

Strain selection, production, and formulation of the biological plant vitality enhancing agent FZB24[®] *Bacillus subtilis*

H. Junge, B. Krebs, M. Kilian

1 Development concept for biological products

Bacillus subtilis, known as hay bacterium, is a well known micro-organism, and various strains are already used industrially in diverse fields. The aim of this project was to develop marketable products from micro-organisms of the genus *Bacillus* for use in biological and integrated plant protection. The necessary criteria for the successful development and marketing of biological products are: reliable effect, suitability for application by conventional techniques, and competitive treatment costs per ha for both the farmer and the gardener. A low-cost production method, constant product quality, satisfactory storage stability, and formulation capability are indispensable too. FZB24[®] meets these criteria, as explained below.

The strain selection process that led to FZB24[®] took several years. Numerous experiments had shown that FZB24[®] was the strain that reliably promoted growth and yields and reduced losses due to soil-borne pathogens in many species of plants. In addition, this strain also fulfilled all the requirements on the production and formulation sides.

2 Strain selection and systematic identification of FZB24[®] *Bacillus subtilis*

So far 65 fully characterized species have been assigned to the genus *Bacillus*. With new diagnostic methods, it is now possible to recognize greater genetic and physiological diversity within individual species. As a consequence, new taxonomic groups could be separated in the future, and some species could be assigned to different groups.

The *Bacillus subtilis* group includes e.g. the species *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. firmus*, which are characterized by acid formation from various sugars, including glucose, under aerobic conditions, and which have ellipsoid spores that are not larger than the parent cell (Priest 1993).

FZB24[®] *Bacillus subtilis*, which had been isolated from natural habitats in Brandenburg, Germany, was identified as *B. subtilis* by the methods of Bergey (1986) on the basis of its morphological, biochemical, and serological properties (Juhr, 1992). The identification as *Bacillus subtilis* has been confirmed by computer-assisted diagnostic systems that are also based on biochemical paramete-

ters, such as the VITEK® system (anonymous, 1996) (Table 1). However, fatty acid analysis (Hoffmann-Hergarten, 1996; Verburg, 1996) also showed correlations with the fatty acid pattern of *Paenibacillus macerans* and *B. amyloliquefaciens*.

The *Bacillus subtilis* strain FZB24® forms the typical wrinkled colonies of *Bacillus subtilis* in surface cultures on solid PD-agar (Fig. 1). Vegetative cells or spores are found in liquid cultures, depending on the age of the culture (Fig. 2).

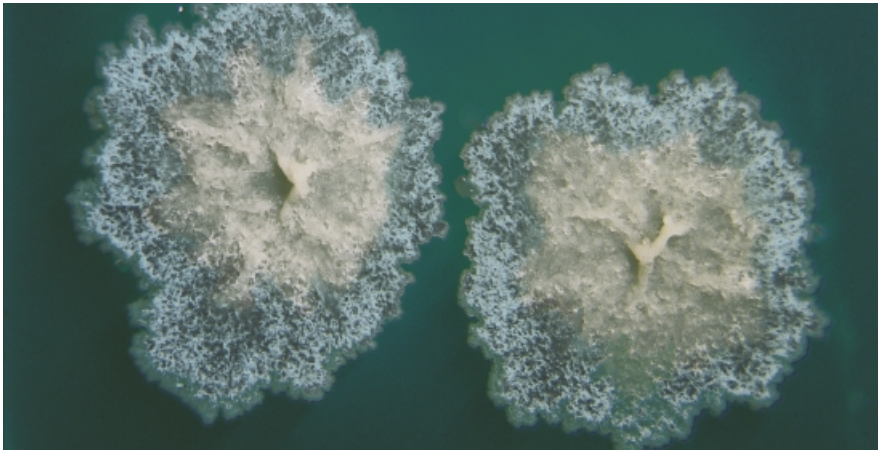


Fig. 1: Colony forms of FZB24® *Bacillus subtilis* on solid PD agar after incubation for 5 days at 25°C (Photo: Dr. U. Steiner, University of Bonn)

