REVIEW / SYNTHÈSE

Developments in the use of *Bacillus* species for industrial production

Marcus Schallmey, Ajay Singh, and Owen P. Ward

Abstract: *Bacillus* species continue to be dominant bacterial workhorses in microbial fermentations. *Bacillus subtilis* (natto) is the key microbial participant in the ongoing production of the soya-based traditional natto fermentation, and some *Bacillus* species are on the Food and Drug Administration's GRAS (generally regarded as safe) list. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability of different species to ferment in the acid, neutral, and alkaline pH ranges, combined with the presence of thermophiles in the genus, has lead to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address a variety of specific applications. Classical mutation and (or) selection techniques, together with advanced cloning and protein engineering strategies, have been exploited to develop these products. Efforts to produce and secrete high yields of foreign recombinant proteins in *Bacillus* hosts initially appeared to be hampered by the degradation of the products by the host proteases. Recent studies have revealed that the slow folding of heterologous proteins at the membrane – cell wall interface of Gram-positive bacteria renders them vulnerable to attack by wall-associated proteases. In addition, the presence of thiol-disulphide oxidoreductases in *B. subtilis* may be beneficial in the secretion of disulphide-bond-containing proteins. Such developments from our understanding of the complex protein translocation machinery of Gram-positive bacteria should allow the resolution of current secretion challenges and make *Bacillus* species preeminent hosts for heterologous protein production. *Bacillus* strains have also been developed and engineered as industrial producers of nucleotides, the vitamin riboflavin, the flavor agent ribose, and the supplement poly-γ-glutamic acid. With the recent characterization of the genome of *B. subtilis* 168 and of some related strains, *Bacillus* species are poised to become the preferred hosts for the production of many new and improved products as we move through the genomic and proteomic era.

Key words: Bacillus, fermentation, enzymes, insecticides, vitamins, antibiotics, D-ribose.

Résumé : Les espèces de *Bacillus* sont encore et toujours les usines bactériennes dominantes dans les fermentations microbiennes. *Bacillus subtilis* (natto) est un participant microbien clé de la production actuelle de la fermentation traditionnelle du soya et certaines espèces de *Bacillus* sont sur la liste 'GRAS' (considéré généralement comme sûr) de la FDA. La capacité de souches sélectionnées de *Bacillus* à produire et sécréter de grandes quantités (20–25 g/L) d'enzymes extracellulaires l'a positionnée comme l'un des plus importants producteur d'enzymes industriels. L'aptitude qu'ont différentes espèces à fermenter dans les zones de pH acide, neutre et alcalin, de concert avec la présence de thermophiles chez ce genre, ont mené à la conception d'une foule de nouveau produits enzymatiques commerciaux ayant l'activité à température et à pH désirés de même que des propriétés de stabilité qui leurs permettent de se prêter à une variété d'usages spécifiques. Les techniques classiques de mutation et (ou) sélection, en conjonction avec des stratégies de pointe en clonage et en génie des protéines, ont été exploitées afin de développer ces produits. Les efforts initiaux dans le but de produire et de sécréter à haut rendement des protéines recombinantes étrangères chez des hôtes *Bacillus* ont été freinés par la dégradation des produits par les protéases de l'hôte. Des études récentes ont révélé que le lent repliement de protéines hétérologues à l'interface membrane-paroi cellulaire des bactéries Gram-positives les rend vulnérables à une attaque par les protéases associées à la paroi. De plus, la présence de thiol-disulfure oxydoréductases chez *B. subtilis* pourrait favoriser la sécrétion de protéines contenants des ponts disulfure. De tels progrès dans notre compréhension de la machinerie complexe de translocation des protéines des bactéries Gram-positives devrait nous permettre de relever les défis actuels en rapport à la sécrétion et de faire des espèces de *Bacillus* des hôtes de premier plan dans la production de protéines hétérologues. Des souches de *Bacillus* ont également été

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conçues et façonnées pour devenir des producteurs industriels de nucléotides, de la vitamine riboflavine, de l'agent aromatique ribose et du supplément acide poly-γ-glutamique. Avec la caractérisation récente du génome de *B. subtilis* 168 et de certaines souches apparentées, les espèces de *Bacillus* sont destinées à devenir les hôtes privilégiés pour la production de plusieurs produits nouveaux ou améliorés, au fur et à mesure que nous progresserons au travers de l'ère de la génomique et de la protéomique.

Mots clés : Bacillus, fermentation, enzymes, insecticides, vitamines, antibiotiques, D-ribose.

