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Research Article

Enzyme Kinetic of Caseinase Produced by Thermophilic *Bacillus licheniformis*

S. M. More

Department of Microbiology, Yeshwant Mahavidyalaya,
Nanded- 431602. (Maharashtra), India
E-mail: drsanmore@gmail.com

ABSTRACT :

Bacillus licheniformis and products produced by the microorganism are inhibitory to the growth of numerous other microorganisms in the environment. The low- molecular- mass microbial secondary metabolites produced by *Bacillus licheniformis* inhibit the growth of other microorganisms at low concentration. *B. licheniformis* has been shown to be inhibitory to the growth of various fungi and has recently been investigated for its use as a biocontrol agent of several fungal pathogens. Therefore, in the present study *Bacillus licheniformis* was further studied in detail for its ability to produce antifungal compound. Mutant and wild strain of *B. licheniformis* were used for production of antifungal compound, the antifungal compound produced by 5-Bromo uracil and Ethidium Bromide mutant isolate separately showed broad spectrum activity against the test organisms *Alternaria alternata*, *Helminthosporium sp.*, *Fusarium moniliforme*, *Aspergillus niger*, *Rhizoctonia solani.* Out of these five fungal plant pathogen fusarium species was more sensitive to antifungal compound produced by *B. licheniformis*.

Key Words: *Bacillus licheniformis* Antifungal compounds, Biocontrol agent.

INTRODUCTION:

In natural habitat microbe interacts with numerous other microorganism. During their interaction microorganism produce a variety of secondary metabolite, many of which posses therapeutic application. These low- molecular- mass microbial secondary metabolites at low concentration inhibit the growth of other microorganisms.

The hot springs, with boiling mudpots and sulfurous are some of the most extreme life supporting environments on earth with temperature from 50^0C-115^0C and pH upto 9. However, little microbiological work has been done to elucidate the microbial life in these hot water spring, one of such spot is at unkeshwar, Nanded. These hot water springs offers organisms for industrial purpose.

B. licheniformis has been shown to be inhibitory to the growth of various fungi and has recently been investigated for its use as a biocontrol agent of several fungal pathogens. Metabolites of *Bacillus licheniformis* produced in culture were antagonistic to *Pyrenophora teres*, the cause of net blotch of barley. Sadfi et al (2001)[7] a total of 83 spore- forming bacteria, belonging to the genus *Bacillus*, was isolated from Tunisian salty soils were tested in vitro and in vivo against *F. roseum var. sambucinum*, the causal agent of dry rot of potato tubers. Results of the in vitro dual culture screening revealed that more than 50% of *Bacillus spp*. isolated from salty soils inhibited the growth of the pathogen in vitro. These effective *Bacillus* isolated were identified as belonging to one of the

species *B. cereus, B. lentimorbus* or *B. licheniformis*. The cell- filtrates of *Bacillus sp*. were unable to inhibit the growth of *Fusarium* sp.

The potential of *Bacillus subtilis, Bacillus* cereus, *Bacillus licheniformis* , *Bacillus pumilus, Brevibacillus laterosporus* and *Paenibacillus polymyxa* as biocontrol agents of four foliar necrotrophic pathogens of wheat had been evaluated (Alippi et al., 2000)[1]. The assays included the study of effect of the bacterial antagonists on fungal growth in the central disc test with paired cultures, effect of the antagonists on the germination of fungal spores in the paired suspension assay, and reduction of disease severity on wheat cultivar in greenhouse experiments.

Huang-ShaoNing et al. (1999)[4] studied biocontrol of cucumber seedling damping off (*Pythium aphanidermatum*) in soilless culture with *Bacillus licheniformis* and *Trichoderma spp*.

Trojanowska et al. (1998)[8] identified bacteria from lupin compost and their antagonistic activity against plant pathogenic fungi in vitro was investigated. A total of 31 strains from 9 *Bacillus* sp. were isolated. The most common species observed were *B. pumilus, B. licheniformis, B. subtilis* and *B. polymyxa*.

