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SCREENING OF SECONDARY METABOLITES FOR ANTIMICROBIAL APPLICATIONS FROM *Bacillus tequilensis* RG2 (SOIL ISOLATE)

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ABSTRACT

Recent decades have witnessed an up-rise in the field of exploiting properties of natural products for drug discovery. The natural soil hosts a diversity of micro-organisms. A number of these soil isolates have been identified for producing various antibiotics but only a few of them have been commercially exploited to treat various diseases. The microorganisms tend to develop multidrug resistance or resistance to an antibiotic(s) after considerable exposure. Thus, there is an increasing demand to find novel antibiotics to which the microorganism(s) is/are sensitive. In the present study, a soil isolate Bacillus tequilensis RG2 was used for production of secondary metabolites. The different media source was used for secondary metabolites production. These metabolites were extracted at regular time interval of 12h, 18h, 24h and 48h. The extraction of compounds was carried out using ethyl acetate and chloroform. The extracted compounds were tested for antimicrobial activity against 5 different test microorganisms (Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Vibrio cholerae, Enterococcus faecalis). These compounds were found to be active against Listeria monocytogenes and Staphylococcus aureus. Hence, extracts can be further purified, characterized and can be exploited at the larger scale for higher production.

KEY WORDS

Antibiotics, Antimicrobial activity, Bacillus tequilensis RG2, Extraction, Secondary metabolites, Resistance

INTRODUCTION

Natural soil habitat is a source of variety of microorganisms [1-3] with the potential to secrete metabolites that could be used as potential drugs. Secondary metabolites are produced by microorganisms in order to tolerate adverse conditions. The metabolites production is regulated by the availability of nutrients and the growth rate of the micro-organisms [4]. These metabolites are also helpful for the microorganism in competing successfully in a natural habitat with other organisms [5]. The production of these metabolites with respect to change in the environment plays a major role in the development of various antibiotics [6]. The ability of the micro-organisms to become multidrug resistant to the available drugs has diverted the attention of the scientists to find new therapeutic agents from nature [7]. Numerous reports suggested the role of genus *Bacillus* in production of various antimicrobial compounds [8,9].

In developing countries, the infectious diseases and multidrug resistance of their causative agents are further aggravating the health problems leading to high mortality. This situation shed light on the urgency to

search for newer antimicrobial sources. Few such diseases and the associated micro-organisms are:

Listeria monocytogenes, a foodborne pathogen causes listeriosis a serious illness in susceptible human hosts like meningitis and abortion [10]. Last few years have witnessed a gradual increase in the patients suffering from this disease primarily due to change in the food habits of consumers. *Listeria monocytogenes* can grow at low temperatures [11]. Raw vegetables, raw meat and ready-to-eat food items also habitat serve a rich and favourable environment for its growth at low temperatures [12]. Therefore, this highlights the importance to find alternatives for the control of *Listeria monocytogenes* in the food industry.

Infections due to *Staphylococcus aureus* have also become endemic within the past two decades [13]. There is increasing mortality [14, 15] associated with prolonged hospital stay and higher cost of treatment. The effective treatment is unreliable due to resistant to multiple antibiotics [16].

Various reports suggest that species from *Bacillus* genus show antimicrobial activity against several clinical bacteria including *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi* and *Listeria monocytogenes* [17]. *Bacillus* sp., a Gram-positive bacterium, is found especially in soil. To combat harsh or unsuitable conditions (radiation, chemical reagents and temperature variations) these can produce secondary metabolite products [18] and can form endospores for their survival [19]. It has been observed that *Bacillus* spp. in different environments produces relatively abundant antimicrobial molecules which could be further purified and tested for their activity [20, 21].

Antibiotics are low molecular-weight secondary metabolites. Soil microbes produce antibiotics in their natural habitat, providing them with a great advantage for competition in their natural habitat. Although various antibiotics produced by bacteria from *Bacillus* genus are also toxic to mammalian cells still these are in the focus of attention of scientists [22] due to the high demand of antibiotics in the pharmaceutical market. The amount of antibiotics produced by these *Bacilli* is approximately 167. From these more, than 66 have been derived from *Bacillus subtilis* and about 23 have originated from *Bacillus brevis* [23].

Generally, different culture media are used to produce different bioactive molecules by *Bacillus* strains. The most commonly used ones are; brain heart infusion (BHI), nutrient broth (NB), trypticase soya broth and Luria bertani [24]. Various solvents in different combinations have been reported to be useful for the extraction, purification and isolation of the antimicrobial metabolities [25].

