WFL Publisher
Science and Technology

Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.8 (2): 1166-1173. 2010 *www.world-food.net*

Screening for some *Bacillus* spp. inhabiting Egyptian soil for the biosynthesis of biologically active metabolites

N. M. Morsi [1], N. M. Atef [1*] and H. El-Hendawy [2]

*[1] Department of Botany, Faculty of Science, Cairo University, Giza 12613, Egypt. [2] Department of Botany, Faculty of Science, Helwan University, Helwan, Egypt. *e-mail: drnmatef@gmail.com*

Received 27 January 2010, accepted 11 April 2010.

Abstract

In this study, a number of *Bacillus* strains were isolated from soil collected from different areas of the experimental fields belonging to the faculty of Agriculture, Cairo University. The isolates which showed antimicrobial effects were identified as *Bacillus licheniformis, B subtilis, B. sphaericus, B. circulans* (3 isolates), *B. badius, B. azotoformans, Brevibacillus brevis* (2 isolates), and an unidentified isolate. The activity of crude filtrate, obtained from the aerobically grown isolates, using four different media, was evaluated *in vitro* against indicator microorganisms including phytopathogenic bacteria and fungi as well as food spoiling bacteria namely *Xanthomonas campestris, Pseudomonas marginalis, Erwinia carotovora, E. chrysanthemi, Fusarium moniliforme, F. equiseti, Staphylococcus aureus* and *Escherichia coli*. The antimicrobial activity of each of the *Bacillus* isolates differed greatly towards the indicator organisms regarding the medium used for growth. This activity was potent when the isolates were grown in nutrient broth, while nearly no activity was detected when molasses-yeast extract medium was used. Phytopathogenic bacteria were the most susceptible microorganisms compared to phytopathogenic fungi and food-spoiling bacteria. Some of the antimicrobial compounds produced by *Bacillus* spp. were identified by using GC-MS analysis. These compounds may be dibutyl phthalate, phenyl acetic acid, phthalic acid and kasugamycin, production of these compounds were by the bacterial isolates B_2, B_3, B_{16} and B_6, respectively.

Key words: *Bacillus* spp., phytopathogenic microorganisms, biologically active compounds.

Introduction

Soil bacteria are sources of high number of natural products with biological activities which are extensively used as pharmaceuticals and agrochemicals [1]. Many antibiotics and/or biological active agents are known to exist, but efforts to discover new ones still continue. Therefore, many species of *Streptomyces, Bacillus* and *Penicillium* have been studied continuously for their ability to produce antibiotics [2].

Bacillus is an interesting genus to be investigated; members of this genus are often considered microbial factories for the production of a vast array of biologically active secondary metabolites, potentially inhibitory for phytopathogenic and food-borne organisms [3-7].

The genus *Bacillus* includes a variety of important species having history of safe use in the fermentation industry, being non- pathogenic, good secretors of proteins and metabolites and easy to cultivate. Products currently available include enzymes, oligo- and lipo-peptides, antibiotics, food additives and flavour enhancers, surfactants and other products [8-10]. Their spore-forming ability also makes these bacteria some of the best candidates for developing efficient bioinsecticide products, from a technological point of view. *Bacillus* spores have a high level of resistance to dryness necessary for the formulation into stable products.

The objective of this study was to screen and evaluate, *in vitro*, the potential antimicrobial activity of some *Bacillus* spp. isolated from different locations in a field in Cairo, Egypt, as well as morphological and biochemical identification of these isolates.

Materials and Methods

Isolation of microorganisms: Soil samples were collected from fields in the Faculty of Agriculture, Cairo University. Samples were obtained by removing the leaf litter and collecting the top 10 cm; the weight of the individual soil sample was approximately 100 g. Isolation of the required *Bacillus* strains was achieved according to the method described by Claus and Berkeley [11] as follows:

Air-dried soil sample (4 g) placed in a beaker to which 20 ml sterile water is added. The beaker is heated in a water bath for 10 min at 80°C, while the content is carefully agitated. The soil sample (1ml) is then ready for inoculation on nutrient agar plate and incubated for 24 h. Purification using streak method was used to obtain pure cultures.

Indicator microorganisms: Different microorganisms were used for the characterization of antimicrobial activity of soil isolates. They include Gram-negative phytopathogenic bacteria namely *Pseudomonas marginalis* [12]; *Erwinia carotovora, E. chrysanthemi* [13] and *Xanthomonas campestris* [14].

The phytopathogenic fungi namely *Fusarium equiseti (corda) saccharoda* and *F. moniliforme* were isolated from infested soils and were identified in the Botany Department, Faculty of Science, Cairo University.

Food and water spoiling bacteria, i.e. *Staphylococcus aureus* and *Escherichia coli.* were provided by Dr. Saeed Dabour, Microbiology Lab., National Institute of Oceanography and Fishery, Cairo, Egypt.

Preparation of the inocula used for screening Bacillus isolates: Bacterial inocula were prepared by suspending freshly cultivated culture in 2 ml, 0.85% NaCl medium. The density was adjusted to 0.5 McFarland unit. This suspension was used for direct inoculation of nutrient agar [15].The inocula of fungi were made from freshly prepared cultures by harvesting conidia according to the method of Leifert *et al*. [16].

Maintenance of cultures: Soil isolates and bacterial indicator organisms were maintained as stock cultures frozen at -21°C in nutrient broth supplemented with 20% glycerol. The pathogenic fungi were cultured on PDA at 25°C for one week and stored at 4°C for long preservations. Strains were propagated twice before used in experiments.

In vitro screening of soil isolates for antimicrobial activity: *Preliminary screening:* Soil isolates were spotted on indicator organisms swabbed in nutrient agar plates and incubated for 24-48 h under optimum temperature. The inhibitory effect was determined by measuring the diameter of inhibition zones.

Secondary screening: The test was done by agar well diffusion assay using cell-free extract resulting from fermentation of the isolates. The wells were cut in seeded nutrient agar plates using sterilized cork borer of 8 mm diameter, each well received 0.1 ml of the extract. Plates were incubated for 24-48 h under optimum temperature. The efficiency of the extracts was determined by measuring the diameter of developed inhibition zones in mm.

