South African Avocado Growers' Association Yearbook 1996. 19:54-58

Optimizing Culturing Conditions for Bacillus Subtilis

L. Korsten[1] N. Cook[2]
[1]*Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002*
[2]*Research Institute for Plant Protection, Private Bag X134, Pretoria 0001*

ABSTRACT

Optimization of *Bacillus subtilis* (isolate B246) growth conditions for mass cell production under laboratory conditions was investigated as part of a biological control programme. Aspects such as increasing yield using various starter culture volumes, various media and incubation time and temperatures, were studied. No significant differences between starter culture volumes were found. Potato media gave the highest yield and was the most economical. A significant drop in viable and in total cell counts was found when *B. subtilis was* harvested through centrifugation. Freezing *B. subtilis* at -78 °C as well as using Ringer-Glycerol as preservation medium was more effective than freezing at -20 °C, or skim-milk medium. Conversion of fresh cultures to dry forms resulted in a 60 % reduction in c.f.u. The lyophilized antagonist remained viable for up to seven months storage at room temperature, although a significant drop in viable cells was observed after the first month's storage.

INTRODUCTION

The genus *Bacillus* comprises a diverse and commercially useful variety of species widely distributed in nature (Harwood, 1989). Apart from their application in industry (Harwood, 1989) and as bio-insecticides (Deacon, 1983), *Bacillus subtilis* (Ehrenberg) Cohn has, for instance, been used for centuries as a food source in Japan (Djien & Hesseltine, 1979) and, has GRAS (generally regarded as safe) clearance in the United States. *Bacillus* spp. are also commonly utilized in biological control of plant diseases, e.g. *Bacillus cereus* Frankland and Frankland (Doherty & Preece, 1978), *Bacillus megaterium* de Bary (French *et al.,* 1964; Purkayastha & Bhattacharyya, 1982; Islam & Nandi, 1985), *Bacillus mycoides* Flügge (McBride, 1969; Spurr, 1981), *Bacillus polymyxa* (Prazmowski) Macé (French *et al.,* 1964), *Bacillus pumilus* Meyer & Gottheil (Morgan, 1963), and especially *Bacillus subtilis* (Nanda & Gangopadyay, 1983; Pusey & Wilson, 1984; Singh & Deverall, 1984; Baker *et al,* 1985).

Avocado *(Persea Americana* Mill.) is susceptible to various fruit diseases, including black spot, anthracnose, *Dothiorella / Colletotrichum* fruit rot complex and stem-end rot. Limited control of these diseases is achieved primarily with pre-harvest fungicide sprays such as copper oxychloride and benomyl. However, growing international concern over the excessive use of fungicides and its detrimental effect on the environment and mankind has forced researchers to evaluate alternative control measures. One such alternative, biological control, has previously been shown to be effective in controlling

avocado fruit diseases (Korsten *et al.,* 1995). Subsequently, the South African avocado industry showed interest in using the antagonist in integrated disease control programmes. The antagonist will therefore have to be commercialized and registered for use at pre and post-harvest level (Korsten *et al.,* 1995). This inevitably entails mass production of the antagonist for commercial use.

For commercial production, the antagonist must first be cultivated and all processes involved, optimized. Optimization takes place under laboratory conditions before up scaling for mass production. Although there is considerable information available on laboratory-scale fermentation *oí Bacillus,* published literature has declined as processes became commercially more significant. (Sharp *et al.,* 1989). Available literature on culturing *Bacillus* spp mainly describes selective media (Norris *et al.,* 1981), or laboratory-scale fermentations (Sharp *et al,* 1989). Optimum conditions for culturing, harvesting, lyophilizing and storing of *Bacillus* antagonists for eventual use against avocado fruit pathogens, had to be determined in order to obtain maximum yield for each batch processed. The most promising avocado biocontrol antagonist *(B. subtilis* isolate B246) was therefore selected for optimization of cultivation conditions and to determine the effect of preservation and long-term storage on cell viability. Results of this investigation are reported here.

