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Study of Antagonistic Action of Antifungal Compound Produced by *Bacillus licheniformis*

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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.

The numbers of hot springs occurrence are large but the knowledge on hot spring microflora is scanty[1]. Thermophiles were the first extremophile to be discovered. They are defined as groups of microorganisms which grow at a temperature above 50^0C, some of them actively grow at 80^0C[2]. 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. For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential in addition to scientific curiosity. It is reported that thermophilic strains produce interesting biological molecules including unusual enzymes, antibiotics, anti algal compounds, anti-cancer substances and secreted sugars[3]. Thermal hot springs in different parts of the world have been studied for their thermophilic microbial diversity and often serve as a source of novel microorganisms for various biotechnological applications[4, 5]. Traditional cheese locally called "*Wagashi*" is produced and preserved using rudimentary methods under unsanitary conditions which may lead to the contamination of the product by toxinogenic or pathogenic microorganisms especially fungi[6, 7]. Furthermore, fungi produce allergenic compounds and toxic metabolites which may penetrate the cheese and affect the consumer's health[8]. It was reported that occurrence of Aflatoxin M1 in cheese could probably increase the risk of developing cancer or toxic and carcinogenic effects[9]. *B. licheniformis* has been shown to be inhibitory to

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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. 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[10]. 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[11]. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance[12]. Drug resistance is more frequently encountered in hospital-acquired pathogens; however the incidence of antibiotic resistant pathogens in community-acquired infections has been also on the rise in recent years[13]. Antibiotic resistance is a global public health problem. Although all countries are affected, the extent of the problem in the developing nations is unknown. With increasing travel and patient movement throughout the world, transmission of drug resistant organisms from one country to another became a possibility[14]. 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. Some workers studied biocontrol of cucumber seedling damping off (*Pythium aphanidermatum*) in soilless culture with *Bacillus licheniformis* and *Trichoderma spp*[15]. Some scientists 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*[16]. Some workers 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[17]. Some workers 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[18]. Some scientists investigate the suppressive effect of 10 compost on 4 phytopathogenic fungi in vitro, of the composts, 4 inhibited fungal growth[19]. Bacteria associated with the effect were isolated and identified as *B. subtilis*. Some scientists 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[20]. Some workers 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[21].

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 of antifungal compound with parameters temperature, pH and salts etc.

Materials and Methodology

Collection of water sample

Water sample was collected from hot water spring[4, 5]at Unkeshwar. Temperature of hot water spring was recorded. Same sample was used for further study.

Media

Nutrient agar & broth was used for isolation & production of *B. licheniformis*. Same media was used for cultivation of bacteria from water sample and was incubated at 48^0C for 48 hrs.

Isolation & identification of bacteria

Nutrient agar was used for isolation of Bacillus species. Colonies showing characteristic feature were selected & confirmed by colony

character and biochemical test. These strains were selected for further study. Bergey's manual of systematic bacteriology 9th edition was followed for confirmation[22].

Inoculum

Bacterial suspension was prepared by adding 10 ml sterile water to a 4- day- old slant culture and 5 ml of this was used as inoculum in all experiments unless and otherwise stated. In each case the bacterial suspension was standardized to have 0.5 O. D. at A_{600} (McFarland Standards). All experiments were conducted in the triplicate and results are presented.

Induction of mutation

Mutation was induced by 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. Maximum number of colony showing petriplate was selected for isolation of mutant strain. Same mutant strain was used for the production of Antifungal compound.

By 5-Bromo-Uracil

Wild isolate of *B. licheniformis* was spread over nutrient agar medium containing 0.3 µg/ml 5- Bromo- Uracil. Same mutant strain was used for production of Antifungal compound along with different parameter which are affecting on activity of Antifungal compound.

By Ethidium bromide

Wild isolate of *Bacillus licheniformis* was spread over nutrient agar medium containing (0.3 mg/ml std. stock) Ethidium bromide and it was incubated at 48^{0}C for 48 hrs for isolation of mutant strain. Mutant strain and wild isolate were inoculated in cultivation media for the production of Antifungal compound to study different parameters affecting Antifungal compound synthesis[23].