Here we report the extraction of secondary metabolites using chloroform and ethyl acetate as solvents from *Bacillus tequilensis* RG2, a soil isolate that can be commercially exploited for the production of antibiotics. Secondary metabolites available from this microorganism can be further purified and used for production at industrial scale.

MATERIALS AND METHODS

Preliminary test

Overnight grown culture of *Bacillus tequilensis* RG2 (Accession number KX014909) was used for the antimicrobial activity assay against test pathogens.

Test microorganisms

Escherichia coli (MTCC 2961), *Vibrio cholera* (MTCC 3906), *Staphylococcus aureus* (MTCC 3160), *Listeria monocytogenes* (MTCC 839), *Enterococcus faecalis* (MTCC 439) were obtained from the MTCC, IMTECH, Chandigarh was used for this study.

Antimicrobial activity of *Bacillus tequilensis* RG2 against test pathogens

The antimicrobial activity of *Bacillus tequilensis* RG2 has been tested by agar well diffusion method [26] on Muller Hinton Agar (MHA) plates. MHA medium (25ml) was poured into pre-sterilized petri dishes. The test micro-organisms was suspended in normal saline. The confluent lawn of approximately 10^5 CFU (Colony Forming Unit) was obtained on MHA plate by spreading the test pathogens. The wells of 6 mm diameter were punched on MHA plates and 100µl of RG2 (A_{600} 0.8) were added in well. The plates were incubated at 37°C and observed for zone of clearance after 24h. The clear zone indicates the zone of inhibition (mm).

Extraction of crude metabolites from *Bacillus tequilensis* RG2 using solvents

For the extraction of metabolites 1% inoculum of RG2 with A_{600} 0.8 was used NB and MHB (Muller Hilton Broth). For the extraction of secondary metabolites culture was harvested at 12h, 18h, 24h, 48h.The grown culture was then centrifuged for 20min at 10,000rpm. The supernatant was further used for extraction process using chloroform and ethyl acetate as solvents (Figure

1) where equal volumes of the solvent and supernatant were taken in a separating funnel and shaked, then the two separated layers were collected in different beakers. The solvents were then evaporated. The resulting mixture was then used for testing the antimicrobial activity [27].

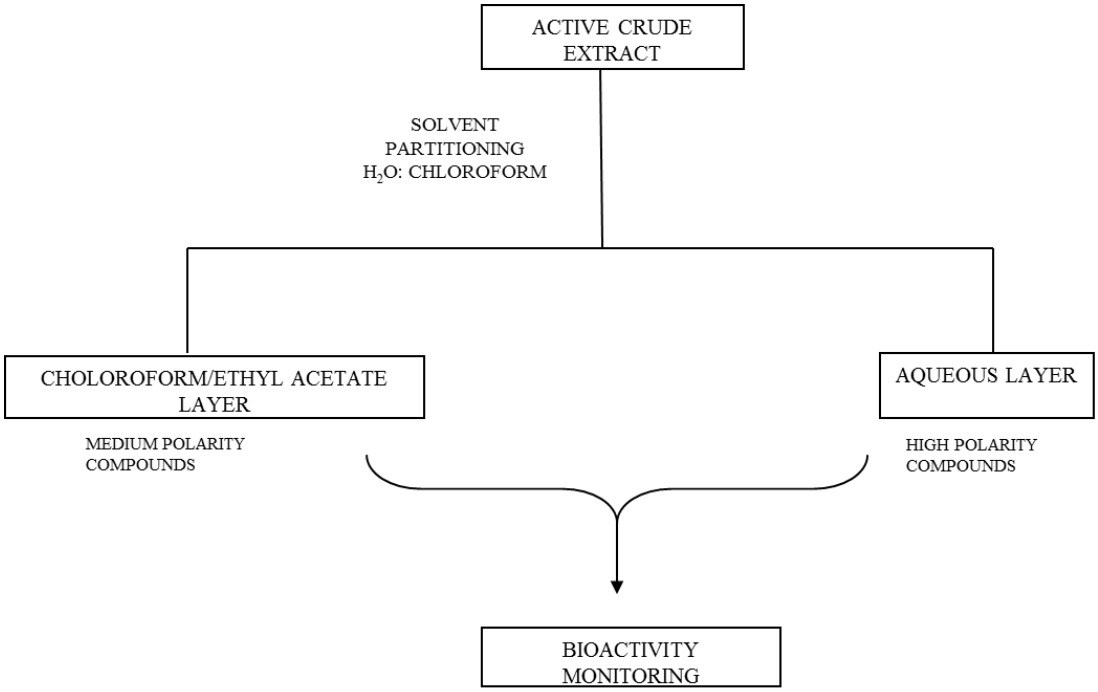

Figure 1: Flow chart for extraction of crude metabolites from *Bacillus tequilensis* RG2

Antimicrobial activity of extracts from *Bacillus tequilensis* RG2

The antimicrobial activity of extracts from RG2 grown in NB and MHB was checked against test pathogens.