MATERIALS AND METHODS

Bacterial cultures

B. subtilis (B246), originally isolated from the avocado phylloplane (Korsten *et al.,* 1988), was selected for optimizing mass cell production due to its strong inhibitory action against economically important avocado post-harvest pathogens. The antagonists were maintained on standard 1 nutrient agar (STD) (Biolab) slants and lyophilized in 30 % v/v glycerol-Ringer's solution at -78 °C.

Optimizing starter cultures

Six replicates were made for each starter volume used, namely 20; 50; 100 and 200 ml STD broth. Each starter volume was inoculated with 1 ml *B. subtilis* 10^7 cells/ml, obtained by washing the 24-hold STD slant with sterile quarter-strength Ringer's solution (Merck). Counts were made using a Petroff-Hausser counting chamber under a Zeiss phase contrast microscope and adjusted accordingly. Inoculated starter volumes were placed on a rotary shaker (76 r.p.m) at ambient room temperature (22-28 °C). After 24 h shake incubation, each starter culture volume was added in its entirety to separate 1,3 *(* STD broth contained in 2,5 *i* Erlenmeyer flasks. These flasks were shake incubated for 66 h before making a viable cell count using serial dilutions (Harrigan & McCance, 1966) as well as a total cell count (as described) from each starter volume. Data was statistically analysed using Tukey's multiple range test.

Optimum incubation time

Twenty one 200 ml starter volumes of *B. subtilis* were shake incubated for 24 h, before

adding each starter volume to 1,3 ℓ STD broth contained in a 2,5 ℓ Erlenmeyer flask. After additional shake incubation, three culture flasks were removed after either 8; 24; 32; 48; 54; 72 or 80 h for viable cell counts. In addition, growth was determined spectrophotometrically with a multiscan ultraviolet/visible spectrophotometer at 560 nm. Data were statistically analysed using Tukey's multiple range test.

Optimum growth temperature

Optimum growth-temperature curves were determined for *B. subtilis* in a temperature gradient incubator Thermocon (New Brunswick) (Du Preez, 1980). Temperatures were set between 660 °C and each Thermocon culture tube containing STD broth was inoculated with 1 ml of antagonist broth culture (10^7 cells/ml). The culture tubes were removed sequentially without stopping the shaker to determine growth spectrophotometrically after 24 h and 48 h. The temperature-growth curve for each antagonist was determined in duplicate.

Growth media

Fourteen different standard, selective or semi-selective media (table 1) were used to determine the most effective medium for mass cell production of *B. subtilis.* Five 200 ml starter cultures were prepared as described from each medium listed in table 1. After 24 h shake incubation at room temperature (22-28 °C), each starter culture was added in its entirety to 1,3 ℓ of the same medium as was used in the starter culture. After additional 48 h shake incubation, total cell counts were made, and data were statistically compared using Tukey's multiple range test.

Table 1
Various growth media for optimizing *Bacillus subtilis* growth

Medium	*Code*	*Reference*
Brain heart	BH	Brooks *et al.*, 1954
Bacillus growth	Bac	Katz & Demain, 1977
Pennassay	Penn	Difco manual
Manganese	Ma	Norris *et al.*, 1981
Milk	MM	Meyer *et al.*, 1985
Nutrient broth	NB	Biolab catalogue
Potato	PM	Boyliss *et al.*, 1981
Antibiotic	Ant	Meyer *et al.*, 1985
Buffered peptone	BP	Meyer *et al.*, 1985
Lactose milk	LM	Meyer *et al.*, 1985
Yeast glucose	G	Norris *et al.*, 1981
Peptone-water	PW	Oxoid manual
L. medium	L	Amary *et al.*, 1987
Standard I	STD	Biolab catalogue

Harvesting

The effect of harvesting on yield was determined. 200 ml *B. subtilis* starter culture, prepared as described for optimizing starter cultures, was added to 1,3 ℓ. STD medium for mass cell production. After 48 h shake incubation, viable counts were made as

previously described. Each growth flask was separately harvested by means of centrifugation in a Sorvall RC-5b refrigerated Superspeed centrifuge (Du Pont Instruments) using the GSA rotor at 11 080,64 g for 20 min. Total solids in the cultures were determined by weighing each final cell pellet. ISO ml sterile Ringer's solution was added to each pellet before mixing it on a Vortex and making a dilution series for viable cell counts. The effect harvesting had on viability was determined by statistically comparing the difference in cell counts from the three replicates before and after harvest using Tukey's multiple range tests.