Identification of soil isolates: Method of identification was based on that described by Reva *et al.* [18] and Claus and Berkeley [11]. Identification tests included Gram staining, cell, and spore morphology, growth in nutrient broth with different concentrations of sodium chloride, determination of the optimum temperature, growth under anaerobic condition and growth at pH 5.7. Biochemical tests such as VP, MR, gelatine liquefaction and starch hydrolysis, production of acids from carbohydrates, catalase and indole tests as well as nitrate reduction and utilization of citrate.

Fermentation media: Four different media were used for growing soil isolates namely nutrient broth (NB), trypticase soy broth (TSB), molasses-urea and molasses-yeast extract, K_2HPO_4 was added to the last two media.

Cultivation of isolates for secondary metabolites production: *Preparation of inocula for fermentation:* Different *Bacillus* isolates were transferred from culture stock into 250 ml Erlenmeyer flask containing 25 ml sterilized nutrient broth. Flasks were incubated on rotary shaker under 120 rpm at 30°C for 24 h.

Preparation of crude secondary metabolites: Production was assayed on liquid medium using the above mentioned media. To 250 ml Erlenmeyer flasks, 50 ml of each medium was added, autoclaved and inoculated with the freshly prepared inocula in the ratio of 10%. Flasks were placed on a rotary shaker at 120 rpm and left for 24h under optimum temperature.

The culture broth was harvested by centrifugation at 10000 xg for 15 min, and the culture supernatant was filtered through bacterial filter and stored in sterile flasks at 4°C until used for antimicrobial assay. Unless otherwise stated, pH was adjusted to 7; incubation temperature was 30°C. Tests were made in triplicate.

Biologically active metabolite assay: Cell-free extract resulting from fermentation of the most potent *Bacillus* spp. studied was brought to qualitative analysis of antimicrobial compounds, using the GC-MS (GC 5980): Column HP-50 (cross linked 65 cm/s, 8.5 psi; oven 45°C (1.5 min) to 300°C (6 min); injection splittless 1µl/ until 275°C; detector mass selective.

The crude active supernatants from the fermentation of B_2, B_3, B_{16} and B_6 grown on NB medium as it exhibited the highest inhibitory effects against the test organisms, were brought to GC-MS analysis. Sterilized NB medium was subjected to GC-MS analysis as control. All the detected compounds in the GC-MS analysis in control results were excluded from the crude filtrates of samples under analysis.

Results and Discussion

Isolation of soil bacteria: The method adopted for isolation was appropriate for finding almost exclusively Gram positive aerobic spore forming bacteria. Twenty pure isolates were collected namely B1 to B20.

Preliminary screening of antimicrobial activity of isolates: Purified *Bacillus* isolates were screened for the presence of antimicrobial activity. Table 1 shows that each of the Bacillus isolates affected at least one of the indicator organisms. Those bacteria producing inhibition zones higher than 10 mm against at least two microorganisms were selected for identification and further characterization of their antimicrobial activity. Based on this criteria 11 bacterial isolates were selected namely B2, B3, B6, B8, B10, B13, B14, B15, B16, B19 and B20.

Identification of Bacillus strains: Identification of *Bacillus* isolates was done using different standard schemes in an attempt to gather the different phenotype characteristics of all known species of *Bacillus*. This was due to the fact that identification of spore forming bacteria was always a difficult task, as many species and genus names used routinely in the current scientific literature are not present in Bergey's Manual [18]. The morphological description and physiological and biochemical characteristics are presented in Table 2a-b, following primary subdivision of isolates into strict aerobic and facultative anaerobes, respectively. From the results we deduced that isolates B2, B15 and B19 may be different strains of *Bacillus circulans;* B8 a strain of *B. subtilis*; B16 *B. sphaericus*; B3 *B. licheniformis*; B10 *B. badius*; B20 *B. azotoformans;* B13 and B14 two strains of *Brevibacillus brevis* and B6 a *Bacillus* isolate which we could not identify.

Cultivation of Bacillus spp. for production of active metabolites: Four different media were used for cultivation under conditions previously mentioned. The purpose of using different fermentation media in this study was to detect the most suitable one for bacterial growth and also the production of active secondary metabolites. The biosynthesis of active compounds by *Bacillus* is controlled by several external (cultivation conditions) and internal factors (medium composition) as mentioned by Akpa *et al.* [19], Duffy and Defago [20] and Slininger and Jackson [21].

The crude active supernatants for each *Bacillus* strain were

Table 1. Preliminary screening of inhibitory effect of bacillus isolates.

Bacillus isolates	Inhibition zones in mm							
	Xanthomonas campestris	*Pseudomonas smarginalis.*	*Erwinia carotovora*	*Erwinia chrysanthemi*	*Staphylococcus sp.*	*E. coli*	*Fusarium equiseti*	*Fusarium moniliforme*
B1	-	-	-	-	-	-	-	-
B2	-	-	11	-	10	-	11	5
B3	12	-	-	-	-	-	11	-
B4	-	-	-	-	-	-	-	-
B5	-	-	-	-	-	-	-	-
B6	-	13	-	-	-	18	-	-
B7	-	-	-	-	-	-	-	-
B8	-	-	11	-	-	-	-	12
B9	-	-	-	-	-	-	-	-
B10	12	-	13	-	-	-	-	-
B11	-	-	-	-	-	-	-	-
B12	-	-	-	-	-	-	-	-
B13	-	-	-	11	-	-	12	-
B14	-	-	13	14	-	-	-	-
B15	20	-	12	-	-	-	-	12
B16	-	-	-	-	-	-	-	12
B17	-	-	-	-	-	-	-	-
B18	-	-	-	-	-	-	-	-
B19	-	-	14	-	-	11	-	-
B20	13	10	20	15	-	-	-	-

Table 2a. Identification of strictly aerobic bacilli.