Antifungal compound production by *B. licheniformis*

Strain of *B. licheniformis* was used for the production of Antifungal compound production 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 drown and assessed for Antifungal compound activity.

Purification

Saturated solution of ammonium sulphate of concentration of 30%, 50% and 75% strength were prepared. These solutions of different concentrations were mixed with equal amount of culture filtrate and the proteins present in the culture filtrate were precipitated. The content was centrifuged at 3000 rpm for 20 min; the pellets dissolved in phosphate buffer and were used for further studies. The culture filtrates (crude or partially purified) were taken in dialysis tube/ bag. The ends were sealed and kept in phosphate buffer pH 7 then transferred to saturated sucrose solution. The resultant content was used for the assay[24].

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^{0}C 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*.

RESULTS AND DISCUSSION

The hot water springs are located at Unkeshwar. The temperature of hot water spring was recorded. Six isolates of *Bacillus species* were isolated and identified on the basis of biochemical characteristics (i.e. Catalase, Anaerobic growth Voges-ProsKauer, Citrate utilization, growth at 55^{0}C are Positive & egg yolk Lecithinase utilization negative; Fermentation of Glucose, Xylose Arabinose, Mannitol). Four isolates were *Bacillus licheniformis* named as BL 1, 3, 4, 6 [25, 26].

These isolates were screened for

antifungal compound production and it was found that BL 4 produced highest quantity of antifungal compound, so this strain was subjected to mutagenesis. wild isolates showed moderate antifungal compound production after 24 to 39 hours, while in mutant strains isolated using different mutation sources 5-Bromo Uracil mutant showed moderate antifungal compound production after 24 hours which is faster than other isolates. These results show that mutation substantially reduced the time of incubation for production of caseinase. One of the important factors in determination of metabolite produced is time[27] (Table 1, Fig. 1).

Table 1. Production of anti- fungal compound by wild & mutant *B. licheniformis* isolates

S. No.	Time in hrs.	Caseinase activity IU / ml			
		Wild isolate	UV radiation	5- Bromo uracil	Ethidium bromide
1	21	4	6	7	4
2	24	5	8	9	6
3	27	7	11	10	8
4	30	9	14	14	11
5	33	11	16	16	14
6	36	14	16	19	17
7	39	13	15	18	16
8	42	13	14	18	14
9	45	12	13	17	13
10	48	4	6	16	12

Table 2. Effect of partial purification on anti - fungal compound produced by wild & mutant *B. licheniformis* isolates

S. No.	Percentage of Ammonium Sulphate	Anti-fungal activity IU / ml			
		Wild isolate	UV radiation	5- Bromo uracil	Ethidium bromide
1	30 %	12	5	6	5
2	50 %	14	6	7	6
3	70 %	15	8	9	8
4	Dialysis after				
5	12 hrs	16	10	11	10
6	24 hrs	17	12	13	12
7	48 hrs	18	14	15	15
8	DEAE- Cellulose	20	17	19	18

Table 3. Antifungal activity produced by wild & mutant *B. licheniformis* isolates tested against some fungal plant pathogen

S. No.	Percentage 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

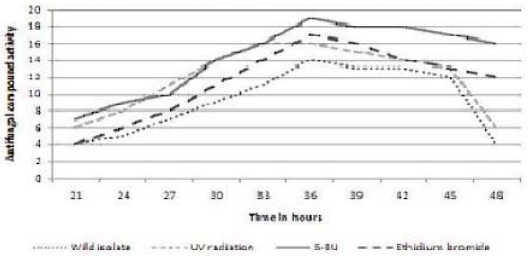

Fig. 1. Production of anti- fungal compound by wild & mutant *B. licheniformis* isolates

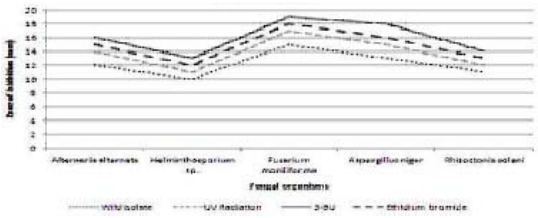

Fig. 2. Antifungal activity produced by wild & mutant *B. licheniformis* isolates tested against some fungal plant pathogen

Partial purification of crude antifungal compound was carried out. The precipitation by 75% saturated ammonium sulphate solution gave relatively more purification (table-2).

