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Research Article

Antibacterial compound of *Bacillus Amyloliquefaciens* and *Bacillus Siamensis*: screening, characterization, and evaluation

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Abstract: This study was aimed at isolating potential antimicrobial compound (AMC) producing bacteria. AMC produced by a *Bacillus* species was evaluated further for its antimicrobial potential. Antimicrobial compound-producing bacteria were isolated from the soil of crop fields from the local region of Satana, Nashik (India), and tested against clinical isolates. Both isolates exhibited remarkable antibacterial potential against Gram-positive and Gram-negative clinical isolates. The AMCs of both SYS 1 and SYS 2 exhibited excellent antibacterial activity against *Salmonella paratyphi B* and *Staphylococcus aureus*. Both AMC-producing isolates were characterized and identified. *Bacillus* species SYS 1 and SYS 2 were identified as *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, respectively. The highest antimicrobial activity of AMC produced by *Bacillus amyloliquefaciens* SYS 1 was exhibited against *Salmonella paratyphi B* (28 mm), followed by *Staphylococcus aureus* (26 mm). *Bacillus siamensis* SYS 2 AMC extracted by the solvent ethyl acetate exerted the highest antimicrobial activity against *Salmonella paratyphi B* (18 mm), followed by *Staphylococcus aureus* (16 mm). A partial characterization of the AMC was conducted and evaluated to contain amino acids and proteins. A higher total protein content of 17.9 µg/mL was estimated in the partially purified AMC of *Bacillus amyloliquefaciens* SYS 1. A detailed evaluation of the structural characteristics of AMC could prove its importance in commercial applications.

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1. INTRODUCTION

The problem of resistance against the present antibiotics in bacteria is increasing day by day (Elmaidomy, 2022). It is an alarming scenario that drug resistance is developing among the pathogenic microbes, and it is important to find effective alternative metabolites and products to overcome it (Peláez, 2003; Huan, 2020). Several studies have reported antimicrobial compounds such as antimicrobial peptides (AMPs) and bacteriocins as potential solutions to the problem of antibiotic resistance (Raheem & Straus, 2019; Moravej *et al.*, 2018; Rotem & Mor, 2009). AMPs (antimicrobial peptides) are considered potential alternatives against such resistant microbial pathogens (Rotem & Mor, 2009). Nearly 2000 varieties of AMPs from living

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organisms have been evaluated (Jenssen *et al.*, 2006; Sang & Blecha, 2008; Donadio *et al.*, 2010; Brogden & Brogden, 2011; Sumi *et al*., 2015), because AMPs are naturally occurring in humans, they serve as effective defences (Huan, 2020). Bacteriocins are ribosomally synthesized antimicrobials, short polypeptides synthesized by a number of different bacteria (Riley & Wertz, 2020; Cherif *et al.,* 2003).

The genus *Bacillus* was evaluated for its ability to produce a range of AMPs. It has considerable weightage for developing new antimicrobial compounds (Bizani *et al.,* 2005; Xie *et al.,* 2009). Many studies have found that AMPs from various *Bacillus* species are effective antimicrobial compounds (Abriouel *et al.*, 2011). *Bacillus* species produce a large number of antimicrobial compounds such as bacteriocins, polyketides, and surfactins (Wang *et al.*, 2014; Cladera-Olivera *et al.,* 2004). Application of surfactins has evolved in food, pharmaceuticals, and cosmetics as a surfactant and emulsifier (Moldes *et al.*, 2021). Bacteriocin-like substances produced by *Bacillus* species were found to be antimicrobials (Wang *et al.*, 2015). These consist of subtilin from *B. subtilis;* coagulin from *Bacillus coagulans*; bacthuricin F4, thuricin 17, entomocin 9, and tochicin from *Bacillus thuringiensis*; cerecin 7 from *Bacillus cereus*; bacillocin 490 from *B. licheniformis* (Cherif *et al.,* 2003; Aunpad & Na-Bangchang 2007; Hammami *et al.*, 2013; Stoica *et al*., 2019 & Sumi *et al*., 2015). *B. subtilis* has been reported to produce gageostatin and difficidin macrolides, that inhibit the growth of many Gram-negative bacteria (Kaspar *et al.*, 2019; Geraldi *et al.*, 2022). Lipopeptides produced by *Bacillus* are considered biosurfactants due to their biodegradability and minimal toxicity to humans, animals, and plants (Makkar & Cameotra, 2002; Jeyakumar & Zhang, 2022). *Bacillus* species is an important bacterium commonly found in soil that produces antimicrobial compounds, etc. In the present study, two *Bacillus* strains were isolated from the soil of different crop fields, and the characterization of an antimicrobial compound produced by them was conducted. The AMC produced by the isolates was evaluated against clinical isolates for antimicrobial potential.