Performed tests		Bacillus isolates					
		B8	B10	B13	B14	B16	B20
Morphological characteristics							
Gram staining		-ve	-ve	+ve	+ve	-ve	+ve
Spores	spherical	-	-	-	-	+	-
	elliptical	+	+	-	-	-	-
	oval	-	-	+	+	-	+
Sporangia swelling		-	-	+	+	+	+
Physiological characteristics							
Growth in NaCl	2%	+	+	+	+	+	+
	5	+	-	+W	+W	+	+W
	7	+	-	+W	+W	-	-
	10	-	-	-	-	-	-
Growth in temperature	5°C	-	-	-	-	-	-
	30	+	+	+	+	+	+W
	55	-	-	-	+	-	-
	65	-	-	-	-	-	-
Growth in NB at pH 5.7		+	+W	+	+	+W	-
Biochemical tests							
Indole		-	-	-	-	-	-
VP		+	-	-	-	-	-
MR		+	+	-	-	+	+
Catalase		+S	+W	+S	+S	+	-
Hydrolysis of	starch	+	-	+	+	-	-
	gelatine	+	+	+	+	+	-
	casein	+	+	+	+	+	-
Acid from	glucose	+S	-	+W	+W	-	-
	mannitol	+S	-	-	-	-	-
	xylose	+	-	-	-	-	-
	arabinose	+	-	-	-	-	-
Nitrate reduced to nitrite		+	-	+	+	-	-
Utilization of citrate		+	-	+	+	+	+

W: weak reaction; S: strong reaction; NB: nutrient broth.

Table 2b. Identification of facultative anaerobes.

Performed tests		Bacillus isolates				
		B2	B3	B6	B15	B19
Morphological characteristics						
Gram staining		-ve	+ve	+ve	-ve	-ve
Spores	spherical	-	-	-	-	-
	elliptical	-	-	-	-	-
	oval	+	+	+	+	+
Sporangia swelling		+	-	-	+	+
Physiological characteristics						
Growth in NaCl	2 %	+	+	+	+	+
	5	+	+	+	+	+
	7	+	+	+	+	+
	10	-	+	-	-	-
Growth in temperature	5° C	-	-	-	-	-
	30	+	+	+	+	+
	55	+W	+	-	+W	-
	65	-	-	-	-	-
Growth in NB at pH 5.7		+	+	-	+W	+W
Biochemical characteristics						
Indole		-	-	-	-	-
VP		-	+	+	-	-
MR		+	+	+	+	-
Catalase		+S	+	+S	+S	+
Hydrolysis of	starch	+	+	+	+	+
	gelatine	+	+	+	+	+
	casein	+	+	+	+	+
Acid from	glucose	+	+W	-	+S	+
	mannitol	-	+W	-	-	-
	xylose	+	+	+	+	+
	arabinose	+	+	+	+	+
Nitrate reduced to nitrite		+	+	-	+	+
Utilization of citrate		+	+	+	+	+

W: weak reaction; S: strong reaction; NB: nutrient broth.

assayed against the indicator organisms, the results were presented in Table 3a-b. The data indicate that *Bacillus* strains showed variable ability of inhibition and inactivation according to the indicator organisms and the type of growth media. Generally speaking *Bacillus circulans* (B2), *B. licheniformis* (B3), *B. sphaericus* (B16) and *Bacillus* sp. (B6) exhibited the highest inhibitory effects against the test organisms, while the rest of *Bacillus* spp. showed a relatively lower one.

Takahashi *et al.* [22] and Howells *et al.* [23] proved that some strains of *B. circulans* are able to produce the broad spectrum nucleoside

Table 3a. Antibacterial activity of *Bacillus* spp. using different fermentation media.

Bacillus spp.	Inhibition zones in mm																							
	Xanthomonas sp.				*Pseudomonas sp.*				*Erwinia carotovora*				*Erwinia chrysanthemi*				*Staphylococcus sp.*				*E. coli*			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
B2	10	-	50	-	-	-	50	-	-	-	15	-	10	10	20	-	11	10	40	-	-	-	40	-
B15	12	12	-	-	-	10	-	-	12	11	-	-	-	10	-	-	-	-	-	-	-	-	-	-
B19	-	-	-	-	-	-	-	-	-	-	15	-	10	10	-	-	11	-	10	-	9	-	15	-
B13	-	-	-	-	-	11	-	-	-	-	-	-	11	10	-	-	-	-	-	-	-	-	--	-
B14	13	11	-	-	-	10	-	-	15	12	-	-	12	10	-	-	-	-	-	-	-	-	-	-
B10	10	-	-	-	12	11	-	-	14	-	-		13	10	-	12	-	-	-	-	-	-	-	
B8	-	-	-	-	14	11	-	-	10	-	-	-	10	11	-	-	-	-	-	10	-	-	-	-
B16	17	10	-	-	16	15	-	-	-	12	20	-	9	14	-	-	-	-	-	-	-	-	-	-
B20	19	35	-	-	10	15	-	-	-	14	10		9	15	13	-	-	-	-	-	-	-	-	-
B3	15	10	-	-	10	11	11	-	11	-	-	-	-	11	10	12	-	-	15	-	-	-	-	-
B6	-	10	-	-	19	11	25	-	10	10	-	-	-	12	-	12	-	-	-	-	42	30	10	-

B2-*Bacillus circulans* strain 1 B15 -*B .circulans* strain 2 B19-*B. circulans* strain 3 a- nutrient broth
B13-*Brevibacillus brevis* strain 1 B14-*B. brevis* strain 2 B10- *Bacillus badius* b- trypticase soy broth
B 8-*Bacillus subtilis* B 16-*Bacillus sphaericus* B 20-*Bacillus azotoformans* c- molasses urea medium
B3-*Bacillus licheniformis* B6-unidentified strain d- molasses - yeast extract medium

Table 3b. Antifungal activity of *Bacillus* spp. using different fermentation media.

