on 25% ZMA plates and were incubated at 37°C in an incubator (Biotechnics, India) for 24 hours. From this plate, the isolated colony was inoculated into 25% ZMB and incubated on rotary shaker incubator at 120 rpm at 37°C for 24 hours. The supernatant obtained after centrifuging the cell suspension was used for further assays. *Fusarium solani* was sub-cultured on PDA plates and incubated at room temperature (28°C ± 2°C) for 4 days.

3.3 Dual culture assay to assess the antifungal activity of *Bacillus* sp. strain SK 23

Assessment of the antifungal activity of the culture was done by dual culture assay. After incubation in 25% ZMB, the culture broth was added into sterile Eppendorf tubes and

centrifuged in Sorvall Lynx 4000 superspeed centrifuge (Sorvall, Germany) at 10,000 rpm at 4°C for 10 minutes. Then, the supernatant was collected and used to carry out the antifungal assay. PDA plates were prepared and kept at room temperature for 24 hours to check for contamination. An agar disc of the fungal pathogen *Fusarium solani* was removed from the master plate using a sterile cork borer and placed in the centre of a PDA plate under sterile conditions. The inoculated PDA plate was then incubated at room temperature (28°C ± 2°C) for 24 hours. After 24 hours, the culture supernatant was ribbon streaked on one side of the fungal disc, 1 cm away from the edge of the agar plate. Sterile, uninoculated media was ribbon streaked on the opposite side as control. The plates were incubated for 3 days at room temperature and the fungal growth was measured on both the sides of the fungal disc in mm and recorded. The experiment was carried out in triplicates and inhibition percentage was calculated using the formula:

$$I\ \% = [(C - T) / C] \times 100$$

where,

I = Inhibition percentage of fungal growth.

C = Growth of fungus on the control side (mm)

T = Growth of fungus on the side inoculated with bacteria (mm)

3.4 Optimization of various media and growth parameters for the growth and production of antifungal compound from *Bacillus* sp. strain SK 23

Optimization of carbon source and concentration, nitrogen source and concentration and sodium chloride (NaCl) concentration in the culture media along with the pH of the medium and temperature of incubation was carried out. Growth of the *Bacillus* sp. strain

SK 23 was recorded in form of turbidity measurement spectrophotometrically. Each flask of the manipulated media was inoculated with a single isolated colony from the master culture plate and the flasks were incubated in a rotary shaker incubator at 37°C ± 2°C (except in media manipulated for determining optimum temperature) at 120 rpm for 24 hours. For each media, a flask without inoculum was maintained as a control. After 24 hours, the Optical Density was measured of each of the media flasks at 600 nm using a UV-Vis spectrophotometer. Also, the antifungal activity was determined in each media using dual culture method as mentioned in previous section.

3.4.1 Effect of various carbon sources on growth and production of antifungal compound

Various carbon sources *viz.* Sucrose, Trehalose, Mannitol, Arabinose, Lactose and Dextrose were supplemented (one at a time) in the production media to optimize the growth of bacterial culture and production of antifungal compound. Each carbon source (1%, w/v) was added into the basal media (20 mL), comprising of a nitrogen source (Peptone, 0.5% w/v) and concentration of NaCl (2%) with pH 7.6 ± 0.2. Assessment of growth was done by spectrophotometric analysis and assessment of antifungal activity was done by dual culture method.

3.4.2 Effect of various nitrogen sources on growth and production of antifungal compound

Various nitrogen sources *viz.* Peptone, Tryptone, Casein, Potassium nitrate, Ammonium chloride and Ammonium sulphate were supplemented (one at a time) in the production

media to optimize the growth of bacterial culture and production of antifungal compound. Each nitrogen source (1%, w/v) was added into the basal media (20 mL), containing a fixed carbon source (Sucrose, 0.5% w/v) and NaCl concentration (2%) with pH 7.6 ± 0.2. Assessment of growth of *Bacillus* sp. strain SK 23 and antifungal activity were carried out.

3.4.3 Determination of the optimum concentration of the selected carbon source

Basal media (20 mL) was prepared in Erlenmeyer flasks with varying concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%) of the optimized carbon source (Sucrose), a fixed nitrogen source (Peptone, 0.5% w/v) and NaCl concentration (2%) with pH 7.6 ± 0.2. Growth and antifungal activity were assessed by spectrophotometry and dual culture assay respectively.

3.4.4 Determination of the optimum concentration of the selected nitrogen source

Basal media (20 mL) was prepared in Erlenmeyer flasks with varying concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%) of the optimized nitrogen source (Tryptone), the optimum concentration of the selected carbon source (Sucrose, 0% w/v) and NaCl concentration (2%) with pH 7.6 ± 0.2. Growth and antifungal activity were assessed by spectrophotometry and dual culture assay respectively.

3.4.5 Determination of the optimum concentration of NaCl for the production of antifungal metabolite

The effect of several NaCl concentrations on the growth and antifungal compound production by *Bacillus* sp. SK 23 was studied by supplementing different concentrations of NaCl in the media where in the optimum carbon source concentration as well as the optimum nitrogen source concentration was already present. NaCl concentrations ranging from 0% to 20% w/v (at intervals of 4%) were supplemented into 20 mL media flasks containing tryptone (2.5%) at pH 7.6 ± 0.2. Growth and antifungal activity were assessed by spectrophotometry and dual culture assay respectively.

3.4.6 Determination of the optimum pH for the production of antifungal compounds

Effect of pH on the growth and production of antifungal compound from *Bacillus* sp. SK 23 was studied by varying the pH of the medium ranging from 4.0 to 14.0 with intervals of 4 using Cyber Scan pH meter 700-E (Eutech, India). The media supplemented with Tryptone (2.5%) and NaCl (4%) was adjusted to different pH by using 1 M HCl and 1 M NaOH. Growth and antifungal activity were assessed by spectrophotometry and dual culture assay respectively.

3.4.7 Determination of the optimum temperature for the production of antifungal compound

Optimum temperature for the growth and production of antifungal compound was determined by varying the incubation temperatures ranging from 10°C to 60°C with intervals of 10°C. The medium was supplemented with Tryptone (2.5%), NaCl (4%) and

pH was maintained at 8.0 ± 0.2. Growth and antifungal activity were assessed by spectrophotometry and dual culture assay respectively.

3.5 Assessment of growth of *Bacillus* sp SK 23 and determination of production of antifungal activity in Standard bacteriological media, Basal media and Optimized media

Bacillus sp. SK 23 was inoculated in 50 ml of Nutrient broth (HiMedia, India), Zobell marine broth (HiMedia, India), and Media D broth respectively and incubated at 37°C and 120 rpm in a rotary incubator shaker (Remi, India) for 24 hours. Basal media (20 mL) and optimized media (20 mL) was prepared in Erlenmeyer flasks and each flask was inoculated with a single colony from the master culture plate and incubated at 37°C for 24 hours. A flask without inoculum was maintained as a control. After 24 hours, the Optical Density was measured at 600 nm using a UV-Vis spectrophotometer. Antifungal activity was assessed by dual culture method.

3.6 Large scale production of antifungal compound under the optimized conditions

Seed culture of *Bacillus* sp. SK 23 was prepared by inoculating a single colony from the master culture plate in 150 ml of the optimized media in an Erlenmeyer flask and incubated overnight at 120 rpm in a rotary shaker incubator. This overnight grown culture (50 mL each) was inoculated into 3 flasks, each containing 650 mL of optimized media and incubated at 40°C in a rotary shaker incubator at 120 rpm for 24 hours.

3.6.1 Extraction of antifungal compound using Acid Precipitation

After 24-hour incubation, the culture broth was centrifuged at 10,000 rpm at 4°C for 15 minutes and the cell-free supernatant was collected. The cell-free culture broth (supernatant) was precipitated by adjusting the pH to 2.0 using concentrated HCl and was stored overnight at 4°C. The precipitate was collected by centrifugation at 10,000 rpm at 4°C for 15 minutes.

3.6.2 Extraction of antifungal compound using Solvent Extraction

The collected precipitate was extracted with 3 different solvents *viz.* Methanol, Ethyl Acetate and N-Hexane. Solvent was added to the precipitate in 1:1 ratio, i.e., the same amount as that of the supernatant, in a separating funnel. The separating funnel was shaken vigorously for 15 minutes and allowed to stand for 1 hour. This was done three times for each solvent. For assessment of antifungal activity, well diffusion assay was carried out. The fungi *Fusarium solani* was inoculated in PDB (50 mL) and incubated on a rotary shaker incubator at room temperature at 120 rpm for 72 hours. After 72 hours, the culture broth was swabbed onto sterile PDA plates and incubated at room temperature for 24 hours. 4 wells were bored into each plate using a sterile cork borer and 100 µl of each of the solvent phase samples were added into 3 wells and the respective solvent was added into the 4^{th} well as the control sample. The plates were incubated at room temperature for 72 hours and the zone of inhibition was measured and recorded.

3.7 Estimation of dry weight and yield of the antifungal compound after extraction

Each of the different solvent samples (70 mL) were dried under vacuum using the rotary vacuum evaporator (Equitron Roteva) at their respective boiling temperatures. The dry weight of the antifungal compound obtained after vacuum evaporation was measured and the yield (mg/mL) was calculated.