Hessenmuller and Zeller (1996)[3] studied fifteen antagonistic bacterial isolates of *Agrobacterium, Bacillus, Enterobacter* and *Pseudomonas* as biocontrol agents *Phytophthora coctorum* and *P. fragariae var. fragariae* (causing crown rot and red core disease) in strawberries (*cv. Elsanta*). All isolates inhibited mycelia growth, and 4 isolates reduced root diseases.

Wijesundera and Herath (1994)[9] isolated a deep purple pigmented of *B. subtilis* obtained from paddy soil inhibited the growth of *R. solani*, the causal agent of sheath blight of rice.

Phae et al. (1990)[6] investigate the suppressive effect of 10 compost on 4 phytopathogenic fungi in vitro, of the composts, 4 inhibited fungal growth. Bacteria associated with the effect were isolated and identified as *B. subtilis*.

Kelemu and Badel (1994)[5] showed that an isolate of *B. subtilis* from the phylloplane of the forage legume *Stylosanthes guianensis* in the Amazon of Peru, exhibited an antifungal activity a wide range of plant pathogenic fungi from various hosts.

Fassouane et al. (1995)[2] studied the antifungal activity of *B. licheniformis* str. FSJ-2, isolated in southern Morocco, against pathogenic fungi and the production of volatile and non- volatile antifungal substances by this bacterium.

Therefore work as whole was undertaken to study efficiency of growth and production of exocellular antifungal compound in *Bacillus licheniformis* , considering different aspects in the chemostate at a variety of specific growth rates, and to correlate the rates of production antifungal compound with parameters temperature, pH and salts etc.

METHODOLOGY :

Collection of water sample :

Water sample was collected from hot water spring which is located at Unkeshwar, Dist. Nanded, Maharashtra in sterile conical flask. Temperature of hot water spring is in the range of 48 to 55^0C throughout the year. Same sample was used for further study.

Isolation & Identification of *Bacillus licheniformis* :

Nutrient broth was used for the cultivation of bacteria from water sample. Which was incubated at 48^0C for 48 hrs then same cultivation broth culture was used for isolation of *Bacillus* sp. On nutrient agar medium specific colonies were selected for confirmation of species by colony character and biochemical test as per the test given in bergey's manuals of systematic bacteriology 9th edition.

Inoculum:

Bacterial suspension was prepared by adding 10 ml sterile water to a 8- day- old slant culture and 5 ml of this was used as inoculum in all experiments unless and otherwise stated. In every case the bacterial suspension was standardized to have 0.8 O. D. (optical density).

Induction of mutation:
Mutation was induced following three methods given below.

Ultra violet radiation:
Suspension of wild isolates was used for mutation. Suspension was spread on nutrient agar medium by spread plate technique and petriplates were exposed to ultra violet radiation by 5 second interval as 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 second. 50 second exposed petriplate was showing measurable number of colony on petriplate. Maximum number of colony showing petriplate was selected for isolation of mutant strain. Same mutant strain was used for the production of antifungal compund.

By 5-Bromo-Uracil:
Wild isolate of *B. lichenifromis* was spreaded over nutrient agar medium containing 0.3 µg/ml 5-Bromo- Uracil. It was incubated at 48^0C for 48 hours. Petriplate showing measurable number of colonies were selected for isolation of mutant strain of *B. licheniformis*. Same mutant strain was used for production of antifungal compund.

By ethidium bromide:
Wild isolate of *Bacillus licheniformis* was spreaded over nutrient agar medium containing (0.3 mg/ml std. stock) Ethidium bromide and it was incubated at 48^0C for 48 hrs for isolation of mutant strain. Petriplate showing maximum number of colonies were selected for isolation of mutant strain. Some mutant strain was inoculated in cultivation media for the production of antifungal compund.

Production of antifungal compound:
Wild & mutant strain of *B. licheniformis* was used for the production of antifungal compound in nutrient medium. For production of antifungal compound 75 ml medium was poured to 250 ml flask. The flask was sterilized 15 lbs for 20 min. the flasks were inoculated with bacterial suspension, the flask were incubated at a specific temperature as stated and (the flask were shaken intermittently) after regular time interval samples were drawn and assessed for antifungal compound activity.