Bacillus spp	Inhibition zones in mm							
	Fusarium equiseti				*Fusarium moniliforme*			
	a	b	c	d	a	b	c	d
B2	34	33	13 h	-	14	20	15	-
B15	-	-	10	-	11	-	-	-
B19	-	-	-	-	-	40	-	-
B13	22	15	13 h	25	-	10	10	11h
B14	-	-	-	-	-	15	-	14 h
B10	-	-	-	-	-	-	-	10 h
B8	-	18	-	-	-	-	11	-
B16	-	-	-	-	10	-	-	14
B20	-	15	-	-	-	-	-	14 h
B3	15	-	15 h	-	10	-	20	-
B6	-	-	12 h	-	-	33	12	10

B2-*Bacillus circulans* strain 1 B15 *B .circulans* strain 2
B13-*Brevibacillus brevis* strain 1 B14-*B. brevis* strain 2
B 8-*Bacillus subtilis* B16-*Bacillus sphaericus*
B3-*Bacillus licheniformis* B6-unidentified strain

B19-*B. circulans* strain 3 a- nutrient broth
B10- *Bacillus badius* b- trypticase soy broth
B 20-*Bacillus azotoformans* c- molasses + urea
d- molasses + yeast extract
h The inhibitory zone was hazy

antibiotics, bagougeramine A and B and the antibiotic butirosin, respectively. Concerning *B. licheniformis*, its different strains are known of producing surfactin [24], bacteriocin like substances [25] and bacitracin [26-30].The effectiveness of our *B. sphaericus* strain was less than expected since this genus is known of producing 167 biologically active compounds against bacteria, fungi, protozoa and virus [31].

Bacillus azotoformans (B20), *B. badius* (B10), *B. subtilis* (B8) and *Brevibacillus brevis* (B13) showed a lesser effect upon the tested indicator organisms than the previously discussed *Bacillus spp.*

B. azotoformans showed an effect against phytopathogenic tested organisms (Table 3a-b). Xie *et al.* [32] isolated from rice rhizosphere *B. azotoformans* together with other *Bacillus* spp., considered as rhizobacteria. Ongena *et al.* [33] indicated that these bacteria have numerous traits, which allow them to act as biocontrol agents, one of those traits, suppression of diseases caused by phytopathogens due to the production of a wide range of antimicrobial compounds.

Brevibacillus brevis was effective against the phytopathogenic microbes tested, though their efficiency was not as much as that described by Katz and Demain [3] who mentioned the production of 23 antibiotics by this genus.

Shoji *et al.* [34] reported that *B. badius* AR-91 produced antibiotics thiocillins II and III active against Gram-positive bacteria. The data given in the current study by our *B. badius* isolate affected the Gram-negative bacteria (Table 3a) which may suggest the production of different compound(s) by this isolate.

Finally come *B. subtilis* with an observed effect upon phytopathogenic bacteria compared to the pathogenic fungi tested (Table 3b). *Bacillus subtilis* is known to produce an array of antimicrobial compounds possessing broad suppressive properties for more than 33 types of plant pathogens [35].

Examination of the effect of each medium alone showed that cultivation of *Bacillus* strains in NB and TSB media favoured the growth and production of antibacterial active compounds as well, by all strains (Table 3a-b, Figs 1and 2). Antifungal active compounds were detected by few *Bacillus* strains when examining *Fusarium equiseti*, but the majority of *Bacillus* strains affected *Fusarium monoliforme*. The effect on food-spoiling organisms was achieved only by *Bacillus circulans* strain (B2) and *Bacillus* sp. (B6).

The antibacterial effect of the different strains decreased to about 50% when molasses-urea medium was used, although the effectiveness against food-spoiling bacteria increased (Table 3a-b, Figs 1 and 2). On the other hand, antifungal activity relatively changed. An increase in the resistance of *Fusarium* spp. has been observed, some of *Bacillus* spp. that earlier showed fungicidal activity (Table 3a-b, Figs 1 and 2), showed later a hazy or repellent action against *Fusarium equiseti*, these were namely *Bacillus circulans* (B2), *B. licheniformis* (B3), *B.* sp.(B6) and *Brevibacillus brevis* (B13).

Cultivation in molasses-yeast extract medium caused a drastic decrease in the antibacterial activity of the strains; only 25% of the strains showed activity, while the effect upon fungi was variable though this effect has not been considered fungicidal against *Fusarium moniliforme*, by the two strains of *Brevibacillus brevis*, *Bacillus badius* and *B. azotoformans* (Table 3a-b, Figs 1 and 2).Our results coincide with those of Bapat and Shah [36], who mentioned that *Brevibacillus brevis* produced extracellular

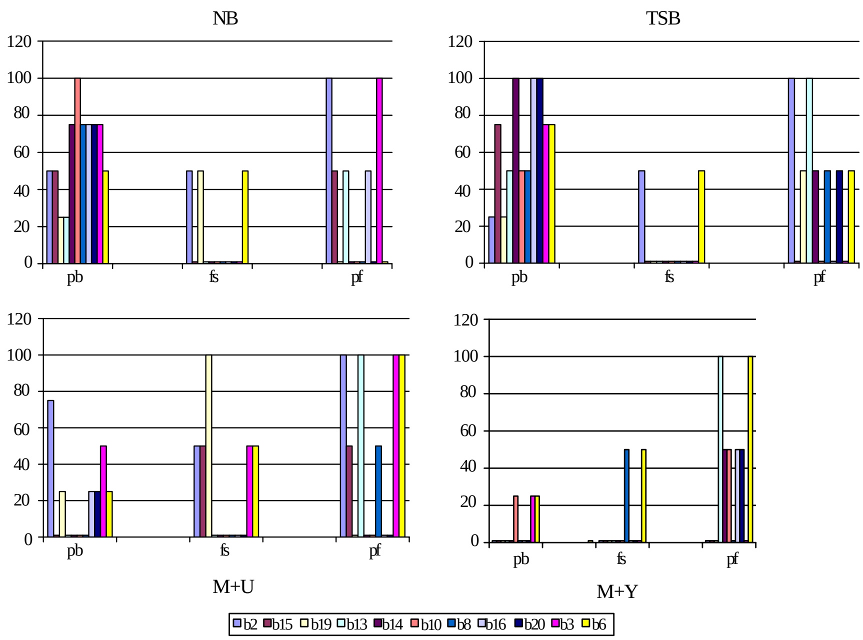

Figure 1. Percentage of affected indicator organisms by different *Bacillus* sp. growing in different media.

Pb: phytopathogenic bacteria; Fs: food and water spoiling bacteria; Pf: phytopathogenic fungi.

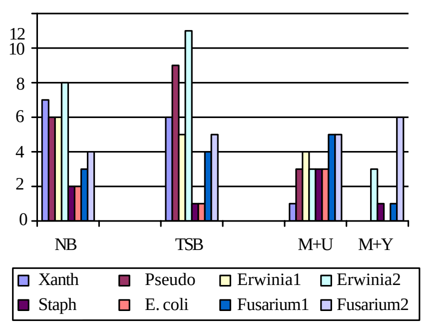

Figure 2. Susceptibility of indicator organisms towards the active compounds.