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Introduction

Bacillus species have been major workhorse industrial microorganisms with roles in applied microbiology, which date back more than a thousand years, since the production of natto by solid-state fermentation of soybeans using *Bacillus subtilis* (natto) was first practiced in Japan (Hara and Ueda 1982). These roles have continually expanded and evolved over the past century. The development of strains and production strategies has recently been influenced or facilitated by the application of molecular biology techniques to strain development. *Bacillus* species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *B. subtilis* and *Bacillus licheniformis*.

In addition, much is now known about the biochemistry, physiology, and genetics of *B. subtilis* and other species, which facilitates further development and greater exploitation of these organisms in industrial processes. The complete genome for *B. subtilis* 168 was recently published, and genomes for a number of other species are at an advanced state of characterization. Advances in our knowledge and interpretation of *Bacillus* genomics will open doors to the further development of products or processes as the genomic information is interpreted and exploited.

Recent applications of phylogenetic analysis, DNA–DNA hybridization, and other molecular techniques to bacterial classification are leading to the reclassification of a range of *Bacillus* species and to the creation of new genera. For example, the former *Bacillus stearothermophilus* and *Bacillus brevis* have been reclassified as *Geobacillus stearothermophilus* and *Brevibacillus brevis*, respectively. We have, nevertheless, included strains reclassified in this way in this review, and the subject of reclassification of the genus *Bacillus* is beyond the scope of this review.

Genomic analysis

Bacillus subtilis and related *Bacillus* strains continue to be the dominant enzyme-producing microorganisms in applied and industrial microbiology. These organisms are an important source of industrial extracellular enzymes, including proteases and amylases, and the biochemistry and physiology of these strains have been investigated in detail. Because of the proven capability of these species to produce and secrete gram quantities per litre of these and other enzymes, they have been considered as potential candidate hosts for the production of heterologous proteins. Consequently, among Gram-positive bacteria, *B. subtilis* was selected as a priority organism for genomic characterization. The complete genome sequence of *B. subtilis* 168 is now published, comprising 4100 protein-coding genes (Kunst et al. 1997). More recently, the genome sequences of *Bacillus cereus* ATCC 14579 and *Bacillus anthracis* A202 were published and the sequence of *Bacillus thuringiensis* subsp. *israelensis* is in press (Ivanova et al. 2003).

Of particular relevance to the high capacity of *B. subtilis* and several relatives, especially *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, to secrete extracellular proteins was the identification of several genes encoding proteins of the major secretion pathway: five type I signal peptidase genes and a type II signal peptidase gene, needed for processing lipid-modified precursors, have been found (Tjalsma 1997).

The sequence confirms the previously characterized intermediary metabolism present in *B. subtilis*, consisting of the Embden–Meyerhof–Parnas (EMP) pathway and the tricarboxylic acid cycle as well as the participation of branched short-chain carboxylic acids in lipid anabolism (Suutari and Laasko 1992). The genomic information related to these pathways will provide the tools to facilitate reconstruction of these pathways and to predict patterns of gene expression. Some of the genes of industrial interest that are responsible for the production of carbohydrate-, lipid-, and protein-degrading enzymes, together with some of the genes having extracellular protein secretory functions, are listed in Table 1.

The analysis of the genome of an alkaliphilic *Bacillus* species *Bacillus halodurans*, assisted by the genomic information from *B. subtilis*, also provided interesting and pertinent information (Takami and Horokoshi 2000). This strain was first reported as a producer of β-galactosidase (Ikura and Horokoshi 1979) and xylanase (Honda et al. 1985). The rationale for investigating this strain relates to the fact that many commercially useful industrial enzymes are produced from alkaliphilic *Bacillus* strains, and *B. halodurans* has already been well characterized physiologically, biochemically, and genetically.

While the completion of these genomes is recognized as a major achievement, characterizing the more variable elements of cell make-up, such as the transcriptome (RNA content) and the proteome (reflecting post translational modifications and protein stability), and identifying the nature and role of uncharacterized proteins represent much greater challenges (Harwood et al. 2002).

Traditional *Bacillus* fermentation

As with other traditional fermented foods that utilize other

Table 1. *Bacillus* spp. genes of industrial interest.