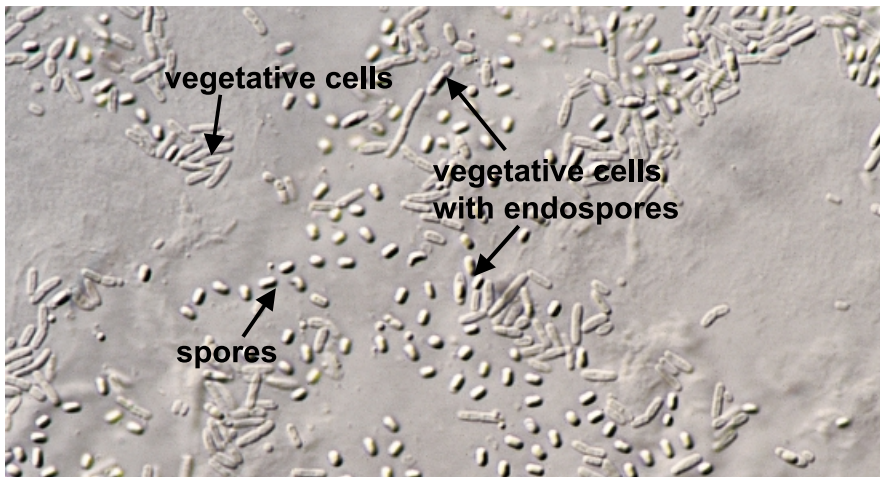


Fig. 2: Vegetative cells and spores of FZB24® *Bacillus subtilis* (Photo: Dr. U. Steiner, University of Bonn)

3 FZB24® *Bacillus subtilis* production process

FZB24® *Bacillus subtilis* is produced in liquid cultures as indicated by the scheme in Figure 3.

The starting cultures are prepared from a lyophilized stock culture. The characteristic properties of the strain are checked on selected single colonies, and only tested colonies are subsequently cultivated. Spore solutions are prepared from those colonies and frozen in portions for the inoculation of precultures. This procedure guarantees a uniform strain identity and a uniform starting material for fermentations over a period of many years.

For a production run, a preculture is prepared in a shaking flask from one of the frozen cultures. After an adequate cultivation time, this preculture is used for the inoculation of a fermenter. The preculture procedure is repeated several times, in accordance with the intended volume of the main fermentation; the fermentation volume can be increased in a ratio of 1:100 in each step.

The main culture takes place in a complex culture medium partly based on soya bean products. Natural starting materials of this type are used only if certificates confirm that they are free from genetically modified constituents. This is important for subsequent use in organic farming. The natural nutrient components are necessary to attain high cell densities and hence high yields of heat-resistant spores of *B. subtilis*. Complex natural nutrients are a source for a number of growth factors and trace elements, mixtures of free amino acids for the exponential growth of the population and for complex organic nitro-

gen sources (proteins, peptides). These provide a continuous supply of the necessary amino acids and small peptides for the growth of the culture in the transition phase. This continuous supply results from the action of the strain-specific extracellular proteases and peptidases, the formation of which is triggered by starvation in respect of individual amino acids after the first restriction of growth (end of the logarithmic phase). In addition to the easily utilizable nitrogen sources, the complex nutrients also provide inorganic phosphate, which is released from these nutrients by the sporulation-specific alkaline phosphatase, and which controls the sporulation process and the development of the heat resistance of the spores. The oxygen-content, the pH, etc. are continuously monitored during the fermentation. Examples of the typical growth curves obtained and the changes in the medium are shown in Figures 4 and 5.

The growth curve is characterized by a logarithmic phase lasting about 4 hours and a prolonged transition phase. The maximum cell density of approximately 1.0×10^{10} CFU/g, with > 95% sporulation of the cells, is reached about 20 hours after inoculation. The fermentation is ended at this point.