Xanth: *Xanthomonas campestris* Staph: *Staphylococcus aureus*
Pseudo: *Pseudomonas marginalis* Fusarium1: *Fusarium equiseti*
Erwinia1: *Erwinia carotovora* Fusarium2: *Fusarium moniliforme*
Erwinia2: *Erwinia chrysanthemi.*

antagonistic substances which inhibit germination of conidia and was fungicidal to the vegetative mycelium of the pathogen. Schmitt *et al.* [37] as well indicated that *B. brevis* inhibited the conidial germination of *Sphaerotheca fuliginea.* San-Lang *et al.* [38] deduced that extensive degradation of *F. oxysporum* hyphae or lysis of the hyphal tips was due to the presence of crude fungicide produced by *B. subtilis* W113.

Secondary metabolism is brought on by exhaustion of nutrient, biosynthesis or addition of inducer and/or by growth rate decrease [39]. Onbasali and Aslim [40] and Page [41] indicated that molasses can be used as substrate for the production of specific metabolites and as source of growth factors; it contains vitamins and other minor constituents. Yeast extract, being a source of organic nitrogen and B complex vitamins [42], increased bacterial growth in molasses media. The weak antimicrobial effect detected in this study by *Bacillus* sp. in molasses media, may be due to the fact that the rate of bacterial growth was still high, so that the production of active metabolites was too low or negligible. Woodruff [43] and Bodansky and Perlman [44] stated that antibiotic accumulation either intracellular or extracellular, normally occurred after the end of logarithmic growth phase and prior to endospore maturation.

The majority of *Bacillus* spp. under study exhibited a moderate antimicrobial effect upon the indicator organisms shown by the diameter of inhibition zones (10-20 mm), while few species like *B. circulans* (B2) upon *Xanthomonas* sp., *Pseudomonas* sp. and food-spoiling organisms; *B. azotoformans (B20)* upon *Xanthomonas* sp. and *Bacillus* sp. (B6) upon *E. coli,* exerted a potent effect as shown by a diameter of inhibition zones higher than 40 mm. The effect upon fungi was detected by *Bacillus circulans* (B2) on *Fusarium* equiseti, *B. circulans* (B19) and *B.* sp. (B6) on *Fusarium* moniliforme (Table 3a-b). This phenomenon has been only observed when cultivation was done in molasses-urea medium, suggesting further investigation.

In the current study, the different *Bacillus* spp. was active against Gram- negative bacteria compared with the Gram-positive ones. *Erwinia chrysanthemi* was the most susceptible organism (Fig. 2) followed by *Pseudomonas* sp. The food-spoiling organisms showed great resistance against most metabolites used. It has been cited by many authors that most of peptide antibiotics produced by *Bacillus* spp. are active mainly against Gram-positive organisms [29]. Katz and Demain [3] stated that polymyxin, circulin, and colistin activity was almost exclusively against Gram-negative bacteria, whereas bacillomycin, mycobacillin and fungistatin are effective agents against molds and yeasts.

GC-MS analysis: All the detected compounds in the GC-MS analysis in control were excluded from the crude filtrate of samples under analysis. When cell free culture supernatant of *Bacillus circulans* (B_2) was subjected to the GC-MS (GC 5890), several peaks were observed (Fig. 3). This suggested that more than one antimicrobial compound was produced by B_2.

The sample contained a peak that corresponded to a peak exhibited by dibutyl phthalate at m/z 528 (Fig. 3). Roy *et al.* [45] found that dibutyl phthalate was produced by a new soil isolate *Streptomyces albidoflavus* 321.2. They also stated that the active compound showed strong activity against Gram-positive and Gram-negative bacteria, as well as unicellular and filamentous fungi.

Fig. 4 shows the antimicrobial activity called bis(2-ethylhexyl) phthalate at m/z 405 detected in the culture supernatant of *Bacillus sphaerius* (B_{16}). It was first reported [46] that a phthalic acid derivative [bis-(2-ethylhexyl) phthalate] was produced biosynthetically by *Streptomyces bangladeshiensis* from the soil of Natore, Bangladesh. They also observed moderate antimicrobial activities against most tested Gram positive and negative bacteria and some pathogenic fungi.

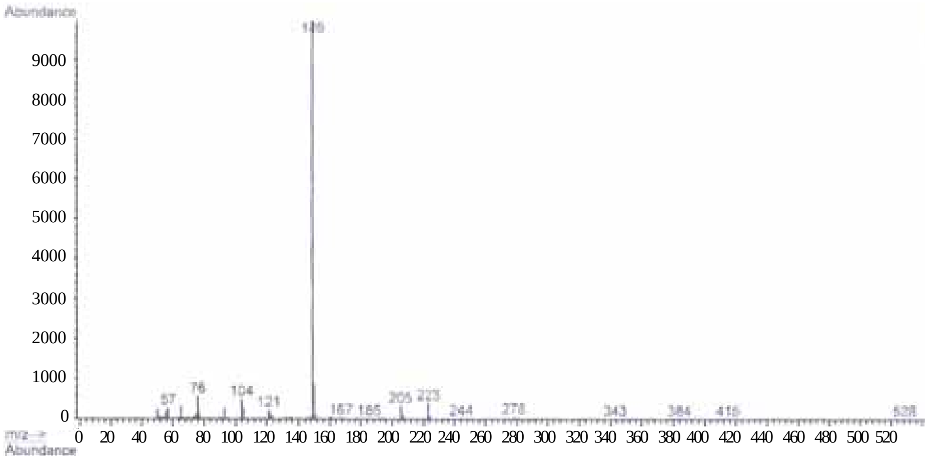

Figure 3. Ms of dibutyl phthalate synthesized by *B. circulans* (B2).