Effect of freezing

The effect temperature and freezing medium had on cell viability after harvesting was determined. *B. subtilis* (10^7 cells/ml) was inoculated into 12 x 250 ml Erlenmeyer flasks containing 100 ml STD broth. After 48 h shake incubation at room temperature, antagonists were separately harvested by centrifugation as described for mass cell production. Harvested pellets were each separately dissolved, either in 100 ml of sterile glycerol-Ringer's solution, or in skim-milk powder (Harrigan & McCance, 1966). Three samples of each medium were stored at -20 °C and -78 °C. Viable cell counts were made from each sample before freezing and 24 h after freezing. The effect of freezing temperature and medium on cell viability was statistically compared using Tukey's multiple range test.

Long-term storage

The effect of long-term storage on cell viability of vacuum-sealed lyophilized antagonists was determined using three freshly prepared 1,5 ℓ batches of *B. subtilis.* Each batch was separately harvested as described for mass cell production, lyophilized and vacuum sealed in separate plastic bags. Viable cell counts were made as described, before, immediately after lyophilization, and at monthly intervals thereafter up to six months. In each case, 1 g lyophilized antagonist was taken for cell counts. The lyophilized antagonists were maintained at ambient room temperature (22-28 °C) throughout the duration of the study. The effect of long-term storage on viable cell counts was statistically compared using Tukey's multiple range test.

RESULTS

Optimizing starter cultures

No significant differences in viable or total cell counts were found, when different initial starter culture volumes were used to inoculate STD growth flasks (table 2).

Table 2
Effect of starter-culture volumes of *Bacillus subtilis* used to inoculate Standard 1 nutrient broth growth flasks[1]

Starter-culture volume	*Total cell count (Cells/ml)*	*Viable cell count (c.f.u./ml)*
20 ml	$1,8 \times 10^8$ a	$1,3 \times 10^8$ a
50 ml	$1,7 \times 10^8$ a	$0,8 \times 10^8$ a
100 ml	$1,9 \times 10^8$ a	$1,0 \times 10^8$ a
200 ml	$1,3 \times 10^8$ a	$1,1 \times 10^8$ a

[1]Mean total cell counts from five replicates. In columns, values followed by the same letter do not differ significantly according to Tukey's multiple range test (P = 0,05).

Optimum incubation time

Optimum incubation time for highest yield of viable cells was 3248 h (table 3), and highest total yield was achieved from 24 h onwards (table 3).

Table 3
Effect of incubation time on *Bacillus subtilis* yield[1]

	Time after inoculation						
	0 h	*8 h*	*24 h*	*32 h*	*48 h*	*54 h*	*72 h*
Viable cells ($\times 10^7$)	—	5,0 bc	8,8 b	19,0 a	13,0 a	7,4 bc	4,5 bc
Absorbance[2]	0,047 c	0,252 b	0,616 a	0,682 a	0,669 a	0,690 a	0,672 a

[1]Mean total cell counts from five replicates. In columns, values followed by the same letter do not differ significantly according to Tukey's multiple range test (P = 0,05).
[2]Determined spectrophotometrically at 560 nm.

Optimum growth temperature

Optimum growth temperature for *B. subtilis* was 30-37 °C, with a minimum temperature of 18 °C and a maximum of 43 °C (figure 1).