RESULTS AND DISCUSSION

It was observed that RG2 is a Gram-positive bacterium when viewed under the microscope after Gram's staining.

Biochemical characteristics

Various biochemical tests were carried out to know the biochemical characteristics of *Bacillus tequilensis*, RG2 (Table 1).

Table 1: Biochemical characteristic of bacterial isolate *Bacillus tequilensis* RG2

S.No	Biochemical test	Positive (+)/Negative (-)
1	Esculetin	(+)
2	Starch	(+)
3	Casein	(+)
4	Catalase	(+)
5	Gelatin	(+)
6	Indole	(+)
7	MR	(-)
8	VP (Voges-Proskauer)	(+)
9	Nitrate	(+)
10	Urease	(-)
11	Oxidase	(+)
12	Citrate	(+)
13	DNase	(-)

Table 2: Zone of inhibition by *Bacillus tequilensis* RG2 against test pathogens

S.No	MTCC Strain	Zone of inhibition
1	EC (*Escherichia coli*) (MTCC-2961)	-
2	SA (*Staphylococcus aureus*) (MTCC-3160)	+ (13mm)
3	VC (*Vibrio cholerae*) (MTCC-3906)	-
4	EF (*Enterococcus faecalis*) (MTCC 439)	-
5	LM (*Listeria monocytogenes*) (MTCC-839)	+ (21mm)

(+) indicates presence of zone of inhibition
(-) indicates absence of zone of inhibition

Table 3: Zone of inhibition of extracts from *Bacillus tequilensis* RG2 grown in NB

	S	C	CA	E	EA
SA	14 mm(12h)	-	15 mm (12h)	-	13 mm (12h)
	15 mm(18h)		15 mm (18h)		14 mm (18h)
	12 mm(24h)		11 mm (24h)		12 mm (24h)
	10 mm(48h)		10 mm (48h)		10 mm (48h)
LM	18 mm(12h)	14 mm (12h)	20 mm (12h)	16 mm (12h)	20 mm (12h)
	21 mm(18h)	15 mm (18h)	20 mm (18h)	14 mm (18h)	19 mm (18h)
	18 mm(24h)	13 mm (24h)	14 mm (24h)	13 mm (24h)	17 mm (24h)
	14 mm(48h)	11 mm (48h)	13 mm (48h)	10 mm (48h)	14 mm (48h)

SA: *Staphylococcus aurues*; LM: *Listeria monocytogenes*; S: Supernatant; C: Chloroform extract; CA: Cholorform aqueous extract; E: Ethyl-acetate extract; EA; Ethyl-acetate aqueous extract

Table 4: Zone of inhibition of extracts *Bacillus tequilensis* RG2 grown in MHB

	S	C	CA	E	EA
SA	14 mm (12h)	12mm (12h)	13 mm (12h)	-	10 mm (12h)
	11 mm (18h)	-	14 mm (18h)		11 mm (18h)
	11 mm (24h)	-	11 mm (24h)		11 mm (24h)
	10 mm (48h)		10 mm (48h)		10 mm (48h)
LM	-	-	-	-	-
	12 mm (18h)	-	14 mm (18h)	-	11 mm (18h)
	18 mm (24h)	-	18 mm (24h)	-	18 mm (24h)
	12 mm (48h)		12 mm (48h)		10 mm (48h)

SA: *Staphylococcus aurues*; LM: *Listeria monocytogenes*; S: Supernatant; C: Chloroform extract; CA: Cholorform aqueous extract; E: Ethyl-acetate extract; EA; Ethyl-acetate aqueous extract

Antimicrobial activity of *Bacillus tequilensis* RG2

The antimicrobial activity of *Bacillus tequilensis* RG2 strain against test pathogens was observed in terms of clear zone (Figure 2) around the well. This indicates the zone of inhibition measured in mm (Table 2).