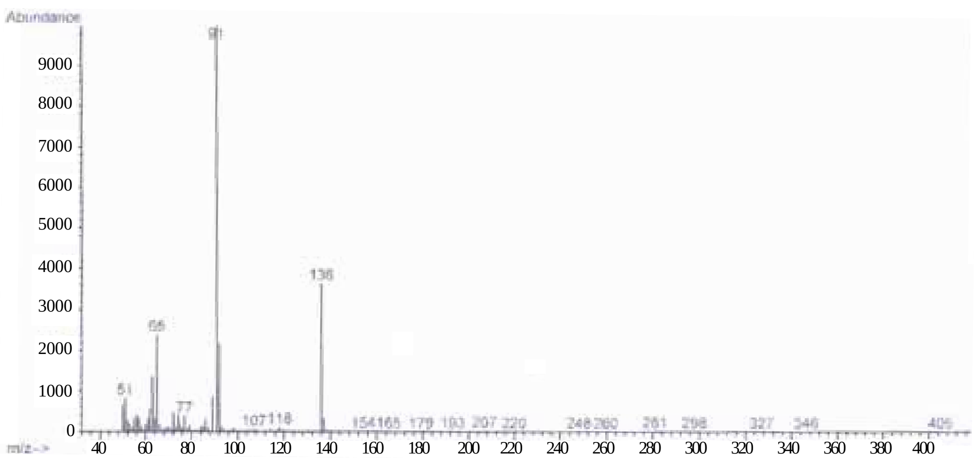

Figure 4. Ms of phenyl acetic acid synthesized by *B. licheniformis* (B3).

Several peaks were also detected by the GC-MS analysis of the culture filtrate of *Bacillus licheniformis* (B_3). One of the antimicrobial substances detected and met agreement with some investigations is phenyl acetic acid at m/z 405 (Fig. 5). Kim *et al.*[47] detected phenyl acetic acid in fermented soybean made with the strain *B. licheniformis* B65-1. They also added that the antibiotic compound was active against bacteria and yeasts such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Phenyl acetic acid and sodium phenyl acetate were isolated by Hwang *et al.*[48] from *Streptomyces humidus* strain S5-55. The authors also found that the compounds completely inhibited the growth of *Pythium ultimum*, *Phytophthora capsiei*, *Rhizoctonia solani*, *Saccharomyces cerevisiae* and *Pseudomonas syringae* pv. *syringae*. The two compounds were as effective as the commercial fungicide metaloxyl in inhibiting spore germination and hyphal growth of *P. capsiei*. Burkhead *et al.*[49] provided preliminary evidence for antifungal activity of phenylacetic acid against *Gibberella pulicaris* produced by the bacterium *Enterobacter coloacae.*

From the several peaks detected by the GC-MS analysis of the fermentation exudates of *Bacillus* isolate (B_6) a peak at m/z 405 was identified as kasugamycin (Fig. 6). Kasugamycin is an aminoglycoside antibiotic isolated from *Streptomyces kasugaensis*[50-52]. Preliminary *in vitro* studies by Hamada *et al.*[53] and Umezawa *et al.*[54] suggested that the antibiotic possesses significant activity against a variety of pathogenic bacteria, including *Pseudomonas* species.

Bacillus spp. have special advantage due to their ability to produce antibiotics in the soluble protein structure directly to the medium, which is found to be cheaper and more effective, so that it is preferable for commercial production[55,56].

To further characterize these antimicrobial substances the purification and characterization is necessary .The mechanisms by which antimicrobial factors inhibit growth of potentially pathogenic bacteria or fungi are also critical. These are currently under investigation.

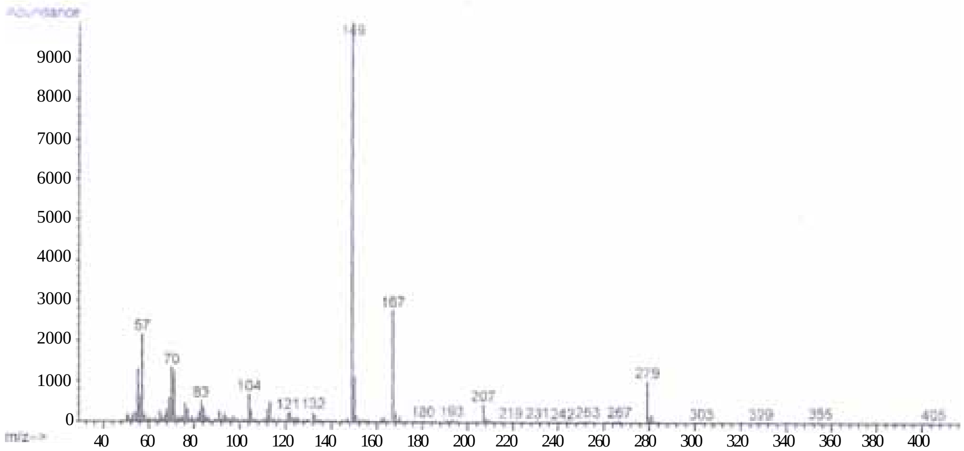

Figure 5. Ms of phthalic acid synthesized by *B. sphaericus* (B16).

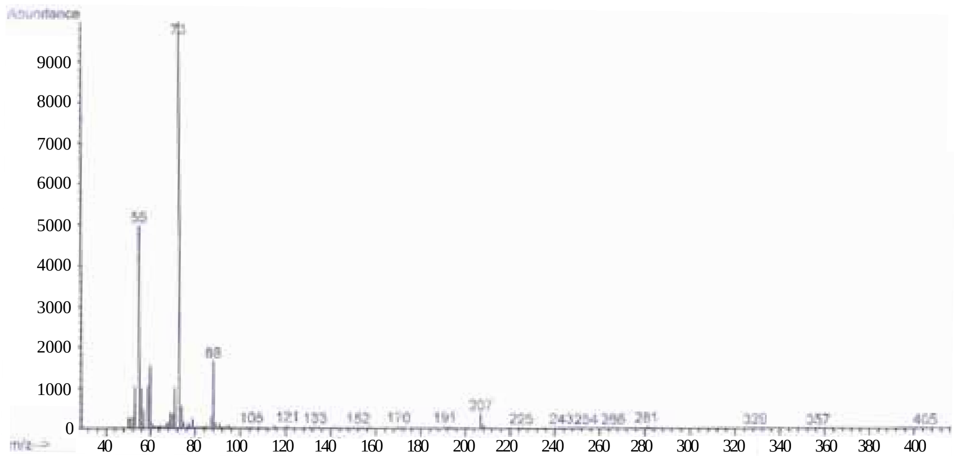

Figure 6. Ms of kasugamycin synthesized by the unidentified *Bacillus* isolate (B6).