2. MATERIALS and METHODS

2.1. Screening of Antimicrobial Compound Producing Organism

A total of 20 soil samples were collected from the rhizosphere of different crops (pomegranate, grape, sugarcane, maize, onion, etc.) in Satana, Nashik. All samples were brought to the laboratory and preserved at low temperature. During isolation, a few plates contained zones of inhibition around a few bacterial colonies in crowded growth. These organisms that produce antimicrobial compounds (AMC) were primarily screened out. From ten isolates, two potential AMC-producing organisms (exhibiting greater zones of inhibition) were further screened out for study.

2.2. Testing of Isolates Against Pathogens by Agar Diffusion Assay

The agar diffusion method has been used for the evaluation of the antibacterial potency of isolates (Piddock, 1990). In this test, an antimicrobial compound is impregnated on paper discs used for assaying (Parish & Davidson, 1993). The disc diffusion assay was used to assess each isolate's antibacterial potential against clinical pathogens. Both AMC producing isolates, SYS 1 and SYS 2, were tested against pathogens such as *E. coli, Staphylococcus aureus, Salmonella paratyphi B,* and *Pseudomonas aeruginosa.* Both AMC producing organisms, SYS 1 and SYS 2, were inoculated in nutrient broth and incubated at 37 °C for 24 hours. After incubation, the broth was centrifuged (5000 rpm for 15 min). The supernatant was collected in sterile tubes and used as crude AMC preparation for the diffusion assay. Mueller Hinton agar containing beef extract: 2.0 g/L, acid hydrolysate of casein: 17.5 g/L, starch: 1.5 g/L, agar-agar: 17.0 g/L, and pH: 7.3±0.1 was used as a basal and seed medium. Sterile basal agar plates and seed agar butts were prepared. After the pouring and solidification of seed agar, sterile filter paper discs were impregnated with the respective crude AMC preparations and placed on the surface of the

medium in each plate. Then all plates were incubated at 37 °C for 24 h. After incubation, the diameters of the zones of inhibition were recorded.

2.3. Identification of Isolates

Both AMC producing organisms were characterized by morphological and biochemical characteristics. Biochemical characterization was conducted according to Bergey's Manual of Determinative Bacteriology (Bergey & Holt, 2000).

2.4. Molecular Characterization of AMC Producing Organisms

DNA was isolated from both bacterial samples using Biopure™ kits for bacterial genomic DNA isolation. The 16S rRNA gene was amplified by PCR from the above-mentioned isolated DNA by using primers such as forward AGAGTTTGATCCTGGCTCAG and reverse TACGGTTACCTTGTTACGACTT. PCR conditions were maintained such that the first cycle was conducted at 94°C for 5 min for initial denaturing, and for 35 cycles conducted, denaturation of DNA was carried out at 94°C for 60 seconds, and 53 °C was maintained for 45 sec for annealing, as well as 68°C for 90 seconds for one extension. The final extension was conducted for one cycle at 68°C for 10 min. Amplified PCR product was subjected to electrophoresis using Agarose gel at 1% along with a 1 kb marker in TAE buffer and visualized by staining with ethidium bromide. The PCR product was purified by washing with sodium acetate and 70% ethanol and eluted from the gel. Forward and reverse sequencing reactions of PCR amplicons were carried out on an ABI 3730XL sequencer to obtain the sequence. The sequences of 16S rRNA were obtained. The assembled DNA sequence was used to carry out BLAST searches with the nr database of the NCBI. The sequences were aligned with NCBI blast sequences, and a phylogenetic tree was constructed. For the identification of both isolates, phylogenetic analysis was conducted.

2.5. Production of Antimicrobial Compound (AMC)

The production of AMC was carried out in a 1000 mL flask containing 500 mL liquid medium. The minimal medium used consists of g/L of glucose (15.00), peptone (5.00), KH_2PO_4 (1.00), $MgSO_4$ (0.1), FeSO4 (0.1), and NaCl (4.00). The medium's pH was adjusted to 7±0.02. After sterilization, the production medium was inoculated with AMC-producing organisms, *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, in their respective flasks. The flasks were incubated at room temperature for 48 h. The broth was centrifuged, and the supernatant was collected for further study.