The oil from *C. odorata* also had been exploited as insecticide, ovicide and larvicide. The leaves extract of *C. odorata* got antifungal property[28, 29]. 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-3, figure-2) 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-3, figure-2) followed by Ethidium bromide (table-3, figure-2) and UV mutants (table-3, figure-2).

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* .

REFERENCES

1. Adhikary S.P. and J. Sahu. Limnology of thermal springs of Orissa. *J. Bom. Nat. His. Soc.*, 1987; **84**: 497- 503.
2. Madigan M.T. and B.L. Mars. Extremophiles. *J. Sci. Am*., 1997; **276**: 66-71.
3. Ladenstein R. and G. Antranikian. Proteins from hyperthermophiles: Stability and catalysis close to the boiling point of water. *Adv. Biochem. Eng. Biotechnol*., 1998; **61**: 37-85.
4. Narayan V.V., Hatha M.A., Morgan H.W. and Rao D. Isolation and characterization of aerobic thermophilic bacteria from the Savusavu Hot spring in Fiji. *Microbes Environ.,* 2008; **23**: 350 – 352.
5. Abou-Shanab R.A.I. Characterization and 16S rDNA identification of thermo-tolerant bacteria isolated from hot springs. *J. Appl. Sci. Res.,* 2007; **3**: 994-1000.
6. Sessou Philippe, Farougou Souaïbou, Alitonou Guy, Djenontin Tindo Sébastien, Yèhouénou Boniface, Azokpota Paulin, Youssao Issaka and Sohounhloué Dominique. Chemical Composition and Antifungal activity of Essential oil of Fresh leaves of *Ocimum gratissimum* from Benin against six Mycotoxigenic Fungi isolated from traditional cheese *Wagashi*. *I. Res. J. Biological Sci.* 2012; **1**(4): 22-27.
7. Filtenborg O., Frisvad J.C. and Thrane U. Moulds in food spoilage. *Int. J. Food Microbiol*., 1996; **33:** 85-102.
8. Nasser A.L. Fungal contamination of white cheese at the stage of consumption in Saudi Arabia, *Pak. J. Biol. Sci*., 2001; **4**(6): 733-735.
9. Elkak A., El-Atat O., Habib J. and Abbas M., Occurrence of aflatoxin M1 in cheese processed

and marketed in Lebanon. *Food Control*, 2012; **25**: 140-143.