Purification of antifungal compound:
Saturated solution ammonium sulphate of concentration of 30%, 50% and 75% was prepared. These solution were mixed with equally amount of culture filtrate at the protein present in the culture filtrate were precipitated. The content was centrifuged at 3000 rpm for 20 min, the pellets dissolved in phosphate buffer and was taken in dialysis tube/ bag. The ends of bag were sealed and bag was kept in phosphate buffer pH 7 then was transferred to saturated sucrose solution. The resultant content were used for the assay. 10 gm of DEAE cellulose was mixed inn 20 ml 1M NaCl solution. It was mixed thoroughly and filled in vertical glass column (2.5X25 cm). the solution for purification was passed through column, later it was eluted with 500 ml glycine-HCl buffer at pH 3.5.

Effect of pH and temperature on antifungal compound:
The pH of the reaction mixture was adjusted at various pH as stated using 0.1 N HCl or NaOH and the activity was assessed at the adjusted temperature. Similarly the temperature of the reaction mixture was made to desire value by placing the content in hot water bath at various temperatures as stated.

Assay of antifungal compound:
Partially purified anti-fungal compound produced by mutant *Bacillus licheniformis* was tested against some phytopathogenic fungi. Rose Bengal medium was prepared and poured in petriplate after solidification different fungal suspension was spread by spread plate technique after spreading sterile paper disc were soaked in anti- fungal compound and placed over the Rose Bengal medium at middle position in aseptic condition. Then plates were incubated at 27 ± 2^0C for 8 days after incubation growth was observed for zone of inhibition. Zone of inhibition was measured for

each fungal pathogen and it was recorded as for *Alternaria alternata, Fusarium moniliforme, Helminthosporium spp., Aspergillus niger* and *Rhizoctonia solani.*

RESULT AND DISCUSSION :

In the present studies effort were made to isolate *Bacillus* species from hot water spring located at Unkeshwar. The ambient temperature of hot spring water is 48 to 55^0C throughout the year. Isolation of moderate thermophilic organisms which can be used at commercial level for production of improved desired product was accomplished. Therefore, water sample collected from the site were analyzed microbiologically for different types of microbial communities. Organisms were isolated by using standard enrichment and isolation techniques proposed in bergey's manuals of systematic bacteriology 9th edition (Table 1).

Studies on antifungal compound production by *Bacillus licheniformis*

Wild isolate of *B. licheniformis* initiates synthesis of antifungal compound in 24 hrs with 0.0004 U/ml and continues to synthesize till 39 hrs synthesizing 0.0014 U/ml and there after the amount decreases (table 2). So time for optimum production of antifungal compound is 39 hrs. The effect of temperature and pH on synthesis of antifungal compound by wild strain of *B. licheniformis* was studied. It was found that maximum antifungal compound was synthesis at 50^0C (Table 4) whereas the optimum pH was 8.5 (Table 3). Partial purification of the crude preparation was carried out. The precipitation by 75% saturated NH_4SO_4 solution gave relatively more purification (Table 5). Similarly the dialysis of antifungal compound preparation for 48 hrs gave maximum activity.

Studies on antifungal compound production by UV mutant *Bacillus licheniformis*

The mutant strain of *B. licheniformis* (exposed to UV radiation) initiates synthesis of antifungal compound in 24 hrs with 0.0006 U/ml and continues to synthesize till 36 hrs synthesizing 0.0016 U/ml and there after the amount decreases (table 2). So time for optimum production of antifungal compound is 36 hrs. The effect of temperature and pH on synthesis of antifungal compound by mutant strain of *B. licheniformis* was studied. It was found that maximum antifungal compound was synthesis at 50^0C (Table 4) whereas the optimum pH was 8.5 (Table 3). Partial purification of the crude preparation was carried out. The precipitation by 75% saturated NH_4SO_4 solution gave relatively more purification (Table 5). Similarly the dialysis of antifungal compound preparation for 48 hrs gave maximum activity.