Growth media

Of the 14 different mediums for optimum *B. subtilis* growth, the potato medium resulted in the highest yield, although not significantly more than STD 1, which in turn did not differ from nutrient broth. The previously mentioned medium was also not more effective than BH, Ma, MM, Ant, BP, LM, YG, PW, and L media, and the Bac and Penn media were the least effective (table 4). However, cost comparisons showed that the potato medium was the least expensive at 81 c/ℓ, whereas STD 1 and nutrient broth were relatively expensive.

Harvesting

Harvesting cells through centrifugation significantly reduced viable cells from 4,2 x 10^8

to 1,4 x 10^s c.f.u./ml. Total solids in the cultures amounted to 1,1 %.

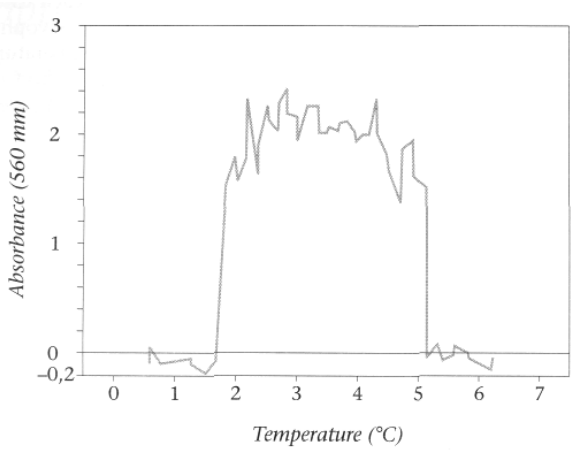

Figure 1
Temperature growth curve of *Bacillus subtilis*

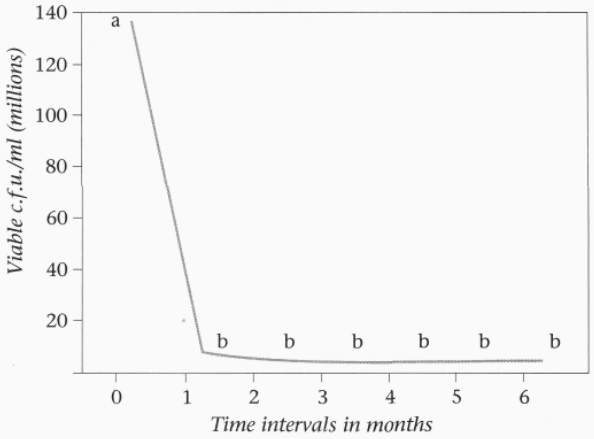

Figure 2
Effect of long-term storage at ambient room temperature of lyophilized *Bacillus subtilis*

Mean viable cell counts from three replicates. Values followed by the same letter do not differ significantly according to Tukey's multiple range test (P = 0,05)

Table 4
Effect of 14 different media on total yield of *Bacillus subtilis*[1]

Media[2]	*Total count in cells/ml*	*Cost/ℓ*[3]
Brain heart	1,2 × 108 cd	R4,88
Bacillus	3,0 × 107 c	R1,41
Pennassay	3,2 × 107 d	R1,72
Manganese	6,0 × 107 cd	R1,62
Milk	2,6 × 108 cd	R1,29
Nutrient B	2,2 × 108 bc	R2,43
Potato	4,9 × 108 a	R0,81
Antibiotic	8,8 × 107 cd	R1,72
Buffered peptone	9,7 × 107 cd	R2,17
Lactose	9,7 × 107 cd	R1,24
Yeast-glucose	4,6 × 107 cd	R1,12
Peptone water	1,5 × 108 cd	R2,87
L medium	9,4 × 107 cd	R2,43
Standard 1	3,6 × 108 ab	R3,40

[1]Mean total cell counts from five replicates. In columns, values followed by the same letter do not differ significantly according to Tukey's multiple range test (P = 0,05).
[2]Medium references are given in table 1
[3]Prices for mediums were calculated from the March 1992 price index from various commercial companies