2.6. Salt Precipitation of AMC

It was characterised as antimicrobial peptides (AMPs) or bacteriocin like substances, admired potential antimicrobial compounds (Rotem and Mor, 2009; Pálffy *et al.*, 2009; Cotter, 2013). The *Bacillus* species have ability to synthesise a range of antimicrobials (Xie *et al.*, 2009; Bizani *et al.*, 2005). After an extensive review of reports, it was noticed that the nature of the antimicrobial compounds produced by various *Bacillus* species belongs to proteinic metabolites that may be antimicrobial peptides (AMPs). The extraction of desirable antimicrobial substances was conducted by salt and organic solvent precipitation. Salt precipitation of the AMC was carried out using the method described by Jayaraman (2007). The culture broth was centrifuged (5000 rpm for 20 min) to remove the cells. The cell free broth was subjected to salt precipitation. Solid ammonium sulphate was added slowly to the culture supernatant to reach 50 %, 60 %, and 70 % saturation and stirred for 24 h at 4 °C. The precipitate was harvested by centrifugation at 5000 rpm for 15 minutes. The precipitate was then dissolved in a 0.07 M sodium acetate buffer and dialyzed overnight at 4 °C against the same buffer. The purified residue was used for further determination of antibacterial activity and preliminary characterization (Gholam *et al.*, 2014).

2.7. Solvent Extraction

For the extraction of an antimicrobial compound, the culture medium was centrifuged at 5000 rpm for 20 minutes. The solvent precipitation of AMC was performed according to Jayaraman (2007). The cell free supernatant was mixed with organic solvents such as ethyl acetate and butanol at a ratio of 1:1 v/v. The mixture was then vigorously shaken and allowed to separate in a separating funnel, resulting in two distinct layers: the organic upper layer and the aqueous lower layer. The organic layer was separated and collected in a clean, sterile flask. Then the organic phase was subjected to evaporation in a rotary vacuum evaporator. The extracts in dry form were collected in clean, sterile containers and preserved at a low temperature in a refrigerator. These purified extracts were used for testing antimicrobial activity by the agar diffusion method.

2.8. Testing of Isolates for Antimicrobial Activity Against Pathogenic Test Organisms

The clinical isolates *E. coli, Salmonella paratyphi B, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were used as test organisms in a preliminary diffusion assay to check antimicrobial potential.

2.9. Preliminary Characterization of AMC

2.9.1. *Thin layer chromatography*

The antimicrobial compounds of the two isolates were analysed by silica gel TLC. The thin layer chromatography was performed using TLC plates (Merck Silica Gel 60 F_{254}). Using a capillary tube, a row of spots of the partially purified antimicrobial compound obtained after salt and solvent extraction and known amino acids was applied along with a line 1.5 cm above the bottom of TLC plates, and the spots were allowed to dry. The TLC plate was placed vertically in a trough (the TLC solvent chamber) containing the solvents n-butanol: methanol: water (3:1:1). Solvents are allowed to travel to 80 percent of the chromatogram, and then the plates were dried in a hot air oven at 120°C for 10 min. Chromatograms were sprayed with ninhydrin solution, dried similarly as mentioned above, and observed for the presence of spots.

2.10. Qualitative Analysis of the Functional Groups of the Antimicrobial Compounds

The detection of amino acids and proteins was performed according to Jayraman (2007). For the detection of amino acids and proteins, the Ninhydrin and Folin-Lowry's tests were performed. According to Jayraman (2007), further quantitative estimation of total proteins was conducted using Folin-Lowry's method. It is a widely used and highly sensitive method for the estimation of proteins. For the total protein content estimation, bovine serum albumin was used as a standard protein. The range of concentrations of bovine serum albumin was prepared from 10 to 100 µg/mL. The reaction was conducted by mixing known concentrations of bovine serum albumin with 5 ml of freshly prepared 2% sodium carbonate in 0.1 N sodium hydroxide and 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartrate in a ratio of 50:1. Then 0.5 ml of diluted, commercially available Folin-Ciocalteau reagent was added to the mixture and incubated for 30 min. in the dark at room temperature. After reaction, the absorbance was measured at 660 nm against a blank (without bovine serum albumin). The standard curve was constructed by plotting the concentration of bovine serum albumin vs. absorbance. The amount of proteins in samples was determined using a standard curve.

3. RESULTS

3.1. Screening and Isolation of AMC Producing Organisms

Using the crowded plate technique, AMC producing organisms were screened out on the basis of the zones of inhibition exhibited from collected soil samples. During the screening, ten potential AMC producing organisms were screened out. All the isolates were bacterial and exhibited higher zones of inhibition. A preliminary disc diffusion assay was conducted to test the AMC producing isolates against clinical pathogens. Among the ten isolates, two organisms showed higher zones of antibacterial activity against all test organisms. Hence isolates designated as SYS 1 and SYS 2 which were screened out and used for further investigations.