4. Results and Discussion

4.1.1 Effect of various carbon sources on growth of *Bacillus* sp. SK 23 and the production of antifungal compound

Bacillus sp. strain SK 23 showed growth in media supplemented with all the carbon sources *viz*. Sucrose, Dextrose, Lactose, Mannitol, Trehalose and Arabinose. However, the absorbance at 600 nm as well as the antifungal activity varied when the carbon sources varied. The absorbance was the highest when the media was supplemented with sucrose as the optical density was found to be 0.347 and the inhibition percentage was also found to be maximum i.e., 59.26% in the same media (Figure 1). The maximum cell number of *Bacillus subtilis* C9 was reported by the use of Mannitol as the optimal carbon source against *Rhizoctonia solani* by Islam et. al. (2012). Mezghanni et. al. (2012) carried out medium optimization of antifungal activity production by *Bacillus amyloliquefaciens* using RSM which showed sucrose as the optimal carbon source to be used in the media. Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT and RSM where sucrose was found to show maximum Iturin A production and high antifungal activity. These reports were found to coincide with the results obtained in this present study.

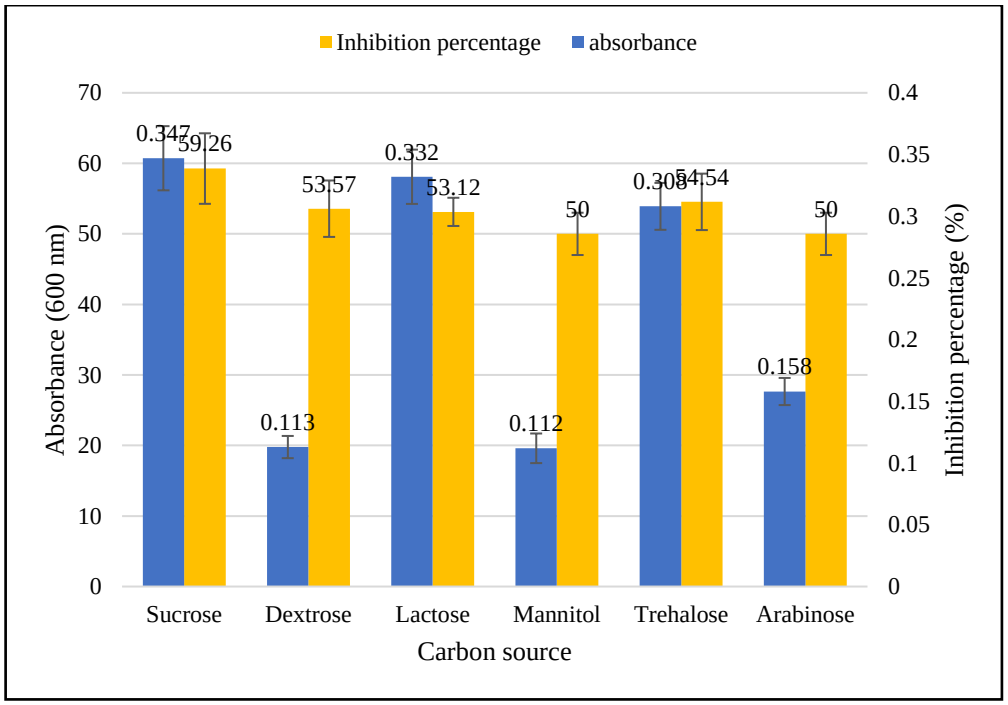


Figure 1: Graph of the Absorbance and Inhibition percentage for utilization of different carbon sources by *Bacillus* sp. strain SK 23

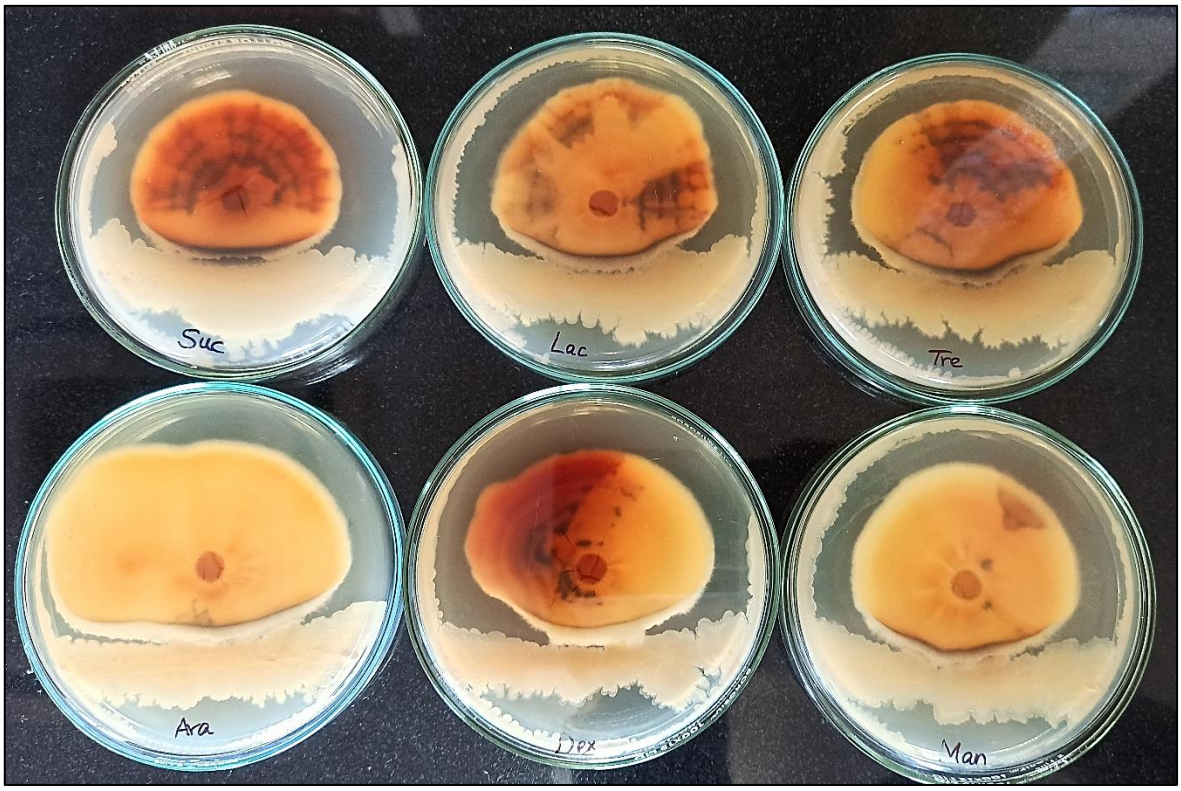


Plate 1: Dual culture assay for the assessment of various carbon sources for the production of *Bacillus* sp. SK 23

4.1.2 Effect of various nitrogen sources on growth of *Bacillus* sp. SK 23 and the production of antifungal compound

Bacillus sp. strain SK 23 showed growth in media supplemented with all the nitrogen sources *viz*. Peptone, Tryptone, Casein, Potassium nitrate, Ammonium sulphate and Ammonium chloride with varying absorbance at 600 nm as well as antifungal activity. The absorbance and antifungal activity were the highest when the media was supplemented with Tryptone as the nitrogen source with optical density 0.384 and inhibition percentage 51.72% respectively (Figure 2). Tryptone and Peptone showed similar antifungal activity as well as similar growth of the *Bacillus* sp. strain SK 23. Soyatone was reported as the nitrogen source for attainment of maximum growth and antibiotic production of *Bacillus subtilis* C9 against *Rhizoctonia solani* by Islam et. al. (2012). Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT and RSM where Peptone was found to show maximum Iturin A production and high antifungal activity. Mezghanni et. al. (2012) optimized the medium of antifungal activity production by *Bacillus amyloliquefaciens* using RSM and the optimal nitrogen source of the medium was found to be Peptone. This shows that the results obtained in this particular study are coinciding with the results obtained by the other researchers in their papers.

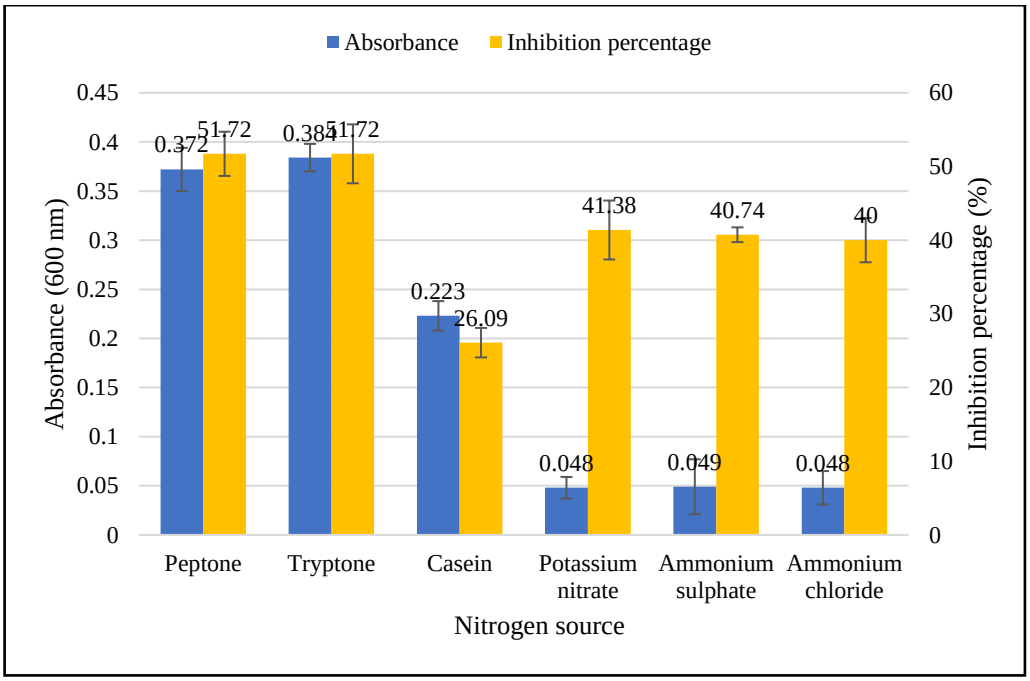


Figure 2: Graph of the Absorbance and Inhibition percentage for utilization of different nitrogen sources by *Bacillus* sp. strain SK 23