Gene	Position on chromosome	Enzyme
abfA	2939	α-L-Arabinofuranosidase
abnA	2949	Arabinan-endo 1,5-L-arabinase
bglC	1940	Endo-1,4-β-glucanase
bglH	4023	β-Glucosidase
bglS	4011	Endo-1,3- and -1,4-β-glucanase
csn	2748	Chitosanase
lacA	3504	β-Galactosidase
sacB	3535	Levansucrase
sacC	2759	Levanase
pel	828	Pectate lyase
pelB	2034	Pectate lyase
yhlE	1095	Glucanase
yjeA	1281	Endo-1,4-β-xylanase
ykfC	1367	Polysugar-degrading enzyme
ykvQ	1445	Chitinase
ynfF	1943	Endo-xylanase
yrhO	2768	Cyclodextrin metabolism
yvdF	3557	Glucan 1,4-α-maltohydrolase
yvfO	3502	Arabinogalactan endo-1,4-β-galactosidase
yveB	3537	Levanase
yvpA	3590	Pectate lyase
yxiA	4040	Arabinan endo-1,5-α-L-arabinosidase
nprE	1541	Extracellular neutral metalloprotease
aprE	1105	Extracellular alkaline serine protease
epr	3939	Minor extracellular serine protease
pepT	3994	Peptidase T
vpr	3907	Minor extracellular serine protease
yrrN	2794	Protease
yrrO	2793	Protease
lipA	292	Lipase
lipB	293	Lipase
csaA	2079	Chaperonin, involved in protein secretion
lsp	1616	Signal peptidase II
prsA	1071	Protein secretion
sipS	2432	Signal peptidase I
sipT	1511	Signal peptidase I
sipU	454	Signal peptidase I
sipV	1122	Signal peptidase I
sipW	2554	Signal peptidase I
yaaT	42	Signal peptidase II

microorganisms, food fermentations mediated by *Bacillus*, can provide insight into some of the potential industrial properties of the genera or species involved. Hence, a brief consideration of Natto production follows. *Bacillus subtilis* (natto) is used in Japan for producing the fermented soybean food, natto (Ueda et al. 1989). Itihiki-natto is an aged product of the *Bacillus* fermentation of steamed soybean. Similar products are produced in Thailand and China. Natto has a characteristic stringy consistency, due to a slime consisting of γ-D-polyglutamic acid (γ-PGA) and a levan-type fructan, and is produced from soybean by *B. subtilis* (natto). The stringy characteristic of the fermented mixture becomes viscous when mixed with sucrose. While *B. subtilis* (natto) produces many enzymes, including amylases and cellulases, the most important enzymes in the production of natto are proteases; two proteases having a pH optima of 8.5 and 10.3–10.8 have been characterized (Yoshimoto et al. 1971). The proteases are responsible for the main flavor, through hydrolysis of soybean protein. Natto or the participating culture, *B. subtilis* (natto), is reported to have a number of beneficial health effects (Ueda 1989). Natto contains antibiotic properties that are reported to be due to a heat unstable bacterial lytic activity and to dipicolinic acid. The consumption of natto during lactation was reported to increase vitamin K in breast milk. *Bacillus subtilis* (natto) cultures have been reported to eliminate pathogenic bacteria from the intestinal canal, and fractions from extracts of natto exhibited anticancer activity and reduced blood pressure in specific laboratory trials.

Industrial enzymes production

Bacillus enzymes

The world market for industrial enzymes is estimated to be 1.6 billion $US, split between food enzymes (29%), feed enzymes (15%), and general technical enzymes (56%) (Outtrup and Jorgensen 2002). The main applications are summarized in Table 2. Most detergent proteases currently used in the market are *Bacillus* spp. serine proteases (Rao et al. 1998). It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market. There are three dominant enzyme suppliers, Novo Nordisk, Genencor International, and DSN N.V., having reported market shares of 41%–44%, 21%, and 8%, respectively, with smaller producers in North America, Europe, Japan, and China making up the remaining 27%–30%.

The alkaline serine proteases (subtilisins) are produced by various *Bacillus* species with a primary application in household detergents, for example Alcalase produced by Novo Nordisk. *Bacillus licheniformis*, *Bacillus pumilus*, and *B. subtilis* produce the subtilisin Carlsberg-type enzyme (Zukowski 1989). Its annual production amounts to about 500 metric tonnes of pure enzyme protein (Rao et al. 1989). Alkalophilic species produce enzymes with greater alkaline tolerance, used in heavy-duty detergent formulations (e.g., esperase from Novo Nordisk). Neutral *Bacillus* spp. proteases are zinc metalloproteinases, with pH optima around 7, which are used in milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing, soy modification for use as flavors, and in animal feeds (Ward 1991).