The solid components of the culture solution, i.e. the spores, cells, and nutrient residues, are separated from the liquid components and the metabolic products formed in the course of the fermentation. The volume is reduced to about 1/10 of its initial value by aseptic separation. The biomass obtained, which is also known as bioslurry because of its consistency, is then dried and formulated by suitable processes.

4 Drying and formulation of FZB24® *Bacillus subtilis*

Various processes have been tested for the drying and formulation of the spores of FZB24® *Bacillus subtilis*. Spray drying, fluidized-bed drying, and freeze drying are fundamentally suitable for drying the *B. subtilis* spores, which are resistant, and for formulating them with the aid of additives. Temperatures of up to 60°C can be used intermittently in the drying process without causing any appreciable losses of viable spores.

Quartz sand was initially used as a carrier for the *B. subtilis* spores, and later soluble fertilizer salts such as potassium nitrate or ammonium sulphate. The granular products yielded by this formulation procedures had spore contents of $> 5.0 \times 10^{10}$ CFU/g and residual moisture contents of about 1%, but showed a relatively wide grain size distribution.

The use of grain starch as a carrier offered the advantage that since this is an organic material, the product could then also be used in organic farming, for which even traces of fertilizer salts in the product are prohibited. However, starch as a carrier leads to a finer grain size, so that only a very fine water-dispersible granulate could be produced. The dispersibility of the formulations on starch was also distinctly better than when potassium nitrate was used as carrier. In addition to its formulation characteristics, the carrier material can also have a certain influence on the biological effect of the product. Conceivable effects are improved adhesion of the treatment suspension to the potato tubers and a support for the bacterium during its establishment in the rhizosphere as a consequence of the small sup-

ply of nutrients. A comparison of KNO₃ and maize starch as carrier materials showed small but statistically non-verifiable advantages for the organic material (Fig. 6).

The residual moisture-content of the product has a decisive influence on the storage stability of the formulation. Particularly under unfavorable storage conditions, if the moisture-content is more than 18%, the spores may die relatively quickly and the product may become mouldy. The dependence of the storage stability on the residual moisture-content is illustrated in Table 2 for storage under extreme conditions at 54°C, which is used to simulate prolonged storage.

In the case of products with starch as the support, the residual moisture-content can be reduced to less than 12% by post-drying under vacuum.

Optimization of the spore-content of the FZB24® formulations, together with low residual moisture-content and diffusion-tight packaging, guarantees that the contents of viable spores in the product remains sufficiently high for at least 2 years. This is confirmed by storage tests at +54°C, at normal room temperature up to 30°C, and at alternating temperatures of -15 to +30°C (Fig. 7).

A second FZB24® formulation with talc as a substantially inert carrier has been specially developed for use as a dry treatment, e.g. for potatoes. The use of a lower spore concentration allows direct application of the undiluted product without any impairment of the adhesion to the surfaces of seeds and seed potatoes.

The result of these studies was the characterization and development of the *Bacillus subtilis* strain having the designation FZB24® (Krebs et al. 1998). Numerous trials had shown that FZB24® is

reliably effective in promoting plant growth and in reducing losses due to soil-borne pathogens. On the basis of its mode of action – growth promotion, stress reduction, and resistance induction combined with weak antifungal effects – FZB24® *Bacillus subtilis* is registered as a 'plant strengthening agent' with the German Biological Institute for Agriculture and Forestry. FZB24® has now also been registered in the USA and in Austria, and registration in other countries is currently being prepared or expected.

5 Summary

The *Bacillus subtilis* strain FZB24® is the result of years of study in which various strains of *Bacillus* were selected on the basis of biological effectivity and suitability for production. FZB24® is produced in a multi-stage liquid fermentation process from a stock culture that guarantees a uniform strain identity. The spores formed in this process are separated from the culture broth and then dried and formulated together with protective colloids, inert material, and other additives. The formulated end product has a storage stability of at least 2 years. FZB24® *Bacillus subtilis* is registered in Germany as a 'plant strengthening agent'. Preparations are being made for its registration in other countries.