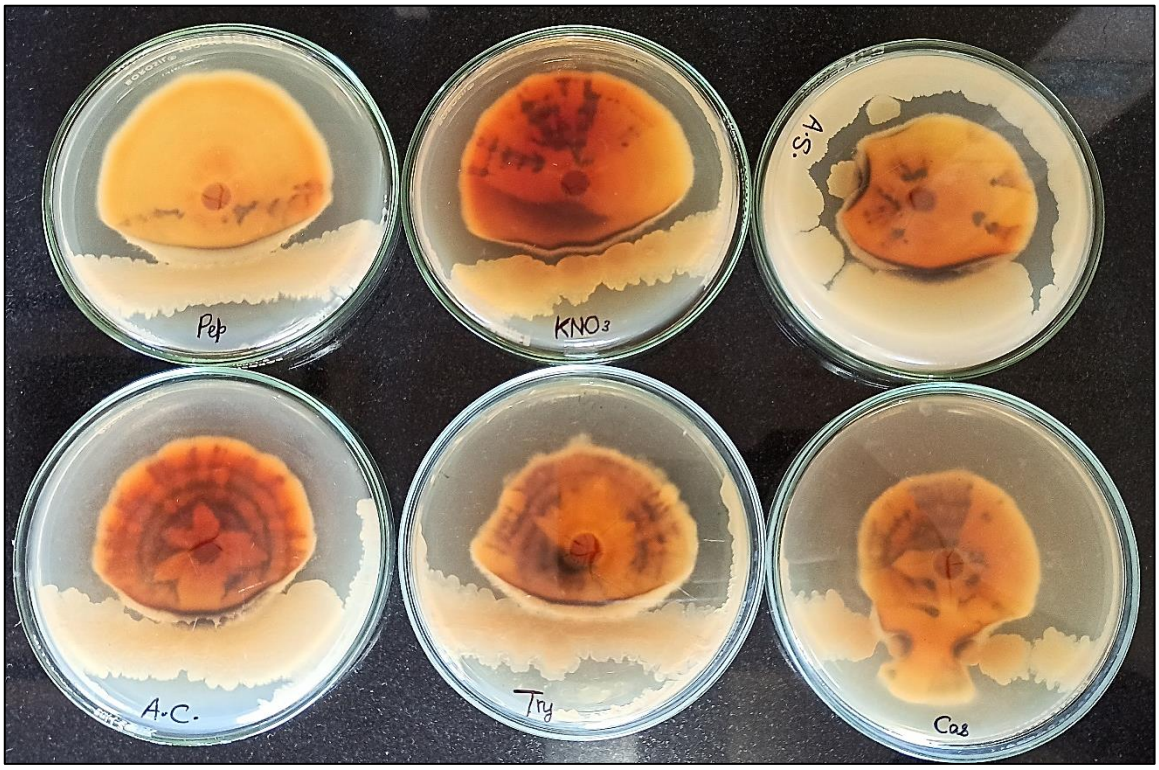


Plate 2: Dual culture assay for the assessment of various nitrogen sources for the production of *Bacillus* sp. SK 23

4.1.3 Determination of the optimum concentration of the selected carbon source

The growth of *Bacillus* sp. strain SK 23 and the antifungal activity varied in the presence of different concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5% and 3%) of sucrose. Growth was seen in each of the media with every different concentration of sucrose. The absorbance and antifungal activity were the highest when the media was supplemented with no sucrose as seen in Figure 3 (optical density 0.355 and inhibition percentage 74.29%). Islam et. al. reported 1% mannitol to be the optimum concentration of the carbon source for maximum cell viability of *Bacillus subtilis* C9. Mezghanni et. al. (2012) carried out the optimization of the medium for antifungal activity production by *Bacillus amyloliquefaciens* using RSM and it suggested that 2.5% of sucrose is required to reach maximum antifungal activity. Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT and RSM where the concentration of sucrose that showed maximum Iturin A production and high antifungal activity was found to be 0.98%. The studies mentioned here show that the optimal sucrose concentration needed for the growth and production of antifungal compound from *Bacillus* sp. is lesser than the nitrogen concentration. In the present study, we found that no sucrose is also suitable for the optimization of the growth and production media.

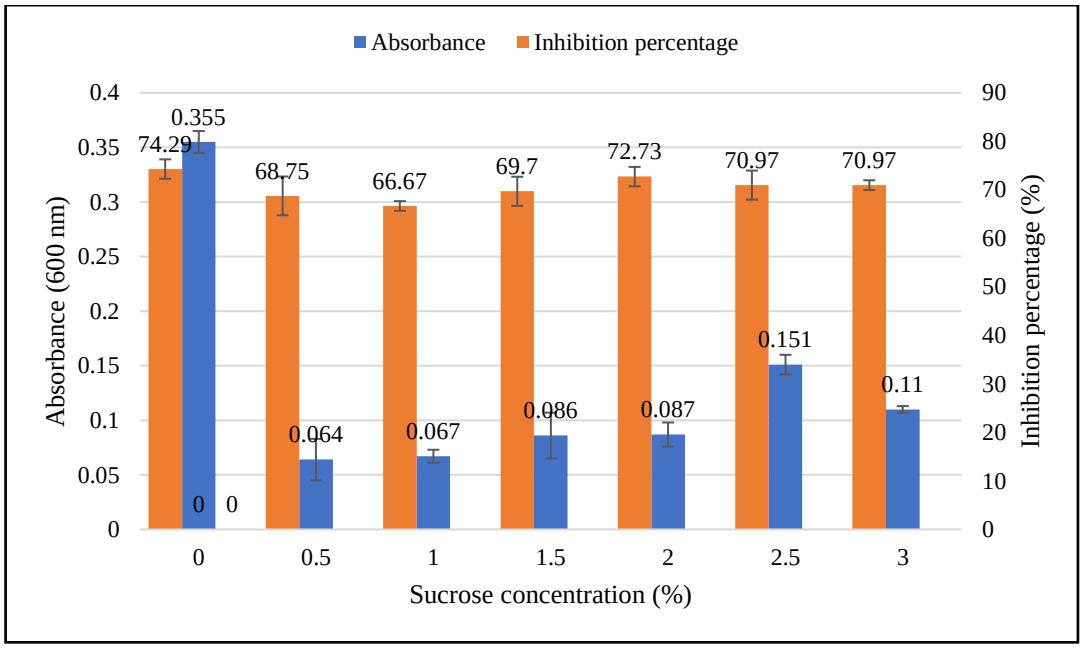
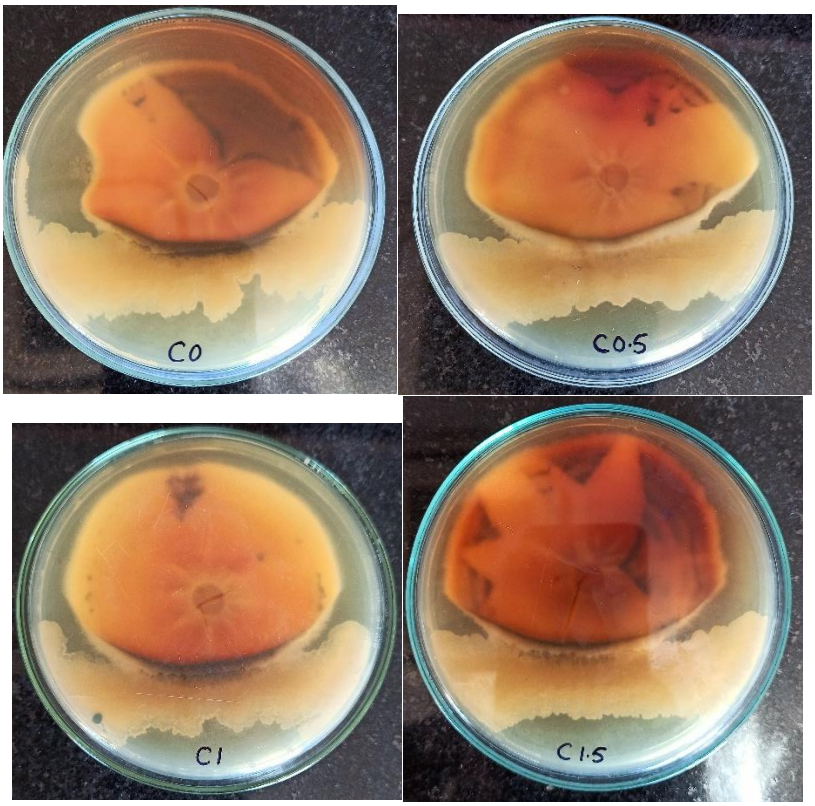