3.2. Characterization of AMC Producing Isolates

The morphological characters detected are depicted in Table 1. The isolate SYS 1 produced mucoid, white colonies, while SYS 2 produced off-white, semi-mucoid colonies. Both isolates SYS 1 and SYS 2 were detected to have the ability to produce enzymes such as amylase, oxidase, and catalase while being unable to produce nitrate reductase. The isolates SYS 1 and SYS 2 were also tested, and they grew in the presence of a higher percentage of salt concentration. Both SYS 1 and SYS 2 isolates were tested for their ability to ferment various sugars and use different sugars as carbon sources. The results of biochemical characterization are depicted in Table 2. Morphological and biochemical characteristics of both isolated organisms suggested that isolated organisms belong to genus *Bacillus.*

Table 1. Morphological characters of the isolates SYS 1 and SYS 2.

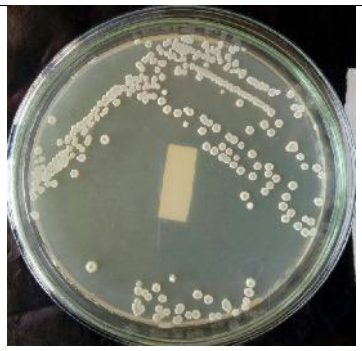
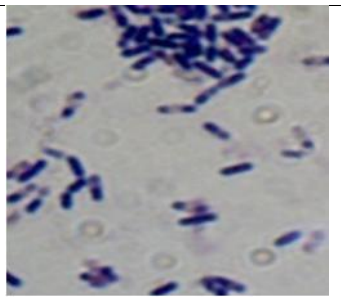
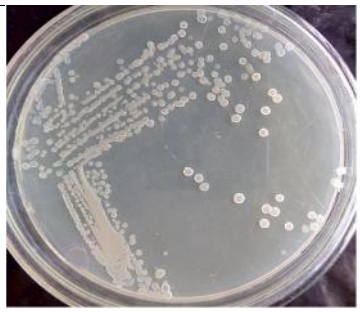
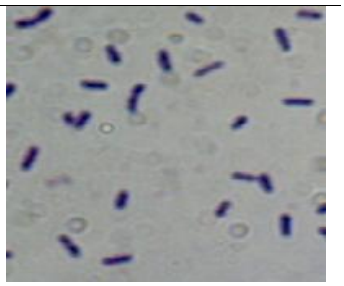
Isolate	Colony Characters		Microscopic Observation (1000x magnified image)	
SYS1	Size-5mm, colour- White circular colonies with entire margined, convex with sticky consistency		Rod shaped, Gram-positive, Motile	
SYS2	Size-4mm, colour- White circular colonies with entire margined, convex with sticky consistency		Rod shaped, Gram-positive, Motile	

Table 2. Biochemical characters of the isolates SYS 1 and SYS 2.

Biochemical Character	Isolate SYS1		Isolate SYS2	
	Sugar Fermentation			
	Acid	Gas	Acid	Gas
Arabinose	-	-	-	-
Mannitol	-	-	-	-
Glucose	+	-	+	-
Sucrose	-	-	-	-
Lactose	-	-	-	-
	Sugar Utilization			
Arabinose	+		+	
Mannitol	+		+	
Glucose	+		+	
Sucrose	+		+	
Lactose	-		-	
	Enzyme production			
Amylase	+		+	
Oxidase	+		+	
Catalase	+		+	
Nitrate reductase	-		-	
Growth in 6.5 % NaCl	+		+	

(+ Positive test; - Negative test)

3.3. Testing of Isolates for Antimicrobial Activity Against Pathogenic Test Organisms

The diameters of the zone of inhibition were measured and recorded, which are summarized in Table 3. Cell-free crude extracts of broth from both isolates (SYS 1 and SYS 2) were prepared. Both isolated organisms were found inhibitory against all pathogenic test organisms. The crude extract SYS 1 inhibited the growth of *E. coli* and *Salmonella paratyphi B* moderately. During the preliminary testing, it was also attempted to determine the inhibitory potential of cell-free crude extracts of broth from both isolates by double dilution (1:1) with sterile distilled water; however, zones of inhibition were unmeasurable against some test organisms.

Table 3. Antimicrobial activity exhibited by AMC of the isolates SYS 1 and SYS 2.