Bacillus spp. amylases have application in a number of industrial processes, such as the food, fermentation, textile, and paper industries (Pandey et al. 2000). α-Amylase from *Bacillus* spp. cleaves internal α-1,4-linkages in an endo fashion. The extraordinary ability of the α-amylase from *B. licheniformis* to operate at 95 °C and withstand temperatures of 105–110 °C for short periods makes this a unique industrial enzyme for liquefying starches, especially cornstarch, which only gelatinizes at 100 °C (Ward 1991). Amylases from *B. amyloliquefaciens*, now known to be a strain of *B. halodurans* (Martinez et al. 2002), also exhibit significant thermostability and are useful in saccharification processes (Breccia et al. 1998). β-Amylases from *Bacillus* species operate to remove maltose units in an exo fashion from starch but cannot bypass the α-1,6-glycosidic linkages. β-Amylases from *B. cereus*, *Bacillus megaterium*, and *B. subtilis* have

Table 2. Industrial application of *Bacillus* spp. enzymes.

Industry (%)*	Major *Bacillus* spp. enzymes	*Bacillus* spp.	Comments	Other enzymes
Detergent (37)	Alkaline proteases	*B. clausii*, *B. amyloliquefaciens*, *B. halodurans*	*Bacillus* proteases dominates the market, particularly the highly alkali stable enzyme from *B. clausii*	Fungal lipases and cellulases
	Alkaline amylase	*B. licheniformis*, *B. halmapalus*	Removal of starch stains	
Starch (13)	α-Amylase	*B. licheniformis*,		Fungal glucoamylase
	Pullulanase	*G. stearothermophilus*†		Glucose isomerase from *Streptomyces*
	Glucose isomerase	*B. acidipullulyticus*, *B. deramificans*		
Textile (6)	Amylase	*B. halodurans*	Desizing	Fungal cellulases
	Pectate lyases, alkaline amylase, mannanase	*Bacillus* sp.	Dehairing hides, stain remover	Oxidases
Dairy (14)	None	Calf and (or) fungal rennets		
Baking (9)	Amylase			
Beverage (6)	Amylase	*B. licheniformis*, *G. stearothermophilus*†, *B. amyloliquefaciens*		Most enzymes fungal
	β-glucanase	*B. subtilis*		

*Animal feed enzymes account for 8% and miscellaneous enzymes 7%.
†*Geobacillus stearothermophilus*, formerly *B. stearothermophilus*.

been available commercially (Fogarty and Kelly 1980; Nigam and Singh 1995; Denner 1996; Pandey et al. 2000). Their moderate thermostabilities have limited their use (Priest 1984).

Bacillus spp. pullulanases hydrolyse the α-1,6-linkages in starch. Glucoamylase can also attack this bond but at a much slower rate. Neopullulanase from *Geobacillus* (formerly *Bacillus) stearothermophillus* TRS40 completely hydrolyzes amylose to produce maltose as the main product (Kamasaka et al. 2002). Cyclomaltodextrin glucanotransferase (CGTase) hydrolyses starch to a series of nonreducing cyclic D-glucosyl polymers called cyclodextrins (Fogarty and Kelly 1980). The α-, β-, and γ-cyclodextrins, with 6, 7, and 8 glucose molecules, respectively, are stable to oxygen, light, and heat but instantly dissolve in the mouth to release flavors (Ostergaard 1982). Industrial production involves use of the corresponding α, β, and γ-CGTases. The α-type enzyme from *Bacillus macerans* has been cloned and sequenced (Takano et al. 1986), and genes from other *Bacillus* species have been isolated and cloned (Schmid 1989).

Glucose isomerase is an important enzyme in the final stages of starch processing to sweeteners, namely the conversion of glucose syrups to high fructose corn syrups (Ward 1989). The enzyme from *Bacillus coagulans* has been used commercially because it has pH and temperature optima (pH 7–9 and 55–65 °C) that are compatible with other elements in the starch hydrolysis process and because it is the only *Bacillus* spp. glucose isomerase in a market dominated by *Streptomyces* spp. enzymes (Zukowski 1989).

Bacillus spp. β-glucanase is used in the enzymatic modification of barley β-1,3/β-1,4-glucan to reduce mash viscosity and aids filtration and yield of wort. The *B. subtilis* enzyme is available commercially, and hybrid enzymes have been developed from the more thermostable *B. macerans* enzyme and the *B. amyloliquefaciens* enzyme, which is similar to the *B. subtilis* enzyme (Borriss et al. 1988, 1989). Thermostable endo-1,5-α-L-arabinase activity from *Geobacillus (*formerly *Bacillus) thermodenitrificans* is suitable for L-arabinose production from sugar beet pulp (Takao et al. 2002).