Figure 3: Graph of the Absorbance and Inhibition percentage for utilization of different concentrations of sucrose by *Bacillus* sp. strain SK 23



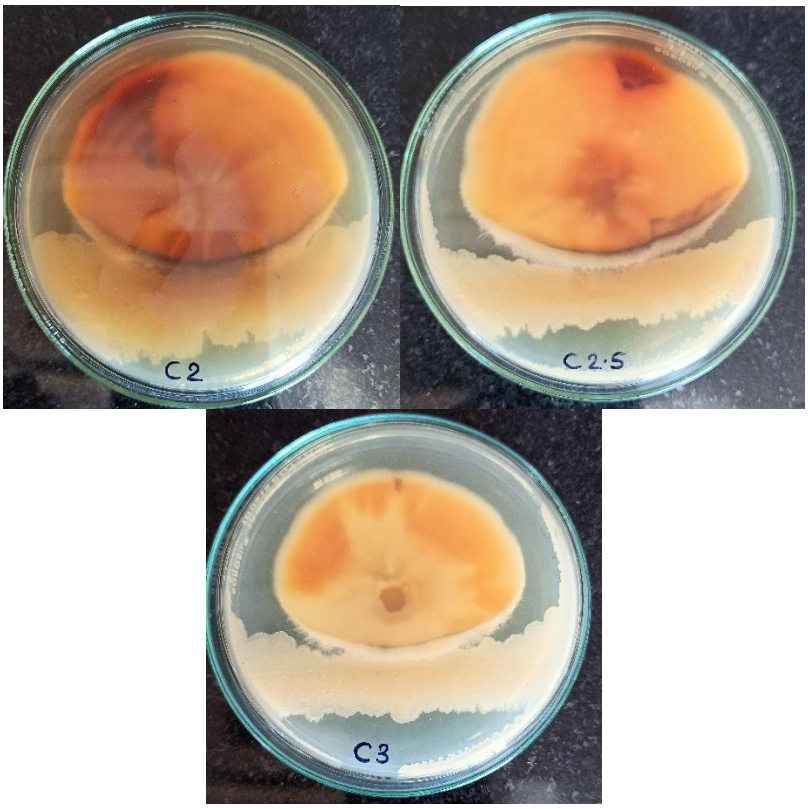


Plate 3: Dual culture assay for the determination of optimum concentration of sucrose for the production of antifungal metabolite

4.1.4 Determination of the optimum concentration of the selected nitrogen source

The growth of *Bacillus* sp. strain SK 23 and the antifungal activity varied in the presence of different concentrations (0%, 0.5%, 1%, 1.5%, 2%, 2.5% and 3%) of tryptone. Growth was seen in each of the media with every concentration of tryptone. The absorbance at 600 nm was the highest at 0.823 when the media was supplemented with 2.5% of tryptone and the inhibition percentage was found to be maximum (78.05%) when the media was supplemented with 2% as well as 2.5% of tryptone concentration as shown in Figure 4. Hence, 2.5% of tryptone concentration was selected as the concentration of the nitrogen source which is necessary for the optimal growth of *Bacillus* sp. strain SK 23. 1% Soytone was reported as the optimum concentration of the nitrogen source for maximum growth and antibiotic production of *Bacillus subtilis* C9 by Islam et. al. (2012). Mezghanni et. al.

(2021) carried out the optimization of the medium for antifungal activity production by *Bacillus amyloliquefaciens* using RSM and it suggested that 2.5% of peptone is required to reach maximum antifungal activity. Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT and RSM where the concentration of peptone that showed maximum Iturin A production and high antifungal activity was found to be 0.94%. The studies mentioned here show that the nitrogen source concentration required for optimization of the growth and production of media is between 1% to 2.5%. The nitrogen source concentration required for the optimization of media for the growth and production of antifungal compound in this study falls in the range of the published reports.

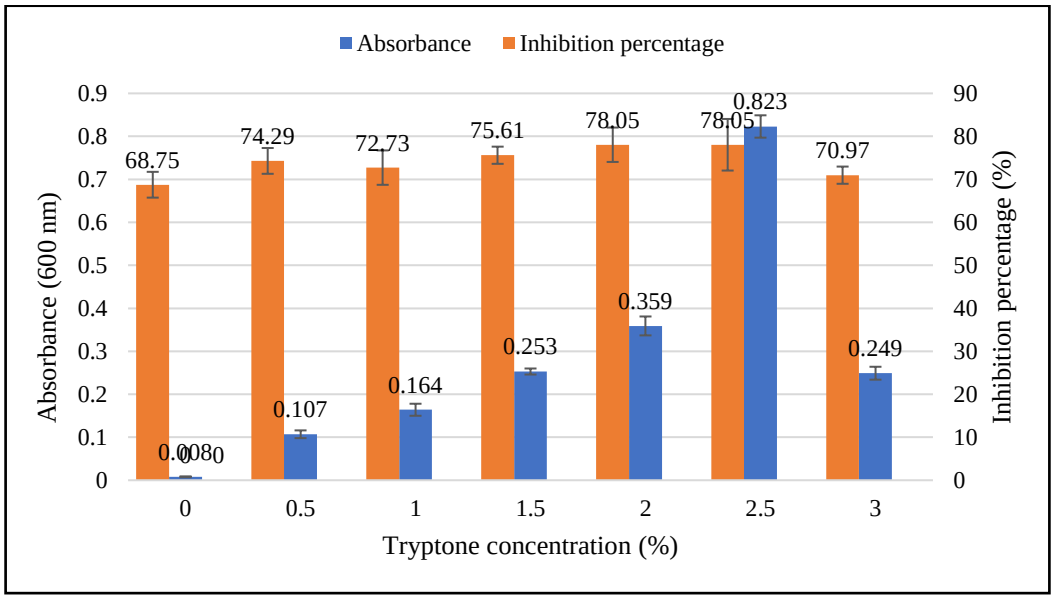


Figure 4: Graph of the Absorbance and Inhibition percentage for utilization of different concentrations of tryptone by *Bacillus* sp. strain SK 23

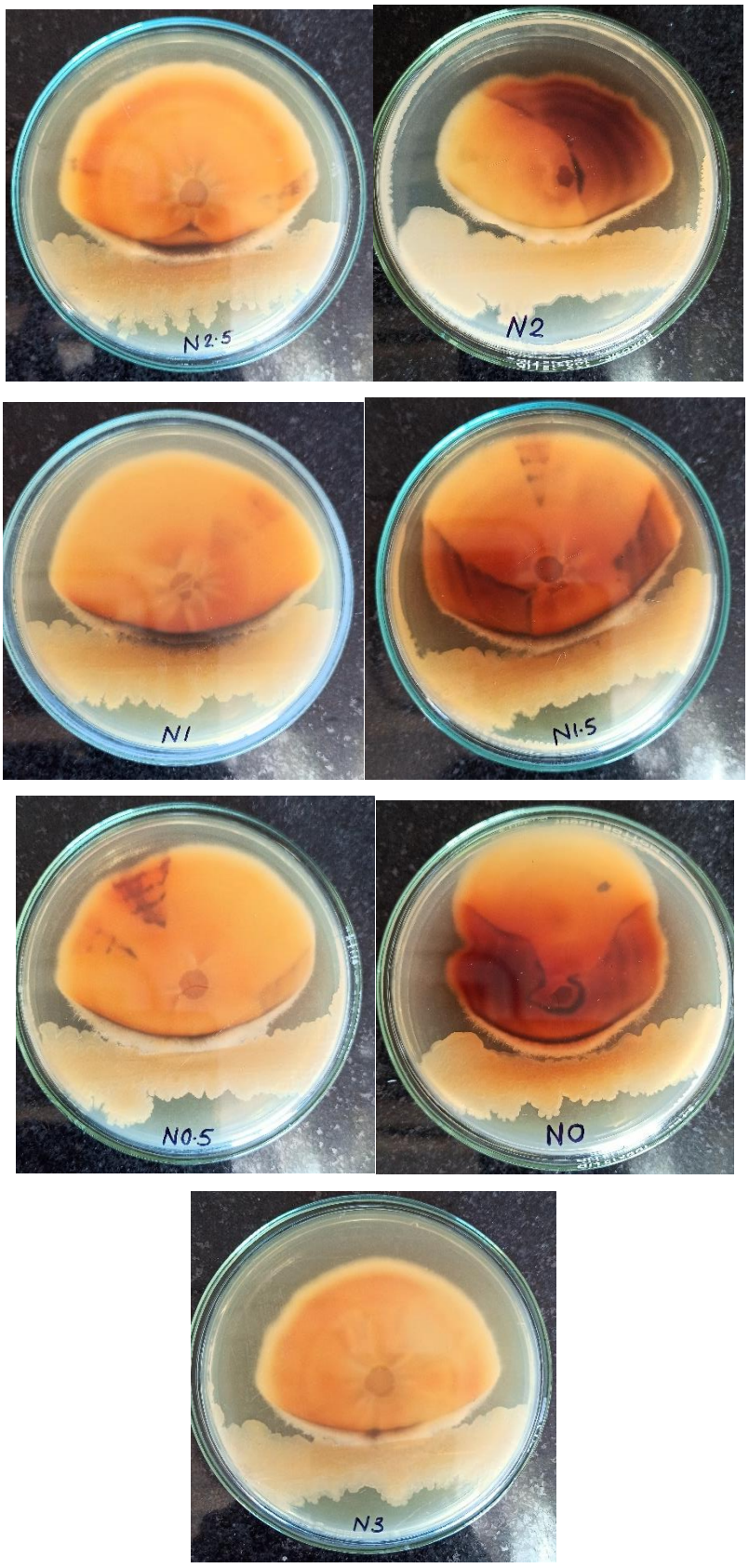


Plate 4: Dual culture assay for the determination of optimum nitrogen source concentration for the production of antifungal metabolite

4.1.5 Determination of the optimum concentration of NaCl for the production of antifungal metabolite

The growth of *Bacillus* sp. strain SK 23 and the antifungal activity varied in the presence of different concentrations (0%, 4%, 8%, 12%, 16% and 20%) of NaCl. Growth was seen in each of the media with every concentration of NaCl. The absorbance at 600 nm was the highest when the media was supplemented with 4% NaCl at 0.921 and the inhibition percentage was found to be maximum (78.38%) in the same media (Figure 5). Al-Sulaimani et. al. (2011) optimized and partially characterized biosurfactants produced by *Bacillus* sp. and found that the biosurfactant retained almost 60% of its activity in a high-saline environment of up to 20% NaCl (w/v).