Studies on antifungal compound production by 5-Bromo-uracil mutant *Bacillus licheniformis*

The mutant strain of *B. licheniformis* (exposed to 5-Bromo-uracil) initiates synthesis of antifungal compound in 21 hrs with 0.0007 U/ml and continues to synthesize till 36 hrs synthesizing 0.0017 U/ml and there after the amount decreases (table 2). So time for optimum production of antifungal compound is 36 hrs. The effect of temperature and pH on synthesis of bacitracin by mutant strain of *B. licheniformis* was studied. It was found that maximum antifungal compound was synthesis at 50^0C (Table 4) whereas the optimum pH was 8.5 (Table 3). Partial purification of the crude preparation was carried out. The precipitation by 75% saturated NH_4SO_4 solution gave relatively more purification (Table 5). Similarly the dialysis of antifungal compound preparation for 48 hrs gave maximum activity.

Studies on antifungal compound production by Ethidium bromide mutant *Bacillus licheniformis*

Mutant strain of *B. licheniformis* (exposed to Ethidium bromide) initiates synthesis of antifungal compound in 21 hrs with 0.0004 U/ml and continues to synthesize till 36 hrs synthesizing 0.0017 U/ml and there after the amount decreases

(table 2); so time for optimum production of antifungal compound in 36 hrs. The effect of temperature and pH on synthesis of antifungal compound by mutant strain of *B. licheniformis* was studied. It was found that maximum antifungal compound was synthesis at 50^0C (Table 4) whereas the optimum pH was 8.5 (Table 3). Partial purification of the crude preparation was carried out. The precipitation by 75% saturated NH_4SO_4 solution gave relatively more purification (Table 5). Similarly the dialysis of antifungal compound preparation for 48 hrs gave maximum activity.

Studies on antifungal compound production by *Bacillus licheniformis* against some plant pathogenic fungi:

The wild & mutant strains of *B. licheniformis* secrets antifungal compound in the culture medium during growth. The activity of the antifungal compound was assessed against five fungi pathogenic to plants. The activity was assayed by disc method. It was found that all the strains of *B. licheniformis* secretes antifungal compound in the medium of growth. The amount of compound varied within the strains. Wild isolate (Table 6) secreted relatively less antifungal compound as expressed by zone of inhibition of five fungi. The maximum activity was expressed by mutant of 5- Bromo-Uracil (Table 6) followed by Ethidium bromide (Table 6) and UV mutants (Table 6). The antifungal compound secreted by wild and mutant strains were tested against five fungal plant pathogen i.e. *Alternaria sp.*, *Helminthosporium sp.*, *Fusarium sp*., *Aspergillus niger*, *Rhizoctonia sp.* Out of these five fungal plant pathogen *Fusarium sp*. was more sensitive to antifungal compound produced by *B. licheniformis*.

CONCLUSION :

The result of this study indicate that the antifungal compound produced by 5-Bromo uracil mutant *Bacillus licheniformis* was found to be strongly effective against all tested fungal species; and *Fusarium* sp.; causative agent of dry rot in potato is the most sensitive species towards the antifungal compound produced by wild & mutant isolates of *Bacillus licheniformis* .

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Table No. 1: Differential Characteristics of the species of genus *Bacillus*

Different Characters of *Bacillus licheniformis*	
Catalase	+ve
Anaerobic growth	+ve
Vogus- proskauer	+ve
Acid from :	
D- Glucose	+ve
L-Arabinose	+ve
D- Xylose	+ve
D- Mannitol	+ve
	(Few gas bubbles may from)
Gas from Glucose	
Hydrolysis of :-	+ve
Casein	+ve
Gelatin	+ve
Starch	+ve
Utilization of Citrate	+ve
Propionate	- ve
Egg- Yolk Lecithinase	
Nitrate Reduced to Nitrate	+ve
Growth at pH 5.7	+ve
Growth at pH 6.8	+ve
Growth in NaCl 2%	+ve
Growth in NaCl 5%	+ve
Growth in NaCl 7%	ND
Growth in NaCl 10%	-ve
Growth at 5^0C	-ve
Growth at 10^0C	+ve
Growth at 30^0C	+ve
Growth at 40^0C	+ve
Growth at 50^0C	+ve
Growth at 55^0C	-ve
Growth at 60^0C	

Table No. 2 : Production of anti- fungal compound by wild & mutant *B. licheniformis* isolates