Table 5
Effect of viable *Bacillus subtilis* cell counts when various freezing temperatures (–20 or –78 °C) and media are used[1]

Freezing medium	*Difference in viable cell count*					
	–20 °C	*V*[2]	*H*[3]	*–78 °C*	*V*	*H*
Skimmed milk[4]	$9,2 \times 10^7$ a A	a	A	$2,8 \times 10^7$	b	A
Glycerol[5]	$2,1 \times 10^7$ a B	a	B	$22,0 \times 10^7$	a	A

[1]Mean viable cell counts from three replicates are given as the difference in cell count before freezing and 24 h after freezing at the specific temperature in c.f.u./ml. Values followed by the same letter do not differ significantly according to Tukey's multiple range test (P = 0,05).
[2]V = Statistical comparison of freezing mediums (vertical comparison)
[3]H = Statistical comparison of freezing temperatures (horizontal comparison)
[4]Skim-milk medium (Harrigan & McCance, 1966)
[5]30 % v/v Glycerol-Ringer's medium

Effect of freezing

Glycerol-Ringer's at -78 °C was found to be the most effective freezing medium and temperature used, resulting in the highest viable yield (table 5).

Long-term storage

Conversion of fresh cultures to dry forms resulted in a 60 % reduction of c.f.u. The lyophilized antagonists remained viable for up to seven months but with a pronounced and significant drop in viability during the first month (figure 2).

DISCUSSION

In this investigation, culturing conditions such as incubation time and growth medium

significantly influenced final yield. From this data, optimized laboratory-scale mass production of *B. subtilis* could be devised, giving optimal yields under these conditions. The fermentation of US-7 in the bench-top fermenter required minor modifications from the conditions which were adapted for production in Erlenmeyer flasks. Therefore optimized physical environmental conditions determined in this study could be useful for scaling-up in future fermentation of the antagonist processes. Furthermore, media used to isolate and screen industrially important micro-organisms are not usually those used for development studies and commercial production. The former type of commercial media often include components which are relatively expensive, often support sub-optimal growth, or may be regarded as undesirable for use on a production scale (Sharp *et al.,* 1989). Therefore, it is anticipated that the potato media could be used in small-scale laboratory conditions, but would probably be replaced in commercial production by an industrial waste material such as citrus peel. This was the case with the optimization and commercialization of *Pichia quilliermondii* (isolate US-7), which controls citrus post-harvest diseases (Hofstein *et al.,* 1991).

Harvesting, freezing and long-term storage of cells also significantly reduced final yield. Therefore, alternative harvesting and preservation methods that do not severely affect final yields should be investigated for commercial production. The importance of nutrition and conditions of the physical environment as well as methods used for product recovery and purification, have previously been described as having a significant effect on the economics and final product yield of mass cell production processes (Sharp *et al.,* 1989). Of the various media tested for laboratory *B. subtilis* production, the potato media was most cost-effective and gave the highest yield. Although the yield obtained in STD I media was not significantly lower than that obtained in potato media, it was more expensive.

The significant reduction in yield as found in this study due to harvesting of cells, is important if viable cells are to be used in future biocontrol programmes. Furthermore, storage of antagonist products is important — our investigation showed a highly significant drop in cell viability within the first month of lyophilization. This could indicate that it might be commercially more feasible to prepare biopesticides on demand, unless a more stabilizing freezing medium or preservation method can be developed. In our investigation, a glycerol-Ringer's medium ensured a less significant drop in viability. Furthermore, spore preparations were a more feasible alternative of preparing and maintaining final yields (unpublished data). If biocontrol is to succeed, it is very important that commercial biopesticide producers should take final product yield into account and ensure that final viable levels are sufficient for effective disease control before application dosages are recommended. Thus, development of smallscale economical laboratory processes requires that the aspects reported on in this investigation should be taken into account, since the process should be cost-effective and therefore optimized (Hofstein *et al.,* 1991).