Sr. No.	Test Organism	Diameter of Zone of Inhibition (in mm)	
		SYS 1	SYS 2
1	*E. coli*	11	10
2	*Salmonella paratyphi B*	13	11
3	*Staphylococcus aureus*	6	8
4	*Pseudomonas aeruginosa*	8	9

3.4. Identification of Isolates

Amplified PCR product was subjected to electrophoresis using Agarose gel 1% along with a 1 kb marker in TAE buffer and visualized by staining with ethidium bromide (Figure 1). The assembled DNA sequence was used to carry out BLAST with the NCBI nucleotide database, and a phylogenetic tree was constructed. From the blast analysis and phylogenetic studies, isolated organisms SYS 1 and SYS 2 were identified as members of the genus *Bacillus* (Figure 2a and Figure 2b). The sequence obtained for SYS 1 was 99% identical to the partial gene sequence of 16S rRNA of *Bacillus amyloliquefaciens* strain C14, and the sequence obtained for SYS 2 was 99% identical to the partial gene sequence of 16S rRNA of *Bacillus siamensis* strain *KCTC13613*. The organisms SYS 1 and SYS 2 were identified as *Bacillus amyloliquefaciens* and SYS 2 as *Bacillus siamensis* SYS 1, respectively, with 99% similarities.

Figure 1. Gel picture of the amplicons.

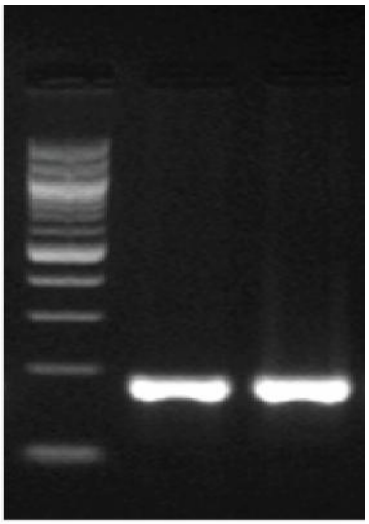


Figure 2a. Phylogenetic tree of SYS 1.

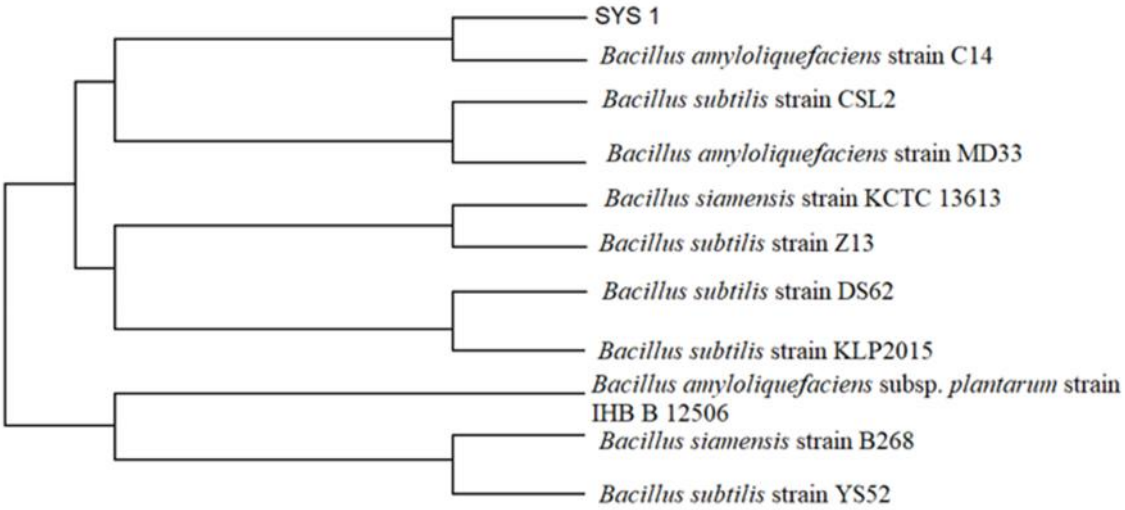
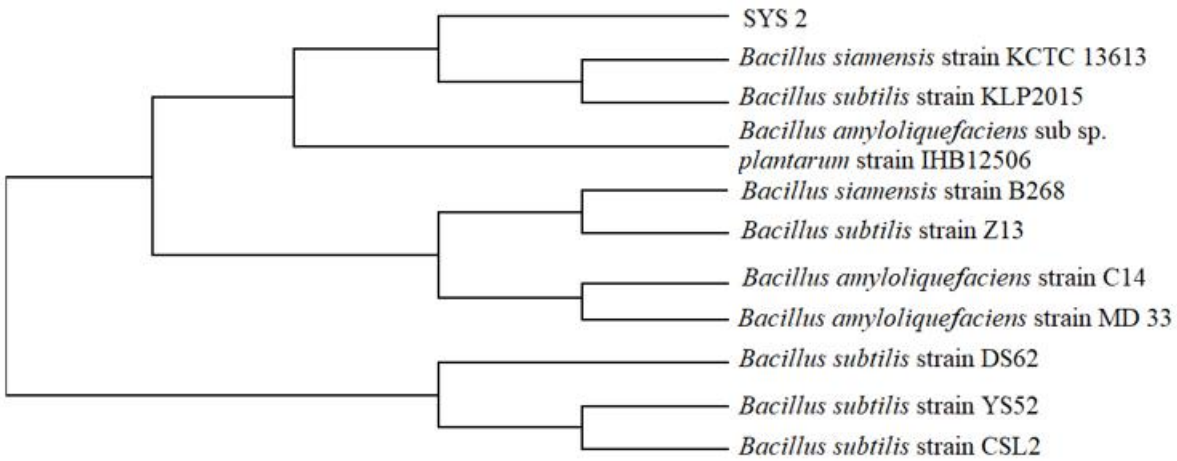