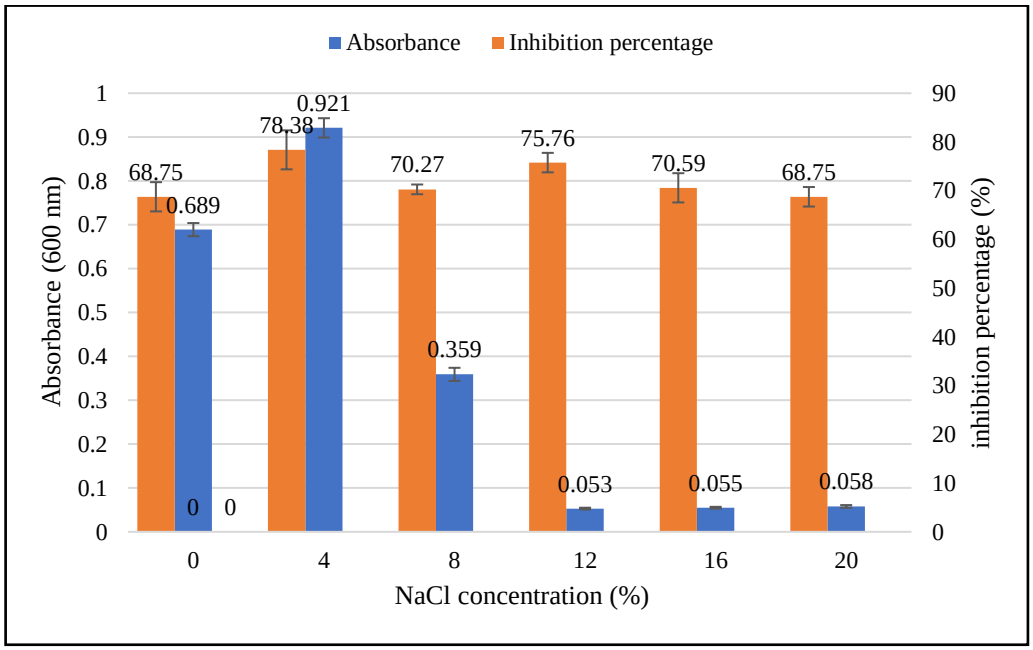


Figure 5: Graph of the Absorbance and Inhibition percentage for utilization of different concentrations of NaCl by *Bacillus* sp. strain SK 23

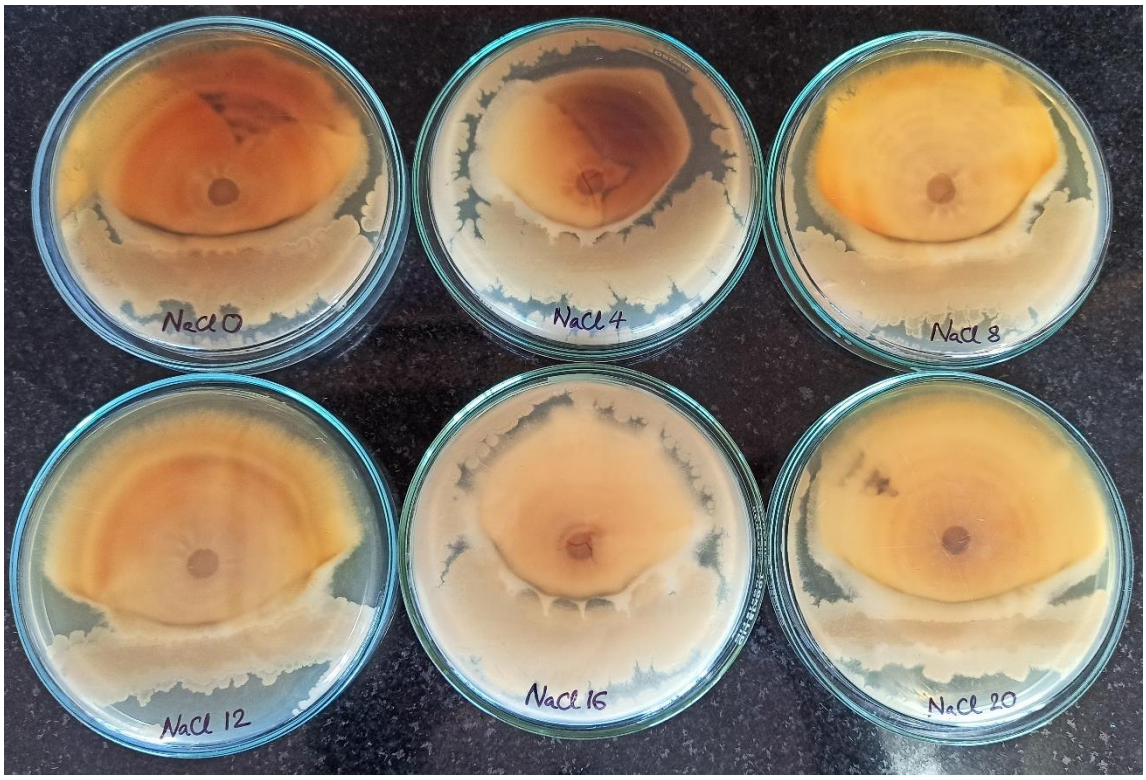


Plate 5: Dual culture assay for effect of NaCl concentration (%) on the production of antifungal metabolite

4.1.6 Determination of the optimum pH for the production of antifungal compounds

The growth of *Bacillus* sp. strain SK 23 and the antifungal activity varied in the presence of different pH (4.0, 6.0, 8.0, 10.0, 12.0 and 14.0). Growth was seen in the media with each of the pH values. The absorbance at 600 nm was the highest at 8.0 pH (0.965) and the inhibition percentage was found to be maximum (66.67%) when the pH of the media was maintained at 4.0 and also at 8.0 as shown in Figure 6. Hence, pH 8.0 was selected as the pH of the media necessary for the optimal growth of *Bacillus* sp. strain SK 23. pH 7.0 was found to be the best for antibiotic production by *Bacillus subtilis* C9 against *Rhizoctonia solani* by Islam et. al. (2012). Al-Sulaimani et. al. (2011) carried out the optimization of media and partial characterization of biosurfactants produced by *Bacillus*

sp. and they found that the cell-free broth produced from strain W19 was found to be stable over a pH range from 6.0 to 10.0 and it was found to be most effective at pH 7.0. Optimization of the antifungal activity by RSM by El-Housseiny et. al. (2021) suggested that pH 8.0 gives the highest antifungal activity by *Bacillus subtilis* isolate CCASU 2021-4. Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT where it showed maximum Iturin A production and high antifungal activity at 6.45. The optimal pH in most of the studies on the optimization of growth and antifungal activity of *Bacillus* species was found to be at 7.0-8.0. *Bacillus* species requires a neutral pH and the results obtained in this study fall in the range of 7.0-8.0.