CONCLUSION

Although we successfully optimized cultivation conditions for *B. subtilis* in the laboratory, commercial production of the antagonist will still be too costly when using these

parameters and growth conditions. Meanwhile, further progress has been made with more economical alternative approaches for mass cell production of the antagonist. (This data is not given in this report and will be referred to only briefly for the benefit of the farmers.) Cheaper waste products were evaluated as growth medium for *B. subtilis.* Dried citrus peel was found to be effective and could sustain cell growth of up to 1 x 10^8 cells/ml under optimal growth conditions (which include aeration in 20 ℓ fermentation tanks). However, harvesting of cells through centrifugation remained a problem, as it was tedious, time consuming and costly. Harvesting by centrifugation also resulted in unacceptably high loss of viable cells, as reported on in this study. Alternative cell-harvesting techniques were subsequently evaluated, including microfiltration (Millipore systems). However, similar filtration systems for large-scale fermentation units are expensive. Eventually, it was decided to move away from liquid fermentation to solid systems, which would eliminate tedious cell harvesting processes. Limited nutrient agar mediums were used in large-scale bacterial growth plates. Harvesting of cells proved to be effective and consisted of scraping the plates, thus effectively eliminating the tedious harvesting of cells through centrifugation. Unfortunately the high costs of sterile plates which could be used once only, forced us to look at gamma irradiation of plates for re-use, but with transport and radiation costs, this also proved expensive in the long run. We eventually moved to more economical bacterial carrier mediums such as beans, which resulted in high cell counts (1 x 10^9 cells/ml). Harvesting by means of decanting also proved easy without any significant loss in yield.

Our final product is now cultivated on a bacterial carrier which is very economical and can yield high cell counts and ensure consistent shelf life without any reduction in viable cells (1 x 10^9 cells/g). This product was stable at room temperature and had a consistent shelf life for several months, without any loss in viability. The product can also dissolve in water or Tag wax and does not result in any residues on the fruit. These alternative approaches have been described in the original and a subsequent patent.

REFERENCES

AMARY, A., KUNST, F., ÄUßERT, E., KLIER, A. & RAPORT, G. 1987. Characterization of the sac Q genes from *B. licheniformis* and *B. subtilis. Journal of Bacteriology.* 169: 324 - 333.

BAKER, C.J., STAVELY, J.R. MOCK, N. 1985. Biocontrol of bean rust by *Bacillus subtilis* under field conditions. *Plant Disease.* 69: 770.

BOYLISS, C.E., WAITES, W.M. & KING, N.R. 1981. Resistance and structure of spores of *B. subtilis. Journal of Applied Bacteriology* 50: 379 - 390.

BROOKS, D.C., HALVORSON, H. & MALVORSON, H.O. 1954. Studies on spore germination its independence from alanine racemase activity. *Journal of Bacteriology* 68: 393 - 399.

DEACON, J.W. 1983. Micobial control of plant pests and diseases. Van Nostrand Reinhold, Woking, U.K.

DIFCO. 1953. *Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures.* 9 ed.

DJIEN, K.S. & HESSELTINE, C.W. 1979. Tempe and related foods. Pages 116 - 140 in: A.H. Rose (Ed.). *Economic Microbiology,* Vol. 4. Academic Press, London.

DOHERTY, M.A. & PREECE, T.F. 1978. *Bacillus cereus* prevents germination of uredospores of *Puccinia allii* and the development of rust disease of leek, *Allium porrum,* in controlled *environment. Physiological Plant Pathology.* 12: 123 - 132.

DU PREEZ, J.C. 1980. *Growth Kinetic Studies of* Acinetobacter calcoaceticus *with Special Reference to Acetate and Ethanol as Carbon Sources.* Unpublished M.Sc. thesis, University of the Orange Free State.

FRENCH, R.C., NOVOTNY, J.F. & SEARLES, R.B. 1964. Properties of bacteria isolated from wheat stem rust spores. *Phytopathology.* 54: 970.

HARRIGAN, W.F. & MCCANCE, M.E. 1966. *Laboratory Methods in Microbiology.* Academic Press, London.