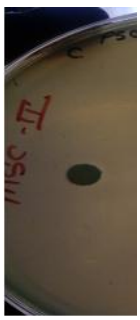
Figure 2b. Phylogenetic tree of SYS 2.

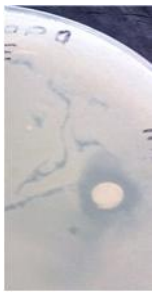


3.5. Evaluation of Antimicrobial Activity of Purified Antimicrobial Substances by *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2

The antimicrobial activity of the purified substance obtained after salt and solvent extraction was evaluated using an agar diffusion assay. Both isolates exhibited higher diameters of zones of inhibition against test organisms as compared to the crude antimicrobial compound used in the earlier assay. The diameters of the zones of inhibition obtained against test organisms are summarised in Table 4. Test organisms, *Salmonella paratyphi B* and *Staphylococcus aureus* were found highly sensitive to the antimicrobial substance produced by both isolates. The isolate, namely *Bacillus amyloliquefaciens* SYS 1 had the highest antimicrobial activity of AMC extracted by solvent ethyl acetate against *Salmonella paratyphi B*, followed by *Staphylococcus aureus*. The isolate, namely *Bacillus siamensis* SYS 2 had the highest antimicrobial activity of AMC extracted by solvent ethyl acetate against *Staphylococcus aureus*, followed by *Salmonella paratyphi B*. The butanol extract of *Bacillus amyloliquefaciens* SYS 1 exhibited maximum antibacterial activity against *Salmonella paratyphi B*, followed by *Pseudomonas aeruginosa*. The butanol extract of *Bacillus siamensis* SYS 2 species exhibited maximum antibacterial activity against *Salmonella paratyphi B*, followed by *E. coli*. The isolate, namely *Bacillus siamensis* SYS 2 (salt purified sample), had maximum antimicrobial activity against *Salmonella paratyphi B*, followed by *Staphylococcus aureus*. AMC produced by the isolate *Bacillus amyloliquefaciens* SYS 1 was detected with high antibacterial potential against *Salmonella paratyphi B* and *Staphylococcus aureus* as compared to the isolate *Bacillus siamensis* SYS 2. AMC produced by isolate *Bacillus siamensis* SYS 2 was found to produce potential antimicrobial activity against *Salmonella paratyphi B* and *Staphylococcus aureus.*

Table 4. Antimicrobial activity exhibited by AMC of the isolates SYS 1 and SYS 2.

Name of Isolate	Purified Sample of AMC	Test organisms / Diameter of Zone of Inhibition (in mm)							
		E. coli		*Staphylococcus aureus*		*Salmonella paratyphi B*		*Pseudomonas aeruginosa*	
Bacillus amyloliquefaciens	Salt Precipitation	8		8		10		6	
	Solvent Precipitation (Ethyl Acetate)	6		26		28		6	

	Solvent Precipitation (Butanol)	7		9		22		12	
Bacillus siamensis	Salt Precipitation	10		16		17		5	
	Solvent Precipitation (Ethyl Acetate)	8		16		18		6	
	Solvent Precipitation (Butanol)	12		8		14		10	

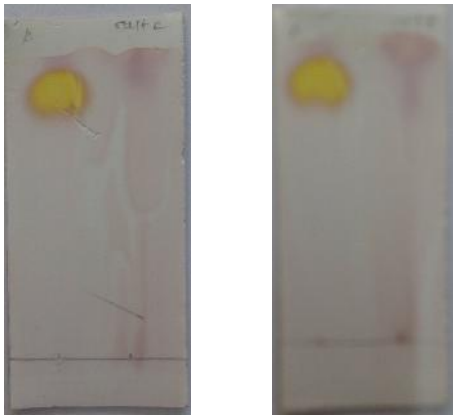
3.6. Preliminary Characterization of AMC Produced by *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2

Bacteriocins are antimicrobial peptides that inhibit or kill sensitive microorganisms (Vijayalakshmi *et al.*, 2011). *Bacillus* species are a rich source of antimicrobial peptides (Von, 1995). Hence, for the preliminary characterization of antimicrobial compounds produced by isolated organisms *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, qualitative tests for the detection of amino acids and proteins were conducted.