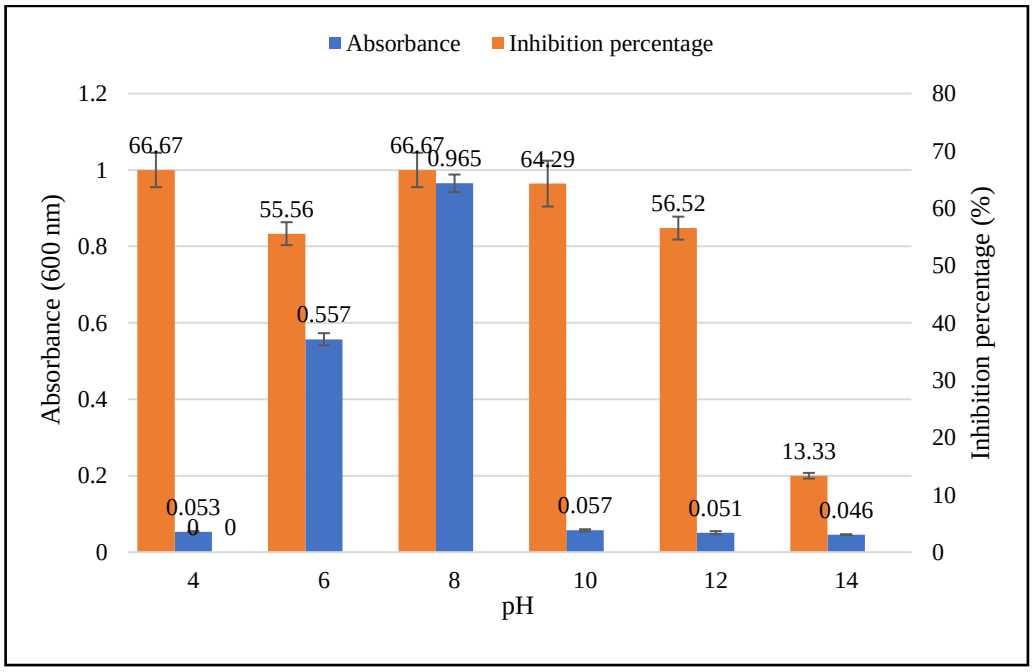


Figure 6: Graph of the Absorbance and Inhibition percentage of different pH values

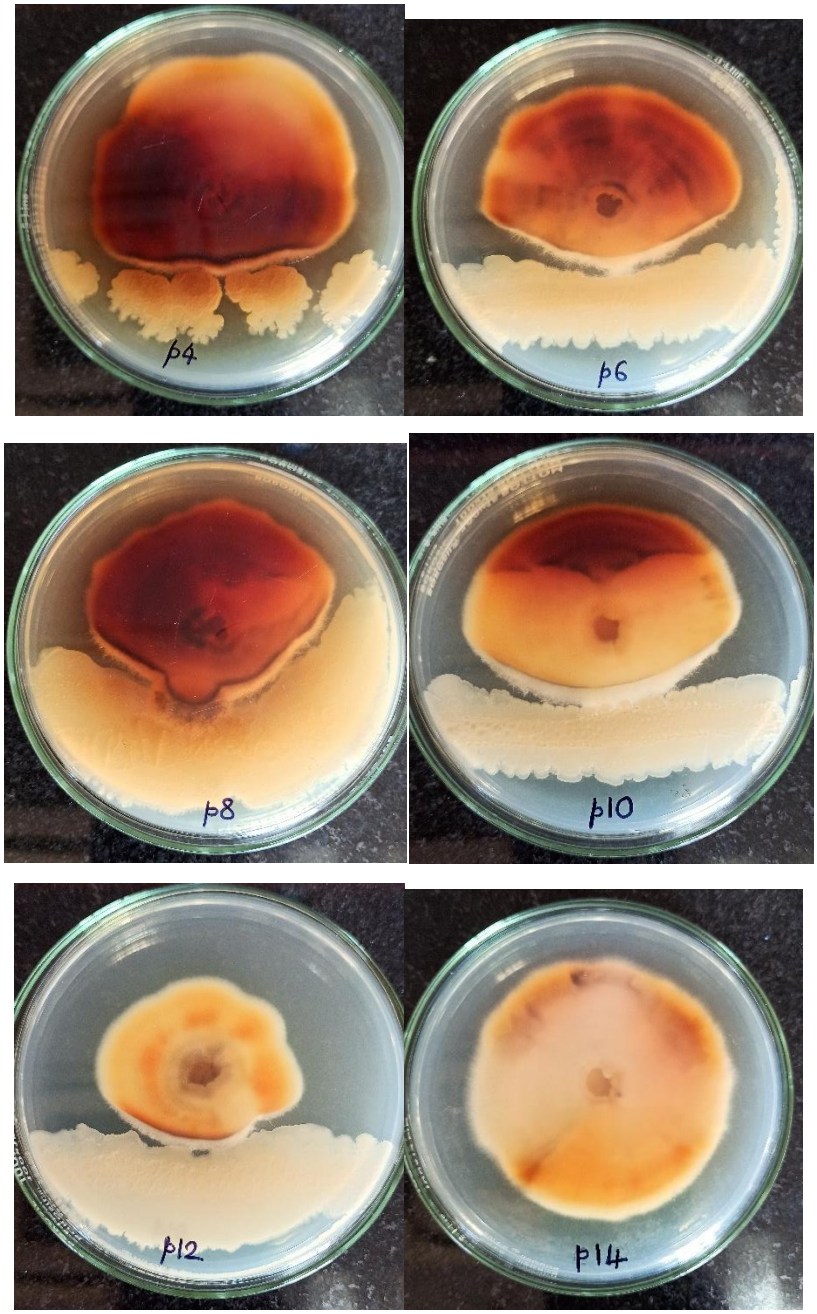


Plate 6: Dual culture assay showing effect of pH of the media on the production of antifungal metabolite

4.1.7 Determination of the optimum temperature for the production of antifungal compound

The growth of *Bacillus* sp. SK 23 and the antifungal activity varied in the presence of different temperatures (10°C, 20°C, 30°C, 40°C, 50°C and 60°C). Growth was seen in the media at each temperature. The absorbance at 600 nm and the inhibition percentage was the highest when the temperature of the media was maintained at 40°C which was 0.916 and 71.87% respectively (Figure 7). The optimum temperature for antibiotic production by *Bacillus subtilis* C9 against *Rhizoctonia solani* was found to be 30°C as reported by Islam et. al. (2012). Zhao et. al. (2014) optimized the production of antifungal lipopeptide from *Bacillus* species BH072 by OFAT where the temperature that showed maximum Iturin A production and high antifungal activity was at 30°C. El-Housseiny et. al. (2021) carried out the optimization of antifungal activity by *Bacillus subtilis* isolate CCASU 2021-4 using RSM and found that the best temperature for the maximum antifungal activity was the temperature at 29.5°C. The studies mentioned show that the temperature required for the optimization of the production of antibiotics and antifungal activity is 30°C.

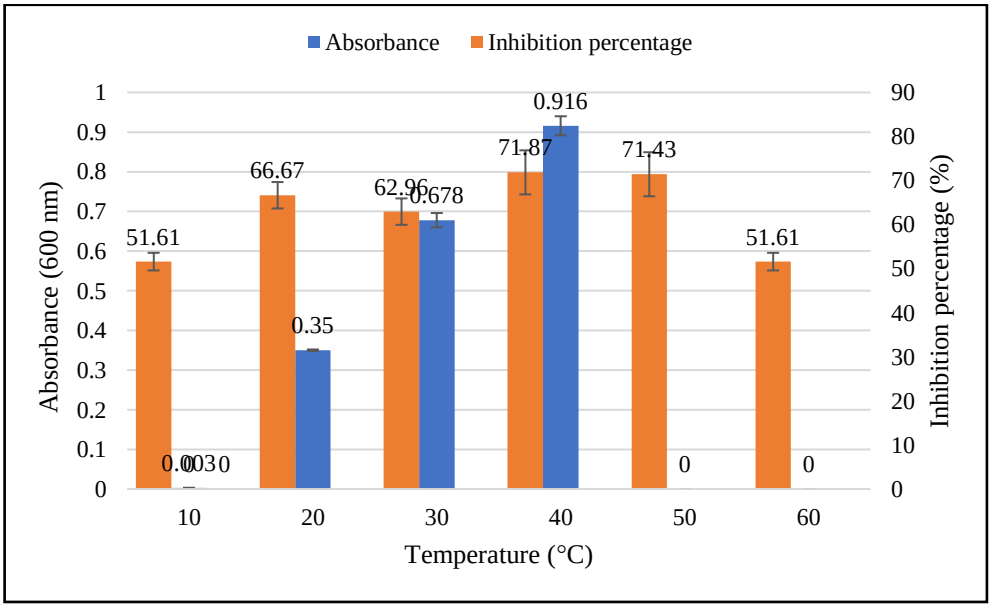


Figure 7: Graph of the Absorbance and Inhibition percentage at different temperatures

Plate 7: Dual culture assay showing effect of different incubation temperatures on the production of antifungal metabolite

4.2 Assessment of growth and production of antifungal compound in Standard bacteriological media, Basal media and Optimized media

Bacillus sp. strain SK 23 showed growth in all three standard bacteriological media *viz.* Zobell marine broth, Nutrient broth and Media D broth. Components of ZMB were selected to use as the basal medium for the growth of *Bacillus* sp. and for the optimization of the production of the antifungal compound as it had the highest absorbance value. Figure 8 shows the results of UV-Vis spectrophotometry, indicating that the bacteria *Bacillus* sp. strain SK 23 grew the best in Zobell marine broth and hence, it required NaCl concentration as well as a nitrogen source for its growth.