Zusammenfassung

Stammselektion, Produktion und Formulierung des biologischen Pflanzenstärkungsmittels FZB24®-*Bacillus subtilis*

Der *Bacillus subtilis* Stamm FZB24® ist das Ergebnis langjähriger Studien, in

denen verschiedene *Bacillus*-Stämme auf Wirkung und Produzierbarkeit hin selektiert wurden. FZB24® wird ausgehend von einer Stammhaltung, die eine gleichbleibende Stammidentität gewährleistet, in einem mehrstufigen Flüssigfermentationsverfahren produziert. Die dort gebildeten Sporen werden durch Separation von der Kulturbrühe abgetrennt und zusammen mit Schutzkolloiden, Inertmaterial und weiteren Zusätzen getrocknet und formuliert. Das formulierte Endprodukt besitzt eine Lagerstabilität von mindestens zwei Jahren. FZB24®-*Bacillus subtilis* ist in Deutschland als Pflanzenstärkungsmittel registriert. Die Registrierung in weiteren Ländern befindet sich in Vorbereitung.

Résumé

Sélection des souches, préparation et formulation de l'agent biologique stimulateur de vigueur végétale FZB24®-*Bacillus subtilis*

La souche FZB24® de *Bacillus subtilis* est le résultat de plusieurs années d'études qui ont permis la sélection de différentes souches de *Bacillus*, en fonction de leur efficacité et des possibilités de leur production. Le FZB24® est produit par un procédé de fermentation en phase liquide en plusieurs étapes, à partir d'une souche mère qui garantit une identité constante de la souche. Les spores qui s'y forment sont isolées par séparation du bouillon de culture et sont séchées et formulées avec des colloïdes protecteurs, un matériau inerte et d'autres additifs. Le produit final formulé a une stabilité au stockage d'au moins 2 ans. Le FZB24®-*Bacillus subtilis* est homologué en Allemagne en tant qu'agent stimulateur

de vigueur végétale. L'homologation dans d'autres pays est en cours de préparation.

Resumen

Selección de cepa, preparación y formulación del vigorizante biológico FZB24®-*Bacillus subtilis*

La cepa FZB24® de *Bacillus subtilis* es el resultado de estudios durante años, en los que se han seleccionado las mejores cepas de *Bacillus* en cuanto a eficacia y producibilidad. FZB24® se produce mediante un proceso de fermentación líquida de varias etapas, partiendo de una cepa original que garantiza una identidad constante. Las esporas formadas se separan del caldo de cultivo y después se secan y formulan junto con coloides protectores, material inerte y otros aditivos. El producto final formulado posee una estabilidad al almacenaje de 2 años como mínimo. FZB24®-*Bacillus subtilis* está registrado en Alemania como vigorizante para vegetales. Se está tramitando el registro en otros países.

Резюме

Селекция штамма, производство и приготовление препаративной формы общеукрепляющего биологического средства FZB24® - *Bacillus subtilis*

Штамм FZB24® бактерии *Bacillus subtilis* является результатом многолетних исследовательских работ, в ходе которых проводилась селекция различных штаммов бактерии в аспекте эффективности и возможностей репродукции. Исходя из условий содер-

жания штамма, обеспечивающих его неизменную идентичность, FZB24® производится по многоступенчатой жидкостной ферментативной технологии. Образовавшиеся споры отделяют путем сепарации от культуральной питательной среды, а затем сушат вместе с защитными коллоидами, инертным материалом и другими добавками и приготавливают препаративную форму. Срок хранения конечного продукта в препаративной форме составляет не менее 2 лет. В Германии препарат FZB24® - *Bacillus subtilis* зарегистрирован как общеукрепляющее средство для растений, его регистрация в других странах находится в стадии подготовки.

6 References

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personal communication

7 Appendix

Table 1: Biochemical properties of FZB24® *Bacillus subtilis* determined on the basis of various identification cards of the Vitek® *in-vitro* diagnosis system. Positive reaction denotes tolerance to or transformation of the test substance. For details see instructions for use (anonymous 1996).