3.6.1. *Thin layer chromatography* (TLC)

The characterization of antimicrobial compounds produced by the isolated organisms *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2 was conducted for the detection of amino acids. The partially purified antimicrobial compound obtained after salt and solvent extraction was analysed using pre-coated silica gel plates. The extracts were spotted on the chromatograms and evaluated as violet-coloured spots with similal Rf values for known amino acids (Figure 3). This indicated that the AMC may contain amino acids.

Figure 3. Chromatograms showing presence of amino acids.



SYS 1 SYS 2

3.6.2. *Qualitative analysis of proteins*

Folin-Lowry's and ninhydrin tests were performed for qualitative analysis of the presence of amino acids and proteins. In Folin-Lowry's and ninhydrin tests, the presence of proteins is indicated by the formation of blue and deep purple to brown colours, respectively. The results obtained are depicted in Table 5 and Figure 4a and Figure 4b.

Table 5. Tests results for detection of proteins.

Test	*Bacillus amyloliquefaciens* SYS 1 (Salt)	*Bacillus siamensis* SYS 2 (Salt)	*Bacillus amyloliquefaciens* SYS 1 (Ethyl acetate)	*Bacillus siamensis* SYS 2 (Ethyl acetate)	*Bacillus amyloliquefaciens* SYS 1 (Butanol)	*Bacillus siamensis* SYS 2 (Butanol)
Lowry Test	+	+	+	+	+	+
Ninhydrin Test	+	+	+	+	+	+

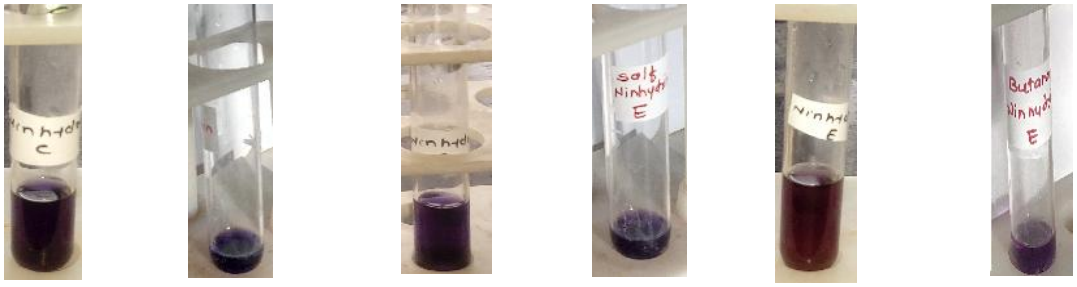
(+ Positive test; - Negative test)

Figure 4a. Detection of proteins in AMC of isolates detected by Folin-Lowry's Test.

Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol) | Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol)

Bacillus amyloliquefaciens SYS 1 *Bacillus siamensis* SYS 2

Figure 4b. Detection of proteins in AMC of isolates detected by Ninhydrin Test.



Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol) | Salt Extract | Solvent Extract (Ethyl acetate) | Solvent Extract (Butanol)

Bacillus amyloliquefaciens SYS 1 — *Bacillus siamensis* SYS 2

3.6.3. Estimation of Total Proteins

Using the Folin Lowrey's method the concentration of total proteins in AMC was determined. The standard curve was constructed by plotting concentration of bovine serum albumin v/s absorbance (Figure 5). The amount of proteins in samples was determined using standard curve. The concentration of total proteins determined in AMC produced by isolates is depicted in Table 6.