References

[1]El Mehalawy, A. A., Gebreel, H. M., Rifaat, H. M., El Kholy, I. M. and Humid, A. A. 2008. Effect of antifungal compounds produced by certain bacteria on physiological activities of human- and plant-pathogenic fungi. Journal of Applied Sciences Research **4**(4):425-432.

[2]Brock, T. D. and Madigan, M. T. 1991. Biology of Microorganisms. 6th edn. Prentice-Hall International Inc., USA.

[3]Katz, E. and Demain, A. L. 1977. The peptide antibiotics of *Bacillus*, chemistry, biogenesis, and possible role. Bacteriological Reviews **41**:449-474.

[4]Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus.* Advances in Applied Microbiology **24**:187-214.

[5]Smirnov, V. V., Reznik, S. R. and Vasilievskaya, I. A. 1986. Aerobe endospore-forming bacteria. Medicina Könyvkiadó, Budapest (in Hungarian).

[6]Emmert, E. A. B. and Handelson, J. 1999. Biocontrol of plant disease: A (Gram-) positive perspective. FEMS Microbiol. Lett. **171**:1-9.

[7]Lisboa, M., Bonatto, D., Bizani, D., Henriques, J. A. P. and Brandelli, A. 2006. Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. Int. Microbiol. **9**:111-118.

[8]De Boer, A. S. and Diderichsen, B. 1999. On the safety of *Bacillus subtilis* and *B. amyloliquefaciens:* A review. Applied Microbiology and Biotechnology **40**:595-598.

[9]Luna, C. L., Mariano, R. L. R. and Souto-Maior, A. M. 2002. Production of biocontrol agent for crucifers black rot disease. Brazilian J. Chemical Engineering **19**(2):133-140.

[10]Awais, M., Pervez, A., Qayyum, S. and Saleem, M. 2008. Effect of glucose, incubation period and pH on the production of peptides antibiotics by *B. pumilus.* African Journal of Microbiology Research **2**:114-119.

[11]Claus, D. and Berkeley, R. C. W. 1986. Genus Bacillus. In Sneath, P. H. A. (ed.). Bergeys Manual of Systematic Bacteriology. Vol. 2. The Williams and Wilkins Company, Baltimore.

[12]El-Hendawy, H. H. 2004. Association of pectolytic fluorescent pseudomonads with post harvest rots of onion. Phytopathol. Mediterr. **43**:369-376.

[13]El-Hendawy, H. H., Osman, M. E. and Ramadan, H. A. 2002. Pectic enzymes produced *in vitro* and *in vivo* by *Erwinia* spp. isolated from carrot and pepper in Egypt. J. Phytopathol. (Berlin) **150**:431-438.

[14]El-Hendawy H. H., Osman, M. E. and Sorour, N. M. 2005. Biological control of bacterial spots caused by *Xanthomonas campestris* pv. *vesicatoria* by using *Rhanella aquatilis*. Microbiological Research **160**:343-352.

[15]Földes, T., Bánhegyi, I., Herpai, Z., Varga, L. and Szigeti, J. 2000. Isolation of *Bacillus s*trains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage microorganisms. Journal of Applied Microbiology **89**:840-846.

[16]Leifert**,** C., Workman, S. and Li, H. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilis* CL45. Journal of Applied Bacteriology **78**:97-108.