Reaction pattern on BACILLUS identification card			
Sucrose	+	Trehalose	+
Tetrazolium Red	-	Palatinose	+/-
Tagatose	-	Sorbitol	+
Glucose	+	N-Acetyl-D-glucosamine	+
Inositol	+	Amylopectin	+
Galactose	+/-	Potassium thiocyanate	+
Arabinose	+/-	7% NaCl	+
Xylose	+/-	Mandelic acid	+
Mannitol	+	Oleandomycin	-
Raffinose	+/-	Sodium acetate	-
Salicin	+	Arabitol	-
Amygdalin	+/-	Polyamidohydrostreptin	+
Inulin	+	Nalidixic acid	-
Ribose	+	Aesculin	+
Maltose	+		
Additional reaction pattern on the GRAM-POSITIVE identification card			
Peptone	+	Raffinose	-
Bacitracin	+	Salicin	+
Ethylhydrocupreine hydrochloride	+	Sorbitol	+
Hemicellulase	+/-	Sucrose	+
6% NaCl	+	Trehalose	+
10% bile	+	Arabinose	-
40% bile	-	Pyruvic acid	-
Aesculin	+	Pullulan	-
Arginine monohydrochloride	-	Inulin	-
Urea	-	Melibiose	-
2,3,5-Triphenyltetrazolium chloride	-	Melezitose	-
Novobiocin sodium salt	-	Cellobiose	+
Dextrose	+	Ribose	-
Lactose	-	Xylose	-
Mannitol	+	Catalase	-

Table 2: Influence of the residual moisture-content of the product on the storage stability of *Bacillus subtilis*, illustrated by the results for three trial batches with swellable starch as carrier material

Moisture-content of the product	5%	11%	18%
Content of viable spores after storage for 6 weeks at 54°C (5% sample = 100%)	100%	62%	29%