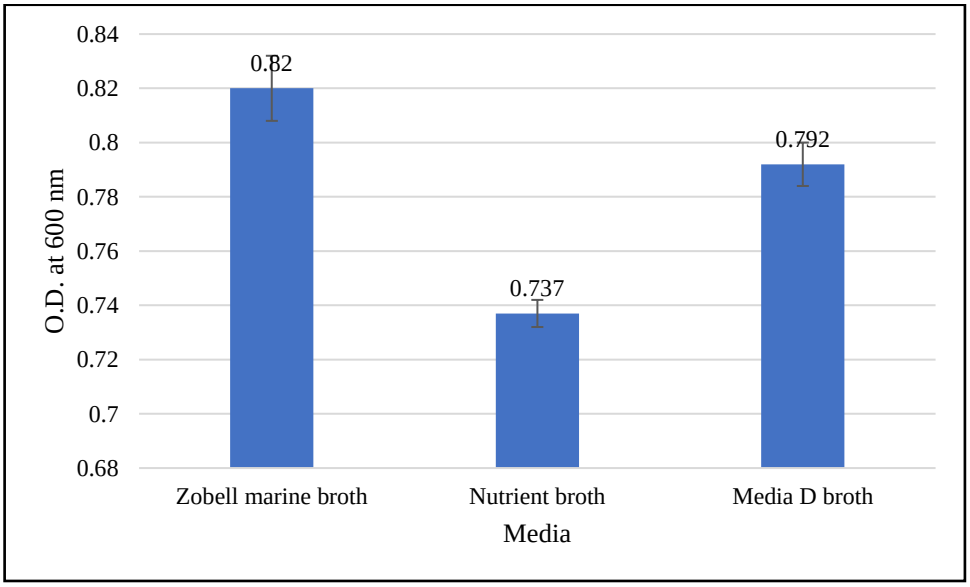


Figure 8: Graph of Absorbance of standard bacteriological media for assessment of growth of *Bacillus* sp. strain SK 23

Growth of *Bacillus* sp. SK 23 in optimized media was found to be higher than the growth of *Bacillus* sp. SK 23 in basal media with absorbance of 0.859 and the inhibition percentage 76.67% as shown in Figure 9. Hence, all the parameters of the media and the

production of antifungal compound were found to be optimized. Zhao et. al. (2014) carried out the optimization of the production of antifungal lipopeptide from *Bacillus* sp. BH072 by OFAT and response surface methodology. The antifungal activity was enhanced by 46.8%. Meena et. al. (2020) aggrandized the *Bacillus subtilis* KLP2015 surfactin metabolite as an antifungal agent using the two factors at a time (TFAT) approach by response surface methodology and it showed potent 75.1% and 41.9% antifungal activity against *Mucor* sp. and *Aspergillus niger* respectively. El-Housseiny et. al. (2021) optimized the antifungal activity of *Bacillus subtilis* isolate CCASU 2021-4 by using response surface methodology resulting in the enhancement of the antifungal activity by 1.2-fold. A study was done by Mezghanni et. al. (2012) on the medium optimization of antifungal activity production by *Bacillus amyloliquefaciens* using response surface methodology and the corresponding bio fungicide production was 250 AU/mL which corresponded to 56% improvement in the production of antifungal compounds. In this study, the inhibition percentage was found to be 76.67% which is higher than all of the other studies that have been mentioned.

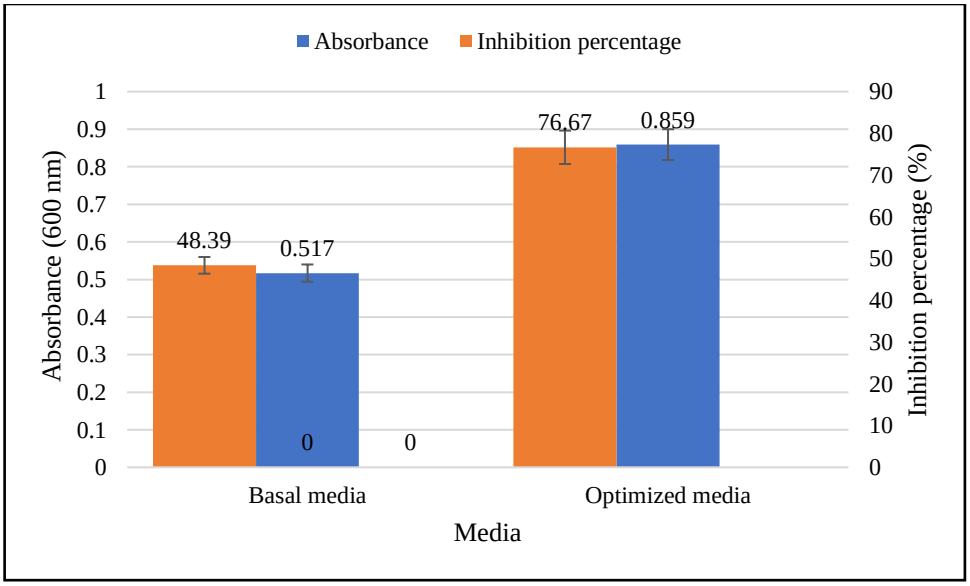
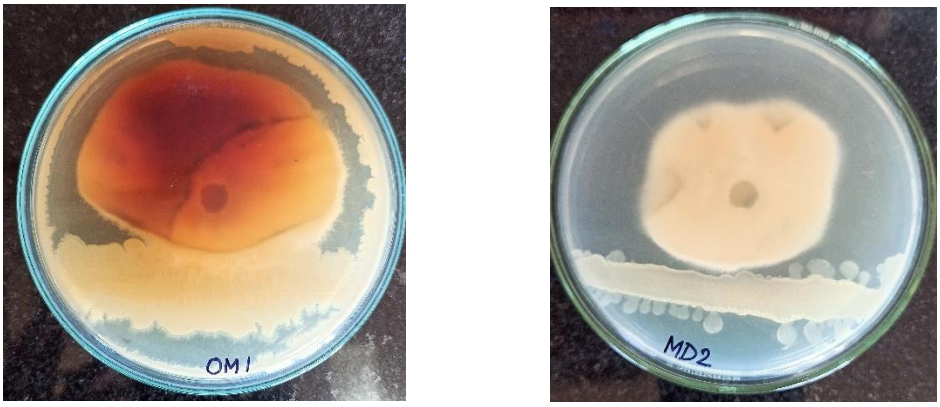


Figure 9: Graph of absorbance and inhibition percentage of *Bacillus* sp. strain SK 23 grown in basal and optimized media



Optimized media Basal media

Plate 8: Dual culture assay of the activity of antifungal metabolite from *Bacillus* sp. grown in Optimized media and Basal media

4.3 Extraction of antifungal compound using Solvent Extraction method

On extraction of the antifungal compound produced by *Bacillus* sp. strain SK 23 by solvent extraction, it was found that the compound dissolved completely in n-Hexane where as it did not dissolve completely in methanol or ethyl acetate. The zone of inhibition was seen to be higher in the solvent extraction by n-Hexane and was found to be 22 mm. Hence, n-Hexane was found to be the best solvent for extraction of antifungal compound by *Bacillus* sp. strain SK 23 with high antifungal activity. The bacterial isolate RHNK22 extracted by Kumar et. al. (2017) by the methods of acid precipitation and methanol extraction showed inhibition of the phytopathogen *Sclerotium rolfsii* and *Macrophomina phaseolina.* Wang et. al. (2012) carried out the purification and characterization of antifungal compounds from *Bacillus coagulans* TQ33 isolated from

skimmed milk powder. Extraction was done using many solvents including n-Hexane and ethyl acetate which showed strong antifungal activity.

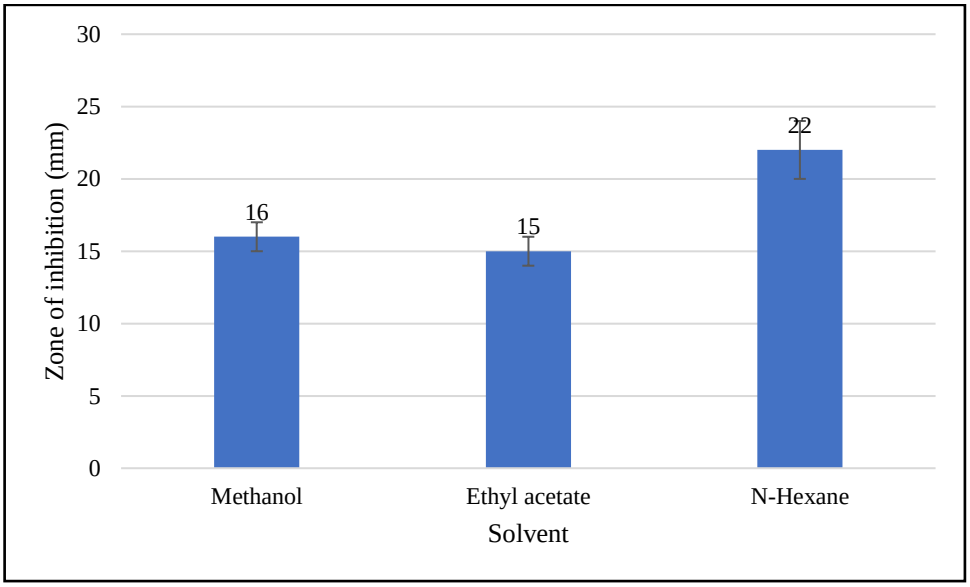


Figure 10: Graph of inhibition percentage of antifungal compound extracted by solvent extraction

Optimized media Basal media

Plate 9: Well diffusion assay for assessment of antifungal activity of antifungal metabolite produced in optimized media and basal media