10. Sadfi N., Cherif M., Fliss I., Boudabbous A. and Antoun H. Evaluation of bacterial isolates from salty soils and *Bacillus thuringiensis* strains for the biocontrol of fusarium dry rot of potato tubers. *Journals of plant pathology.,* 2001; **83**(2): 101-118.
11. Alippi A.M., Perello A.E., Sisterna M.N., Greco N.M. and Cordo C.A. Potential of spore-forming bacteria as biocontrol agents of wheat foliar diseases under laboratory and greenhouse conditions. *Zeitschrift-fur-Pflanzenkrankheiten-und-Pflanzenschutz.,* 2000; **107**(2): 155-169.
12. Huang S.N., Shen H.S., Wu H.M. Biocontrol of cucumber seedling damping off (pythium aphanidermatum) in soilless culture with *Bacillus licheniformis* and trichoderma spp. *Chinese- Journal-of-Biological-Control.* 1999; **15**(1): 45.
13. Trojanowska K., Stachowiak B., Muzquiz M., Burbano C., Gulewicz K. fungistatic activity of bacteria isolated from composts of bitter lupin straw. *3rd European conference on grain legumes. Opportunities for high quality, healthy and added-value crops to meet European demands. Valladolid, Spain.,* 1998; 262.
14. Hessenmuller A. and Zeller W. biological control of soil- borne phytophthora species in strawberry with bacterial antagonists. I. antagonistic effect and colonization of rhizoplane. *Zeitschrift-fur-Pflanzenkrankheiten-und-Pflanzenschutz.,* 1996; **103**(6): 602-609.
15. Wijesundera L.C. and Herath W.H.M.W. An isolate of *Bacillus* subtilis from Sri Lanka inhibitory to *Rhizoctonia solani*. *Journal of the national science council of Sri-lanka.* 1994; **22**(1): 15-21.
16. Radha K., Mahima R., Ramanathan G. and Thangapandian V. Survey on Drug Resistant Pattern of Clinical Isolates and Effect of Plant Extract on the Drug Resistant Pattern. *I. Res. J. Biological Sci.,* 2012; **1**(3): 14-19.
17. Hooton T.M. and Levy S.B., Antimicrobial résistance – a plan of action for community practice, *Ame. Fam. Physician.,* 2001; **63**(6): 1087-1098.
18. Michaelrecht I., Stephen H. and Joseph P. Basis for prokaryotic specificity of action of aminoglycoside antibiotics, *J. Biol. chem.* 1999; **18**(11): 3133-3138.
19. Phae C.G., Sasaki M., Shoda M., and Kubota H. Characteristics of *Bacillus subtilis* isolated from composts suppressing phytopathogenic microorganisms. *Soil-Science-and-plant-nutrition.,* 1990; **36**(4): 575-586.
20. Kelemu S. and Badel J.L. In vitro inhibition of *Colletotrichum gloeosporioides* and other phytopathogenic fungi by an Amazonian isolate of *Bacillus* subtilis and its cell- free culture filtrate. *Australian-Plant-Pathology.,* 1994; **23**(2): 41-45.
21. Fassouane, A., Rachidi, M., Rouffaud M.A., El-Abbouyi A. and Nguyen V.H. In vitro antifungal activity of *Bacillus licheniformis* FSJ-2 products against dermatophytes. *Journal-de-mycologie-medicale.,* 1995; **5**(4): 244-248.
22. Bergey D.H., Holt J.G., Krieg N.R. and Sneath P.H.A. *(Breed RS, Murray EGD and Smith NR, eds.):* Bergey's Mannual of Determinative Bacteriology, 9th edn., *Baltimore: Williams and Wilkins,* 1994.
23. Alikhanian S. I. Induced mutagenesis in the selection of microorganisms. *Adv. Apl. Microbiol.,* 1962; **4**: 1-50.
24. El sawah M.M.A. and El Din M.Z. Production and properties of thermostable alkaline protease from *Bacillus licheniformis* and its importance in Domiati Cheese ripening. *Annals-of-agricultural-Science-Cairo.,* 2000; **45**(c): 113-127.
25. Janstova B. and Lukasova J. Heat resistance of Bacillus spp. spores isolated from cow's milk and farm environment. *Acta. Veterinaria. Brno.,* 2001; **70**(2): 179-184.
26. Zahoor S., Muhammad M.J., Muhammad N.A. and Ikram-ul-haq. Isolation and Molecular Identification of a Facultatively Anaerobic Bacterium From the Hot Spring of Azad Kashmir. *Pak. J. Bot*., 2012; **44:** 329-333.
27. Sztajar H. and Maliszewska I. The effect of culture conditions on lipolytic productivity of microorganism. *Biotech. Lett.,* 1988; **10**(3): 199-204.
28. Avlessi F., Alitonou G.A., Djenontin T. S., Tchobo F., Yèhouénou B., Menut C. and Sohounhloué D. Chemical composition and Biological activities of the Essential oil extracted from the Fresh leaves of *Chromolaena odorata* (L. Robinson) growing in Benin. *Res. J. Biological Sci.,* 2012; **1**(3): 7-13.
29. Kra K.D., Diallo H.A. and Et Kouadio Y.J., Activités antifongiques de l'extrait de *Chromolaena odorata* (L.) King and Robins sur deux isolats de *Fusarium oxysporum* (E.F. Sm.) responsables du jaunissement mortel des feuilles des bananiers, *J. Appl. Biosci.,* 2009; **24**: 1488 – 1496.