Sr. No.	Time in hrs.	Caseinase activity u/ml			
		Wild isolate	UV radiation	5- Bromo uracil	Ethidium bromide
1	21	0.0004	0.0006	0.0007	0.0004
2	24	0.0005	0.0008	0.0009	0.0006
3	27	0.0007	0.0011	0.0010	0.0008
4	30	0.0009	0.0014	0.0014	0.0011
5	33	0.0011	0.0016	0.0016	0.0014
6	36	0.0014	0.0016	0.0019	0.0017
7	39	0.0013	0.0015	0.0018	0.0016
8	42	0.0013	0.0014	0.0018	0.0014
9	45	0.0012	0.0013	0.0017	0.0013
10	48	0.0004	0.0006	0.0016	0.0012

Table No. 3 : Effect of pH on anti- fungal compound produced by wild & mutant *B. licheniformis* isolates

Sr. No.	pH	Caseinase activity u/ml			
		Wild Isolate	UV radiation	5-Bromo uracil	Ethidium bromide
1	3.5	0.0007	0.0009	0.0010	0.0009
2	4.0	0.0008	0.0009	0.0010	0.0010
3	4.5	0.0009	0.0010	0.0011	0.0010
4	5.0	0.0011	0.0012	0.0012	0.0011
5	5.5	0.0011	0.0013	0.0013	0.0012
6	6.0	0.0012	0.0014	0.0014	0.0013
7	6.5	0.0013	0.0015	0.0014	0.0014
8	7.0	0.0013	0.0016	0.0015	0.0015
9	7.5	0.0014	0.0017	0.0016	0.0016
10	8.0	0.0015	0.0017	0.0017	0.0017
11	8.5	0.0016	0.0018	0.0019	0.0018
12	9.0	0.0015	0.0017	0.0018	0.0017
13	9.5	0.0015	0.0016	0.0017	0.0015
14	10.0	0.0014	0.0014	0.0016	0.0014
15	10.5	0.0013	0.0013	0.0014	0.0013
16	11.0	0.0011	0.0011	0.0013	0.0012

Table No. 4 : Effect of temperature on anti- fungal compound produced by wild & mutant *B. licheniformis* isolates

Sr. No.	Temperature in ^{0}C	Caseinase activity u/ml			
		Wild Isolate	UV radiation	5- Bromo uracil	Ethidium bromide
1	25	0.0011	0.0011	0.0012	0.0011
2	30	0.0012	0.0013	0.0014	0.0012
3	35	0.0012	0.0014	0.0015	0.0013
4	40	0.0013	0.0015	0.0016	0.0015
5	45	0.0014	0.0017	0.0018	0.0016
6	50	0.0018	0.0018	0.0019	0.0018
7	55	0.0020	0.0017	0.0018	0.0017
8	60	0.0018	0.0016	0.0017	0.0015
9	65	0.0016	0.0015	0.0016	0.0014
10	70	0.0014	0.0013	0.0014	0.0012

Table No. 5 : Effect of partial purification on anti - fungal compound produced by wild & mutant *B. licheniformis* isolates

Sr. No.	Percentage of Ammonium Sulphate	Anti-fungal activity u/ml			
		Wild Isolate	UV radiation	5-Bromo uracil	Ethidium bromide
1	30 %	0.0012	0.0005	0.0006	0.0005
2	50 %	0.0014	0.0006	0.0007	0.0006
3	70 %	0.0015	0.0008	0.0009	0.0008
4	Dialysis after				
5	12 hrs	0.0016	0.0010	0.0011	0.0010
6	24 hrs	0.0017	0.0012	0.0013	0.0012
7	48 hrs	0.0018	0.0014	0.0015	0.0015
8	DEAE- Cellulose	0.0020	0.0017	0.0019	0.0018

Table No. 6 : Antifungal activity produced by wild & mutant *B. licheniformis* isolates tested against some fungal plant pathogen

Sr. No.	Name of Pathogen	Zone of Inhibition in mm			
		Wild Isolate	UV radiation	5-Bromo uracil	Ethidium bromide
1	*Alternaria alternata*	12	14	16	15
2	*Helminthosporium sp.*	10	11	13	12
3	*Fusarium moniliforme*	15	17	19	18
4	*Aspergillus niger*	13	15	18	16
5	*Rhizoctonia solani*	11	12	14	13