Antimicrobial assay of crude extracts from *Bacillus tequilensis* RG2 grown in NB and MHB

The crude extracts from RG2 grown NB and MHB was prepared at a different time interval (12h, 18h, 24h and 48h) using two different solvents chloroform and ethyl acetate and was directly used for the antimicrobial assay.

Table 3 And Table 4 summarize the zone of inhibition of compounds extracted from *Bacillus tequilensis* RG2 grown in NB and MHB respectively. Figure 3 and Figure 4 shows the antimicrobial activity of extracts from *Bacillus tequilensis* RG2 grown in NB and MHB respectively. The extracts were active against *Listeria monocytogenes* and *Staphylococcus aureus.*

Upon comparing the results of antimicrobial activity of the crude supernatant only it is observed that molecules secreted by the organism when grown in NB broth have a higher chance of being exploited as a drug for *Listeria monocytogenes* and *Staphylococcus aureus* bacteria than when grown in MHB. Also, between the mid-polar

compounds isolated in the chloroform/ethyl acetate layer and the high polarity compounds in the corresponding aqueous layer, high polarity compounds showing a bigger zone of inhibition (in mm) have a better potential for being used as an antibiotic for treating the diseases caused by *Listeria monocytogenes* and *Staphylococcus aureus*. Further work on purification and isolation followed by the bioactivity monitoring and structural elucidation of the molecules will shed light on the types of molecules which could be used to cure diseases. Various reports suggest for the exploiting soil isolate for enzyme and antibiotic production. But provide a single source for the industrial application in pharmaceutical and enzyme sector.

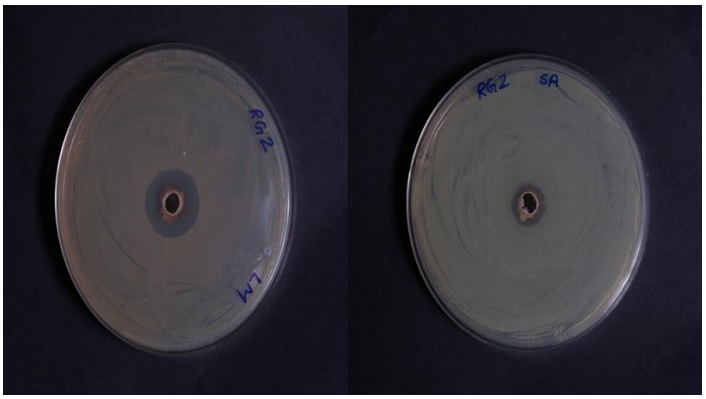

Figure 2: Zone of inhibition by *Bacillus tequilensis* RG2 against *Listeria monocytogenes* and *Staphylococcus aureus*

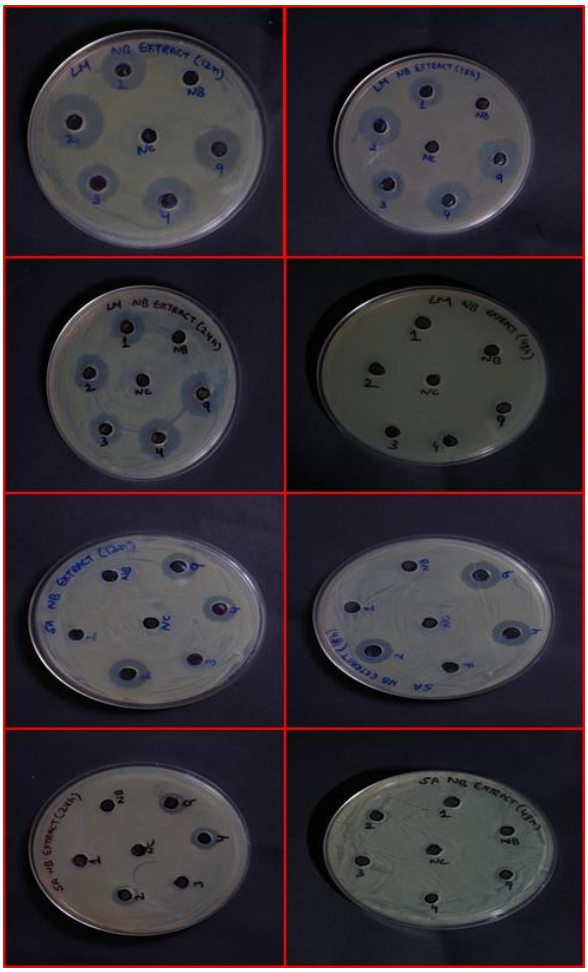

Figure 3: Antimicrobial assay of extracts from *Bacillus tequilensis* grown in NB against *Listeria monocytogenes* and *Staphylococcus aureus*: - 1: C(Chloroform extract); 2 : CA(Chloroform aqueous extract); 3:E(Ethyl acetate extract); 4:EA(Ethyl acetate aqueous extract); 9:S Supernatant; NC (negative control: water); NB: Nutrient Broth also as control