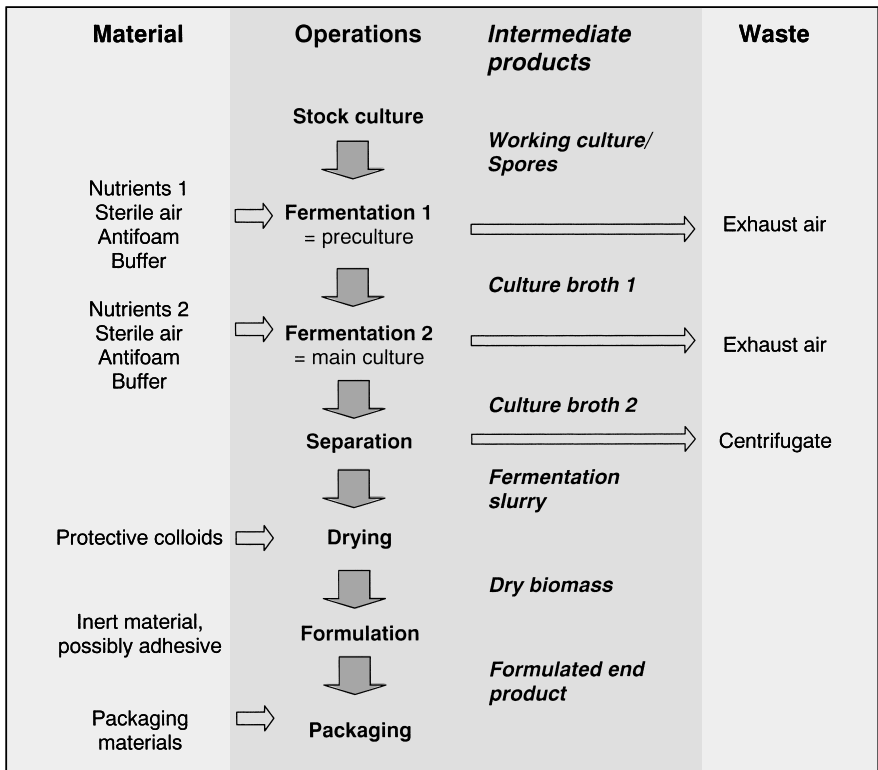


Fig. 3: Scheme of the FZB24® *Bacillus subtilis* fermentation process

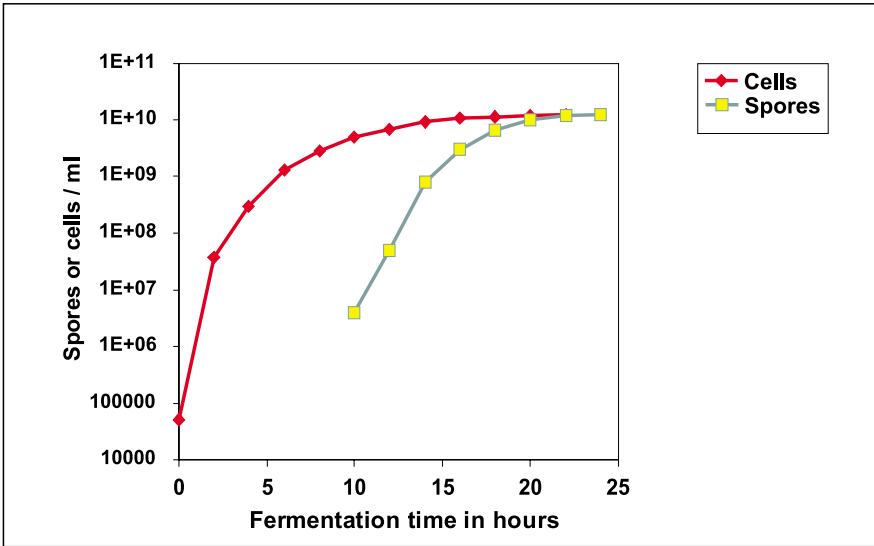


Fig. 4: Typical development of the numbers of vegetative cells and spores of FZB24® during the fermentation process

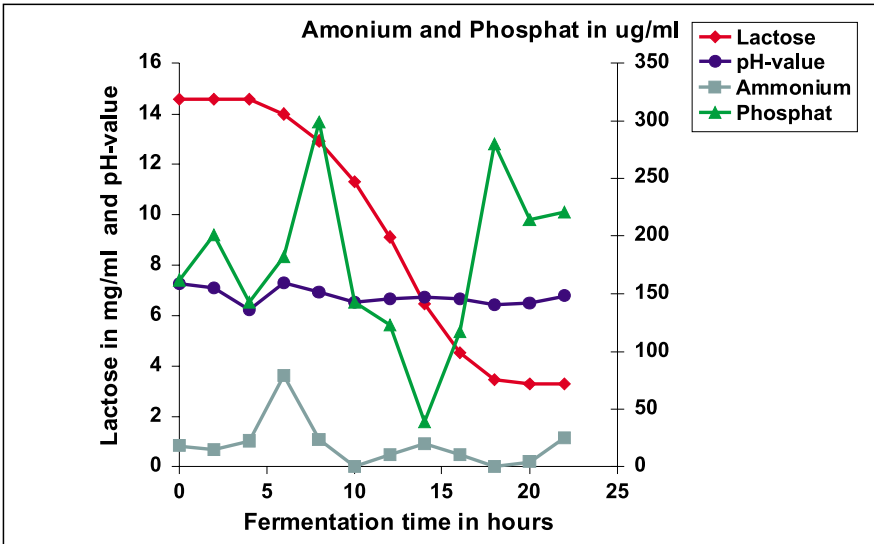


Fig. 5: Typical development of the pH value, lactose, ammonium and phosphate-content of the fermentation broth during the fermentation process of FZB24®