[17]Reva, O. N., Sorokulova, I. B. and Smirnov, V. V. 2001. Simplified

technique for identification of the aerobic spore-forming bacteria by phenotype. International Journal of Systematic and Evolutionary Microbiology **51**:1361-1371.
[17]Asha Devi, N. K., Balakrishnan, K., Gopal, R. and Padmavathy, S. 2008. *Bacillus clausii* MB9 from the east coast regions of India: Isolation, biochemical characterization and antimicrobial potentials. Current Science **95**(5):627-636.
[19]Akpa, E., Jacques, P., Wathelet, B., Paquot, M., Fucks, R., Budzikiewicz, H. and Thonart, P. 2001. Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. Applied Biochemistry and Biotechnology **91**:551-561.
[20]Duffy, B. K. and Defago, G. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Applied Environmental Microbiology **65**:2429-2438.
[21]Slininger, P. J. and Jackson, M. A. 1992. Nutritional factors regulating growth and accumulation of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. Applied Microbiology and Biotechnology **37**:388-392.
[22]Takahashi, A., Saito, N., Hotta, K., Okami, Y. and Umezawa, H. 1986. Bagougeramines A and B, new nucleoside antibiotics produced by a strain of *Bacillus circulans*. I. Taxonomy of the producing organism and isolation and biological properties of the antibiotics. J. Antibiot. (Tokyo) **39**(8):1033-1040.
[23]Howells, J. D., Anderson, L. E., Coffey, G. L., Senos, G. D., Underhill, M. A., Volger, D. L. and Ehrlich, J. 1972. Butirosin, a new amino-glycosidic antibiotic complex: Bacterial origin and some microbiological studies. Antimicrob. Agents Chemother. **2**:79-83.
[24]Tendulkar, S. R., Saikumari, Y. K., Patel, V., Raghotama, S., Munshi, T., Balaram, P. and Chattoo, B. B. 2007. Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*. J. Appl. Microbiol. **103**(6):2331-2339.
[25]Parker, M. T. and Collier, L. H. (1990). Principles of Bacteriology, Virology and Immunity. 7th edn. Vol. 2. D.C. Decker, Philadelphia.
[26]Snell, N., Ijichi, K. and Lewis, J. C. 1955. Paper chromatographic identification of polypeptide gram positive inhibiting antibiotics. Appl. Microbiol. **4**:13-17.
[27]Andrea, M. N., Konz, D. and Marahiel, M. A. 2001. The two component regulatory system BacRSis associated with bacitracin (self resistance) of *B. licheniformis* ATCC10716. Eur. J. Biochem. **268**:3180-3189.
[28]Jamil, B., Hasan, F., Hameed, A. and Ahmed, S. 2007. Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptidic antibiotic production. Pak. J. Sci. **20**(1):26-31.
[29]Ming, L. J. and Epperson, J. D. 2002. Metal binding and structure-activity relationship of the metalloantibiotic peptide bacitracin. Journal of Inorganic Biochemistry **91**:46-58.
[30]Al-Janabi, A. A. S. 2006. Identification of bacitracin production by local isolate of *B. licheniformis*. African J. Biotechnology **5**(18):1600-1601.
[31]Munimbazi, C. and Bullerman, L. B. 1998. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. J. Appl. Microbiol. **84**:959-968.
[32]Xie, G., Su, B. and Cui, Z. 1998. Isolation and identification of N2-fixing strains of *Bacillus* in rice rhizosphere of the Yangtze River Valley. Wei Sheng Wu Xue Bao **38**(6):480-483.
[33]Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonart, P. 2005. *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Appl. Microbiol. Biotechnol. **67**:692-698.
[35]Shoji, J., Hinoo, H., Wakisaka, Y., Koizumi, K., Mayama, M., Matsuura, S. and Matsumoto, K. 1976. Isolation of three new antibiotics, thiocillins I, II, III related to micrococcine. The Journal of Antibiotics **29**(4):366-374.
[35]Stein, T. 2005. *Bacillus subtilis* antibiotics: Structures, synthesis and specific functions. Mol. Microbiol. **56**:845-857.
[36]Bapat**,** S. and Shah, A. K. 2000. Biological control of fusarial wilt of pigeon pea by *Bacillus brevis*. Can. J. Microbiol. **46**:125-132.
[37]Schmitt, A., Eisemann, S., Mackay, N. and Seddon, B. 1998. Antifungal activity of gramicidins and use of *Bacillus brevis* for control of *Sphaerotheca fuliginea.* Modern Fungicides and Antifungal Compounds 2.12 t International Reinhardsbrunn Symposium, Friedrichroda, Thuringia, Germany, 24th -29th May.
[39]Demain**,** A. L. 1998. Induction of microbial secondary metabolism. Int. Microbiol. **1**:259-264.
[40]Onbasali, D. and Aslim, B. 2008. Determination of antimicrobial activity and production of some metabolites by *Pseudomonas aeruginosa* B1 and B2 in sugar beet molasses. African Journal of Biotechnology **7**(24):4614-4619.
[41]Page, W. J. 1989. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. **31**:329-333.
[42]Yoo, I. K., Cheng. N. N., Lee, E. G., Cheng, Y. K. and Moon, S. H. 1997. Effect of B vitamin supplementation on lactic acid production by *Lactobacillus casei*. J. Ferment. Bioeng. **84**:172-175.
[43]Woodruff, H. E. 1966. The physiology of antibiotic production: The role of the producing organism. Symp. Soc. Gen. Microbiol. **16**:22.
[44]Bodansky, M. and Perlman, D. 1969. Peptide antibiotics. Science **163**:94.
[45]Roy, R. N. Lasker, S. and Sen, S. K. 2006. Dibutyl phthalate, the bioactive compound produced by *Streptomyces albidoflavus* 321.2. Microbiological Research **16**(2):121-126.
[46]Alim Al-Bari, M., Abu Sayeed, M., Rahman, M. S. and Mossadile, M. A. 2006. Characterization and antimicrobial activities of a phthalic acid derivative produced by *Streptomyces bangladeshiensis* a novel species collected in Bangladesh. Research J. Medicine and Medical Sciences **1**(2):77-81.
[47]Kim, Y., Cho, J.-Y., Kuk, J.-H., Moon, H., Cho, J. I., Kim, Y.-C. and Park, K.-H. 2004. Identification and antimicrobial activity of phenyl acetic acid produced by *Bacillus licheniformis* isolated from fermented soybean, Chungkook-Jang. Current Microbiology **48**(4):312-317.
[48]Hwang, B. K., Lim, S. W., Kim, B. S., Lee, J. Y. and Moon, S. S. 2001. Isolation and *in vivo* and *in vitro* antifungal activity of phenyl acetic acid and sodium phenyl acetate from *Streptomyces humidus*. Appl. and Environ. Microbiol. **67**(8):3739-3745.
[49]Burkhead, K. D., Slininger, P. J. and Schisler, D. A. 1998. Biological control bacterium *Enterobacter coloacae* S11:T:07 (NRRL 13-21050) produces the antifungal compound phenyl acetic acid. Soil Biol. Biochem. **30**:665-667.
[50]Umezawa, H., Okami, Y., Hashimoto, T., Sukara, Y., Hamada, M. and Takeuchi, T. 1965. A new antibiotic, kasugamycin. J. Antibiotic (Tokyo) Ser. A **18**:101-103.
[51]Hotta, K., Ogata, T., Ishikawa, J., Okanishi, M. and Mizuno, S. 1996. Mechanism of multiple aminoglycoside resistance of kasugamycin-producing *Streptomyces kasugaensis* MB 273: Involvement of two types of acetyl transferases in resistance to astromycin group antibiotics. J. Antibiotics **49**(7):682-688.
[52]Duffin, P. M. and Seifert, H. S. 2009. KsgA mutations confer resistance to kasugamycin in *Neisseria gonorrhoeae*. Int. J. Antimicrob. Agents **33**(4):321-327
[53]Hamada, M., Hashimoto, T., Takahashi, T., Yokoyama, S., Miyake, M., Takeuchi, T., Okami, Y. and Umezewa, H. 1965. Antimicrobial activity of kasugamycin. J. Antibiotic (Tokyo) Ser. A **18**:104-106.
[54]Umezawa, H., Hamada, M., Sukara, Y., Hashimoto, T., Ikekawa, T., Tanaka, N., Maeda, K., Okami, Y. and Takeuichi, T. 1966. Kasugamycin, a new antibiotic 1965. Antimicrobial Agent and Chemotherapy, pp.753-757.
[55]Priest, F. G. 1989. Products and applications. In Bacillus Biotechnology Hand Book Vol. 2. Plenum, New York, London.
[56]Debayov, V. G. 1982. The industrial use of bacilli. In The Molecular Biology of Bacilli 1. Academic Press, FL.