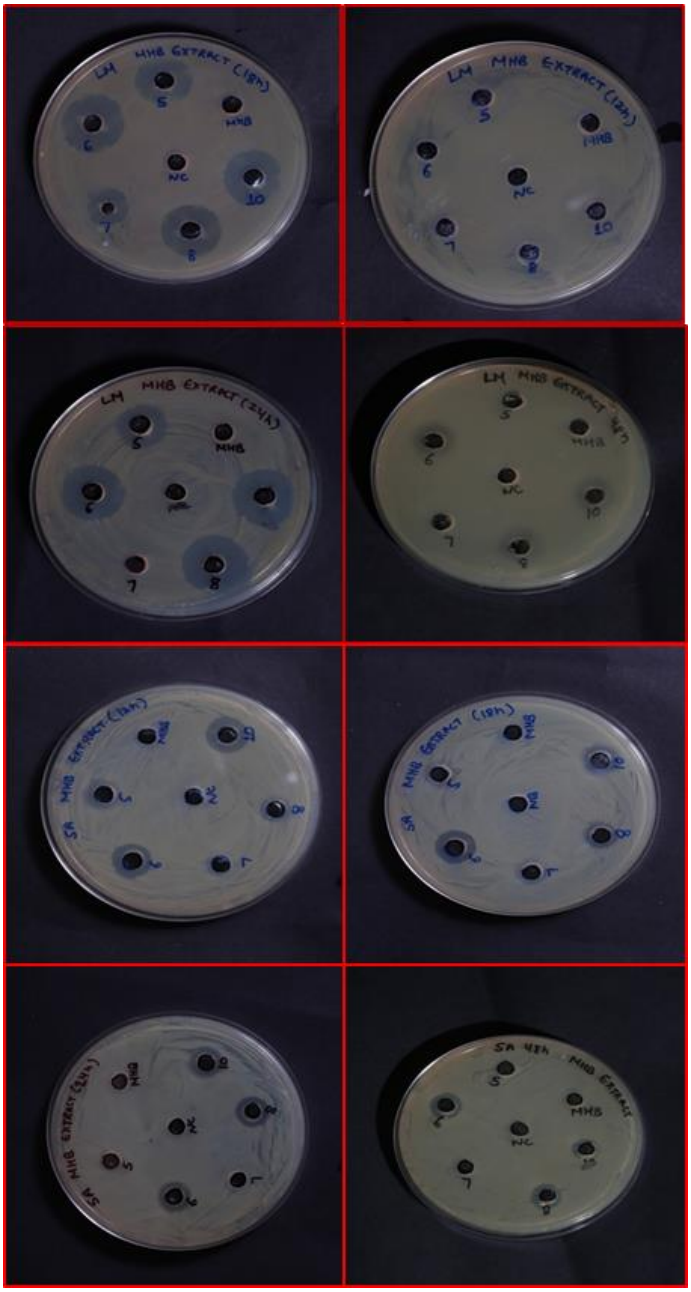

Figure 4: Antimicrobial assay of extracts from *Bacillus tequilensis* RG2 grown in MHB against *Listeria monocytogenes* and *Staphylococcus aureus*: - 5:C(Chloroform extract); 6:CA(Chloroform aqueous extract); 7:E(Ethyl acetate extract); 8:EA(Ethyl acetate aqueous extract); 10:Supernatant; NC (negative control: water); MHB also as negative control.

This study reveals that the isolated *Bacillus tequilensis* RG2 has the capability for antimicrobial activity against the pathogenic organisms.

CONCLUSION

This study was successful in preliminary screening for antibacterial activity by *Bacillus tequilensis* RG2. Further studies for the purification of compounds and its characterization are in progress. The present study reveals the antimicrobial activities of metabolites extracted from *Bacillus tequilensis* RG2. These compounds were found to be active against *Listeria monocytogenes* and *Staphylococcus aureus*. So, antimicrobial compounds from *Bacillus tequilensis* RG2 can be exploited for higher production at industrial scale

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