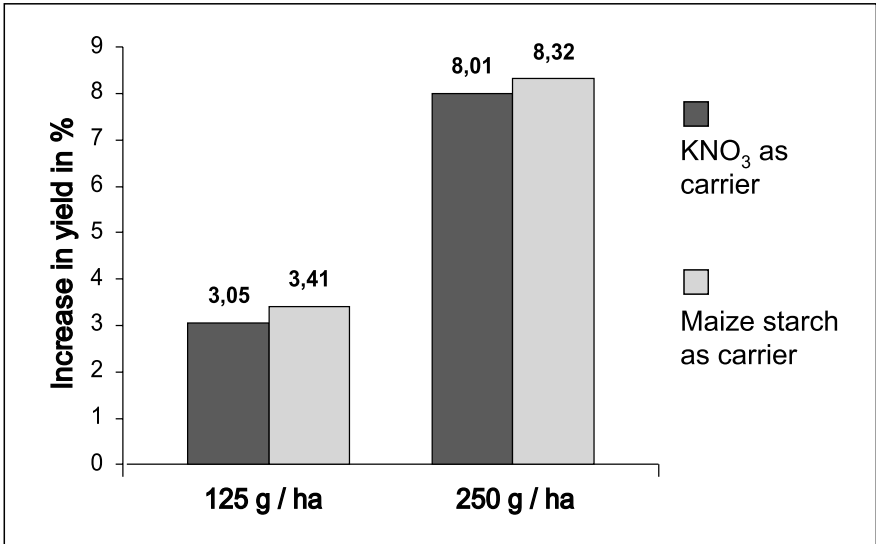


Fig. 6: Influence of the two carrier materials of the FZB24® formulations on the yield of potatoes after seed tuber treatment (Trials University of Bonn, Dr. U. Steiner 1996)

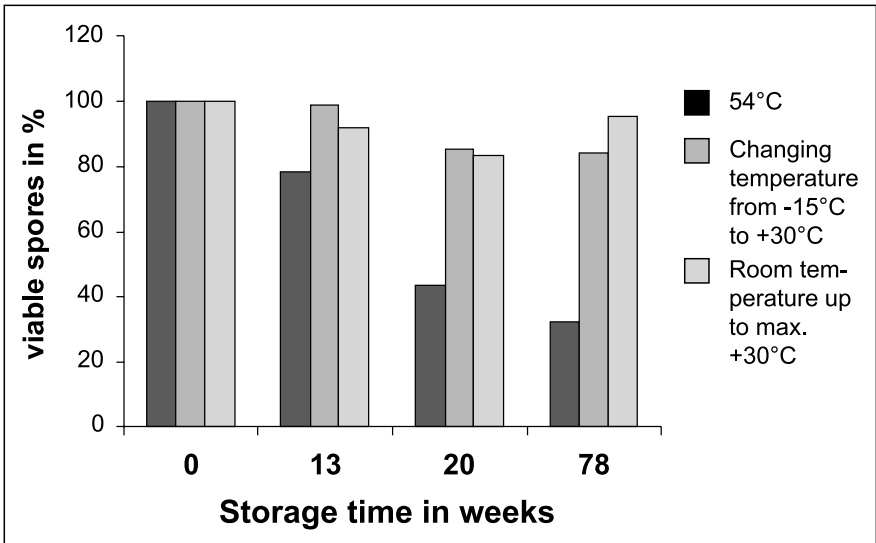


Fig. 7: Storage stability of FZB24® under different temperature conditions

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Dr. Helmut Junge
Dr. Birgit Krebs
FZB Biotechnik GmbH
Glienicke Weg 185
D-12489 Berlin
Tel.: 030-67 057 0
Fax: 030-67 057 233
email: dr.junge.fzb@t-online.de

Dr. Michael Kilian
Bayer AG
Landwirtschaftszentrum Monheim
Geschäftsbereich Pflanzenschutz/
Forschung
D-51368 Leverkusen
Tel.: 02173-383210 (Dr.Kilian)
email: michael.kilian.mk@bayer-ag.de