Figure 5. Standard curve for estimation of total proteins

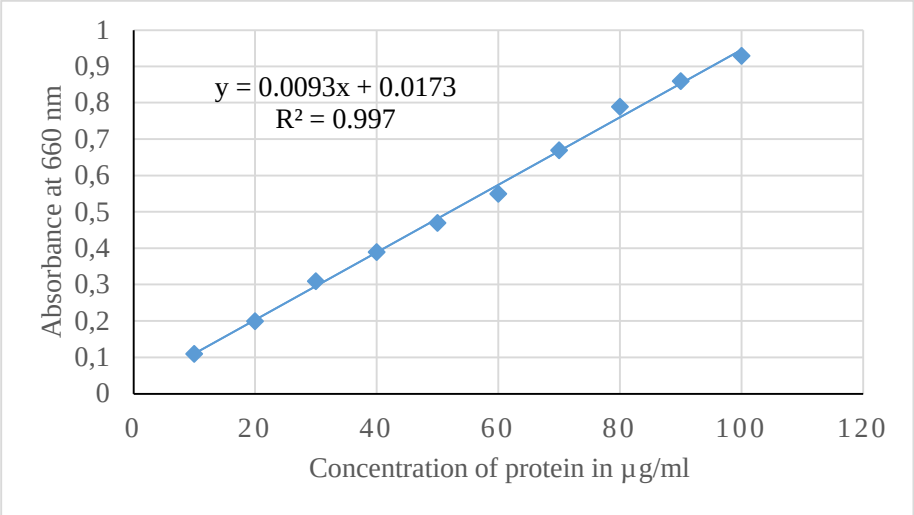


Table 6. Concentration of proteins determined in AMC produced by isolated organisms.

Sr. No.	Name of Isolate/ Sample	Concentration of Proteins (µg/ml)
1	*Bacillus amyloliquefaciens* SYS 1 Salt Extract (Ammonium sulphate)	7.7
2	*Bacillus siamensis* SYS 2 Salt Extract (Ammonium sulphate)	13.7
3	*Bacillus amyloliquefaciens* SYS 1 Solvent extraction (Ethyl acetate)	8.4
4	*Bacillus siamensis* SYS 2 Solvent extraction (Ethyl acetate)	17.9
5	*Bacillus amyloliquefaciens* SYS 1 Solvent extraction (Butanol)	14.7
6	*Bacillus siamensis* SYS 2 Solvent extraction (Butanol)	17.9

4. DISCUSSION

This study was attempted for the isolation and characterization of antimicrobial compound (AMC) producing bacteria from rhizosphere soil samples of different crops cultivated in the local region. Many members of *Bacillus* species have been reported to be capable of producing potential antimicrobial compounds (Caulier *et al.*, 2019). *Bacillus amyloliquefaciens* RO strains that inhibit different microhabitats to generate a range of antibiotics indicate the prospect of using certain strains as prospective biocontrol agents (Jeyakumar & Zhang, 2022*). Bacillus subtilis* produces subtilin and subtilosin (Stein, 2005; Pattnaik *et al.*, 2005), and *Bacillus cereus* produces cerein (Oscariz *et al.*, 1999), all of which have been shown to be effective against Gram-positive bacteria (Stoica *et al.*, 2019). Antimicrobial compound producing *Bacillus* strains was reported to exhibit inhibitory activity against *E. coli*, *P. aeruginosa*, *B. cereus*, and *S. aureus* (Geraldi *et al.*, 2022). *Bacillus megaterium L2* was reported to produce an antimicrobial compound, and an aqueous extract exerted potential antimicrobial activity against three plant pathogens (Xie *et al.*, 2021). *Bacillus* species-derived antimicrobial compounds have perspectives in sectors such as pharma, food, and agriculture (Wang *et al.*, 2014; Lin *et al.*, 2018). In the present investigation, two potential bacteria, *Bacillus amyloliquefaciens* SYS 1 and *Bacillus siamensis* SYS 2, were isolated and exhibited excellent antibacterial activity against clinical isolates, *Salmonella paratyphi B* and *Staphylococcus aureus. E. coli* and *Pseudomonas aeruginosa*, both clinical pathogens, were found to be sensitive to the AMC produced by both isolates. Initially, crude preparations of AMC from both isolated organisms showed moderate antibacterial activity. After partial purification using salt and organic solvent extraction of the AMC produced by both isolates, it exhibited admirable antibacterial activity. Purified bacteriocin from *B. subtilis GAS101* was reported to have strong antibacterial activity against *E. coli* and *S. epidermidis* (Sharma *et al.*, 2018). Preliminary characterization of AMC was conducted for the detection of amino acids and proteins, and partially purified AMC of both isolates contained amino acids and proteins. A higher 17.9 µg/mL of total protein content was estimated in the AMC of *Bacillus siamensis* SYS 2, which was extracted with the solvents-ethyl acetate and butanol.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Rajendrabhai Vasait: designed the study and all authors carried out the sample collection and experiments, wrote the final version of the manuscript. **All authors:** Conducted the data analysis, read and approved the final version of the manuscript.

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