4.4 Estimation of dry weight and yield of the antifungal compound after extraction

The dry weight of each of the solvent samples after rotary evaporation under vacuum was found to be 16.1 mg in 70 mL methanol, 12.3 mg in 70 mL ethyl acetate and 15.7 mg in 70 mL n-Hexane and the yield of the antifungal metabolite in methanol was calculated to be 0.23 mg/mL, in ethyl acetate was 0.176 mg/mL and in n-Hexane was 0.224 mg/mL as seen in Figure 11 and Figure 12. The dry weight of the samples from basal media were found to be 5.2 mg in methanol, 3.9 mg in ethyl acetate and 4.9 mg in n-Hexane and the yield of the antifungal metabolite was found to be 0.074 mg/mL in methanol, 0.056 mg/mL in ethyl acetate and 0.070 mg/mL in n-Hexane. Hence, the yield was seen to increase by 3-fold when *Bacillus* sp. SK 23 was grown in optimized media. In the study reported by Wang et. al. (2012), antifungal compounds from *Bacillus coagulans* TQ33 isolated from skimmed milk were purified and characterized and were found in the ethyl acetate fraction concentrated in vacuum to obtain 12 g of residue. Biosurfactants produced by *Bacillus* species were optimized and partially characterized aiming to test the potential of microbial enhanced oil recovery in Oman oil fields and was found that the yield of biosurfactant produced by *Bacillus subtilis* strain W19 was 2.5 g/L. A study was done by Mezghanni et. al. (2012) on the medium optimization of antifungal activity production by *Bacillus amyloliquefaciens* using response surface methodology and the corresponding bio fungicide production was found to be 250 AU/ml. The yield obtained from this study by extraction was lesser than the other mentioned studies but the antifungal activity surpassed the others indicating that the antifungal metabolite is more effective against the phytopathogen *Fusarium solani.*

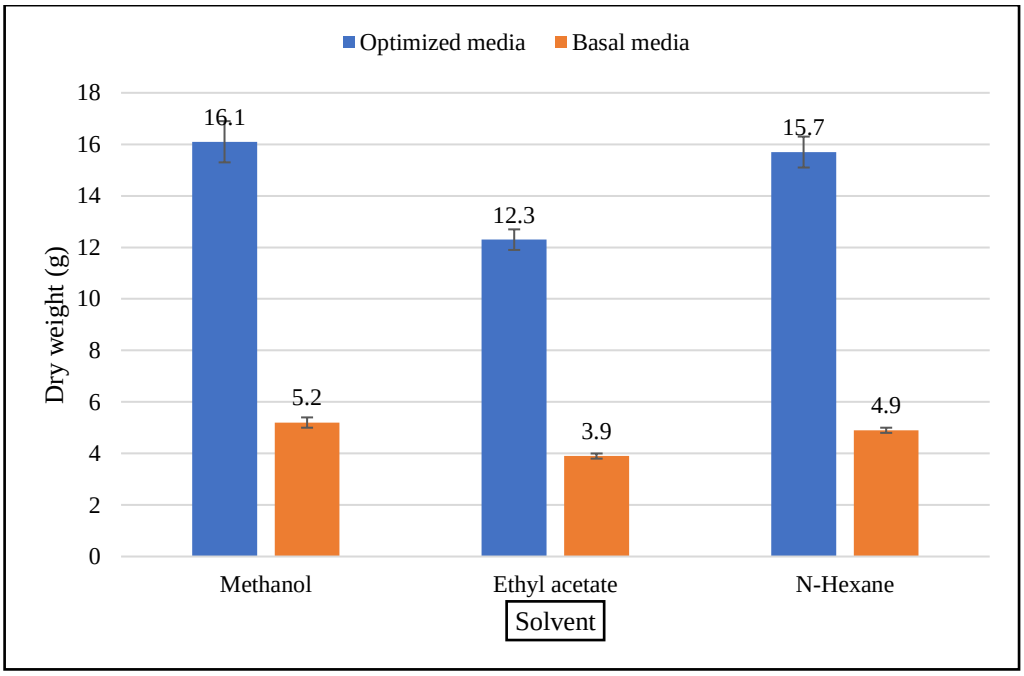


Figure 11: Graph of dry weight of extracted antifungal metabolite from optimized media and basal media

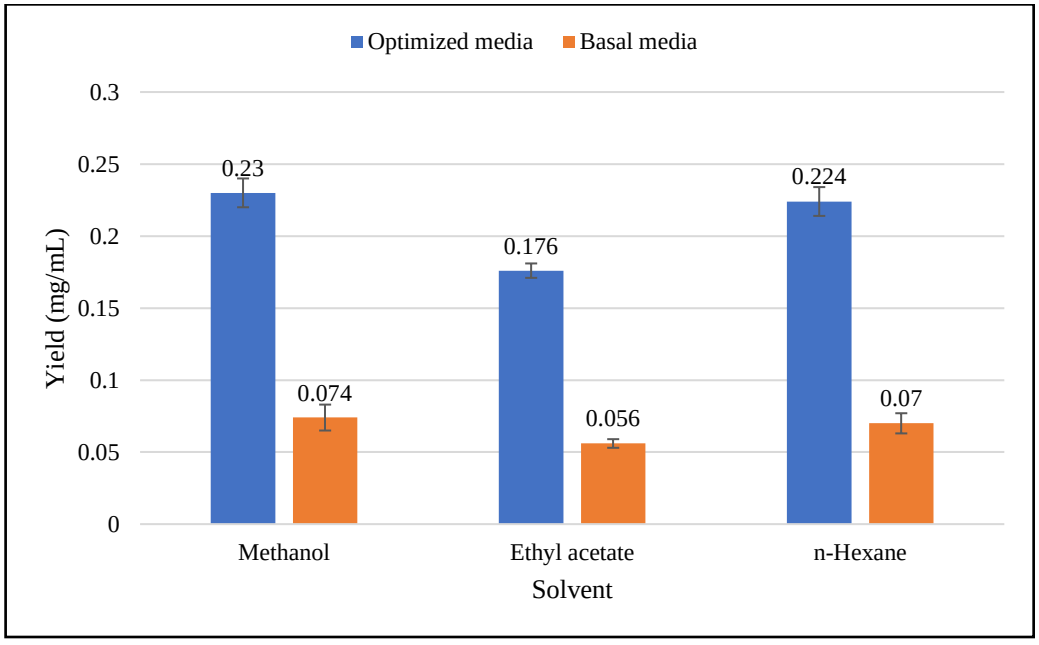


Figure 12: Graph of yield of extracted antifungal metabolite from optimized media and basal media

5. Summary

In the present work, a *Bacillus* sp. strain SK 23 was found to produce antifungal compound against phyto-pathogenic fungi *Fusarium solani*.

The optimized media conditions for the optimum growth of the *Bacillus* sp. SK 23 as well as the production of the antifungal compound were with 2.5% tryptone (nitrogen source concentration), 4% NaCl concentration, pH 8.0 and 40°C incubation temperature.

The optical density of the culture broth at 600 nm under optimized conditions increased from 0.517 to 0.859. The antifungal activity of the antifungal compound under optimized conditions was found to increase from 48.39% to 76.67%.

Extraction of the antifungal compound was carried out using methanol, ethyl acetate and N-hexane. The antifungal compound dissolved completely in N-hexane and gave the maximum inhibition of 22 mm of *F. solani.*

Optimization of the media components and growth conditions increased the yield of the antifungal compound by 3-fold.

The present work provides a pilot scale production for the industrial growth and production of the antifungal compound produced by *Bacillus* sp. SK 23 which can be used for agricultural applications.

6. Conclusion

Optimization of the media components and growth conditions increased the yield of the antifungal compound 3-fold. Hence this study shows the potential of *Bacillus* sp. against fungal plant pathogen *F. solani.*

7. Future Prospects

- Purification and characterization of the antifungal compound from *Bacillus* sp. strain SK 23.

- A scale up process for the industrial production of *Bacillus* sp. strain SK 23 and the antifungal metabolite.

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7. Appendix

1. Media D (MD)

Tryptone	1.5 g
Soya peptone	0.5 g
Sodium chloride	0.5 g
Agar	2.0 g
Distilled water	100 mL
pH	7.2

2. Zobell marine broth components as Basal media

Peptone	0.5 g
Sodium chloride	2.0 g
Agar	2.0 g
Distilled water	100 mL
pH	7.6 ± 0.2

3. Optimized media for *Bacillus* sp.

Tryptone	2.5 g
Sodium chloride	4.0 g
Agar	2.0 g
Distilled water	100 mL
pH	8.0 ± 0.2
Temperature	40°C

4. Potato Dextrose Agar (PDA)

PDA powder	3.9 g
Agar	0.5 g
Distilled water	100 mL
pH	5.6 ± 0.2

5. Zobell Marine Agar (ZMA)

ZMA powder	5.5 g
Agar	0.5 g
Distilled water	100 mL
pH	7.6 ± 0.2

6. Nutrient Broth (NB)

NB powder	1.3 g
Distilled water	100 ml
pH	6.8 ± 0.2

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1. Introduction

Bacillus sp. are microorganisms which are Gram positive, rod-shaped, facultative aerobic, spore forming bacteria which are spread widely in nature. The genus Bacillus have been isolated from many surroundings with probable applications in the field of biotechnology. Bacillus species are a source of many secondary metabolites which have a number of applications as anti-cancer agents, antibiotics, biosurfactants, biopesticides, enzyme inhibitors, etc. Bacillus sp. shows a wide range of physiological abilities; hence it can survive in most of the environmental conditions and compete with other favourable organisms within that environment. It produces stable metabolites which have an inhibitory effect on other microorganisms CITATION Far09 \l 16393 (Faruk Adamu Kuta, 2009). Bacillus sp. synthesize different extracellular metabolites having broad-spectrum activity against various microbes and hence it is important to investigate it for antimicrobic activity CITATION MAw10 \l 16393 (M. Awais, 2010).