HARWOOD, C.R. 1989. Introduction to the biotechnology of *Bacillus.* In: C.R. Harwood (Ed.). *Biotechnology Handbooks,* vol 2: *Bacillus.* 1 - 4. Plenum Press, London.

HOFSTEIN, R., DROBY, S., CHALUTZ, E., WILSON, C. & FRIDLANDER, B. 1991. Scaling-up the production for application of antagonist form — basic research to R & D. In: C. Wilson, & E. Chalutz (Eds.). *Biological Control of Post-harvest Diseases of Fruits and Vegetables.* 197 - 210. Workshop proceedings, U.S. Department of Agriculture, Agricultural Research Services, ARS-92.

ISLAM, K.Z. & NANDI, B. 1985. Control of brown spot of rice by *Bacillus megaterium. ZeitschriftPflanzenkrantzen Pflanzenschutz.* 92: 241.

KATZ, E. & DEMAIN, A.C. 1977. The peptide antibiotics *of Bacillus:* Chemistry, biogenesis and possible functions. *Biological Review.* 41: 449 - 474.

KORSTEN, L., BEZUIDENHOUT, J.J. & KOTZÉ, J.M. 1988. Biological control of post-harvest diseases of avocado. *South African Avocado Growers 'Association Yearbook.* 11: 75.

KORSTEN, L., DE JAGER, E.S. & DE VILLIERS, E.E. 1995. Evaluation of bacterial epiphytes isolated from avocado leaf and fruit surfaces for biocontrol of avocado post-harvest diseases. *Plant Disease.* 79 (11): 1149 - 1156.

MCBRIDE, R.P. 1969. A microbial control *of Melampsom medusae. Canadian Journal of Botany* 47: 711.

MERCK. *Biolab Culture Media Catalogue.* Merck (Pty.) Ltd., South Africa.

MEYER, H.P., KAPPELI, O. & FEICHTER, A. 1985. Growth control in microbial cultures. *Annual Review of Microbiology* 39: 299 - 319.

MORGAN, F.L. 1963. Infection, inhibition and germ-tube lysis of three cereal rusts by *Bacillus pumilus. Phytopathology* 53: 1346.

NANDA, H.P. & GANGOPADHYAY, S. 1983. Control of rice helminthosporiose with *Bacillus subtilis* antagonistic towards *Bipolaris oryzae. International Journal of Tropical Plant Disease* 1: 25.

NORRIS, J.R., BERKELEY, R.C.W., LOGAN, N.A. & O'DONNEL, A.G. 1981. The genera *Bacillus* and *Sporolactobacillus.* In: M. Starr, H. Stolp, H.G. Truper, A. Balows & H. Schlegel *the Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria,* vol 2: 1711 - 1742. Springer-Verlag, Berlin

OXOID. 1979. *Oxoid Manual of Culture Media, Ingredients and other Laboratory Services.* 4 ed

PURKAYASTHA, R.P. & BATTACHARYYA, B. 1982. Antagonism of microorganisms from jute phyllosphere towards *Colletotrichum corchori. Transactions of the British Mycological Society.* 78: 509

PUSEY, PL. & WILSON, C.L. 1984. Post-harvest biological control of stone fruit brown rot by *Bacillus subtilis. Plant Disease.* 68: 753 – 756

SHARP, J., SCAWEN, M.D. & ATKINSON, T. 1989. Fermentation and downstream processing *oí Bacillus.* In: C.R. Harwood (Ed.). *Biotechnology handbooks,* vol *2 Bacillus.* 255 - 292. Plenum Press, London

SINGH, V. & DEVERALL, BJ. 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. *Transactions of the British Mycological Society.* 83: 487.

SPURR, H.N. Jr. 1981. Experiments on foliar disease control using bacterial antagonists. In: J.P. Blakeman (Ed.). *Microbial Ecology of the Phylloplane.* 369 - 381. Academic Press, London.