There are several other enzymes of industrial importance produced by different species of *Bacillus*. Levansucrases from *Bacillus circulans* (Oseguera et al. 1996); cellulases from *B. subtilis* (Singh and Hayashi 1995); xylanases from *B. subtilis*, *B. coagulans*, and *B. circulans* (Kuhad et al. 1997; Bajpai 1997); CGTase from *Bacillus firmus* (Gawande et al. 1999); esterases from *B. circulans* (Kademi et al. 2000); chitinases from *B. thuringiensis* (Thamthiankul et al. 2001); tannase from *B. licheniformis* (Mondal et al. 2000); and restriction endonucleases from various species (Maruro and Yoshikawa 1989; Puchkova et al. 2002), are the examples of important industrial enzymes from *Bacillus* spp. origin.

Importance of alkaliphilic *Bacillus* species and their enzymes

Most alkaliphile bacilli produce various alkaline enzymes, including proteases, amylases, xylanases, pullulanases, and cellulases. Properties of these enzymes from some of the most important alkaliphilic *Bacillus* spp. enzyme producers are presented in Table 3.

Numerous species of *Bacillus* have been explored for alkaline protease production, but most potential alkaline protease producers are strains of *B. amyloliquefaciens*, *B. licheniformis*, *B. mojavensis*, and *B. subtilis* (Rao et al. 1998). The proteases from these strains, having pH optima in the range 9–12 and good thermostability, have found commercial applications in detergents, in abating and (or) dehairing of leather (Gupta et al. 2002), and in the recovery

of silver from X-ray films (Fujiwara et al. 1991). Trypsin is currently used in combination with *Bacillus* spp. and *Aspergillus* spp. proteases in the leather industry.

The starch-degrading enzymes produced by *Bacillus* spp. include alkaline α-amylases and CGTases. α-Amylases are stable in the presence of EDTA and saccharify the majority of starch to mono-, di-, tri-, and tetra-saccharides. The CGTases produce various cyclodextrins with applications in foodstuffs, chemicals, and pharmaceuticals. Alkaline pullulanase has applications in dishwasher detergents for the removal of starches.

The alkaline xylanase has applications in food processing because of its broad pH range for activity, high temperature optima, and thermostability. The alkaline cellulases also exhibit excellent thermostability and have applications in detergents. Some are resistant to detergent components, including proteases, metal ions, chelating agents, and surfactants. The enzyme from *Bacillus* strain KSM-635 is produced constitutively, and a variant of this strain exhibited extraordinary productivity, producing 20–25 g/L of this enzyme (Itoh et al. 1989, 1991).

Engineered genes and enzymes

Knowledge of the crystalline structures of commercial enzymes, availability of cloned genes, and use of random or site-directed mutagenesis have facilitated the production of enzymes with altered pH-activity profiles, substrate specificity, enhanced stability, greater metal binding capacity, and resistance to oxidizing agents (Estell et al. 1985, 1986; Russell and Fersht 1987; Russell et al. 1987; Takagi et al. 1989*b*; Singh 1999).

The development of the engineered enzyme, Termamyl LC, by Novo provides an interesting example in starch hydrolysis processes. The *B. amyloliquefaciens* α-amylase has high specific activity and a desirable starch hydrolysis profile but has low thermostability. The *B. licheniformis* enzyme has good thermostability but low specific activity. Calcium is needed for amylase stabilization but should be minimized, as it inhibits subsequent enzymatic glucose isomerization. A hybrid enzyme was developed by combining the *B. licheniformis* and *B. amyloliquefaciens* enzymes to achieve a desirable pattern of starch hydrolysis, high specific activity, and thermostability at low calcium levels.

Commercial protease variants of the *Bacillus clausii* enzyme have been developed through protein engineering for the detergent market in response to the need for enzymes with improved performance, for example, in low temperature washes, in soft water, and in bleach-containing detergents. Protein-engineered variants of the *B. liquefaciens* and *B. halodurans* α-amylases were commercialized with improved alkali tolerance. Recently a gene encoding exoinulinase (*inu*) from *Bacillus polymyxa* was cloned and expressed in *Escherichia coli* (Kwon et al. 2003). The enzyme hydrolyzed sucrose, levan, and raffinose, in addition to inulin.

The gene *lipA* coding for lipase A from *B. subtilis* was isolated by PCR amplification and expressed in *E. coli*, *Saccharomyces cerevisiae*, and *B. subtilis*, using pBR322-, Yeplac111-, and pUB110-derived vectors, respectively (Sanchez et al. 2002). An increase in the lipase activity in the heterologous host indicated that the described systems could represent a useful tool to enhance enzyme productivity for biotechnological applications.

Tye et al. (2002) described molecular cloning and biochemical characterization of two novel phytases from *B. subtilis* and *B. licheniformis*. The phytases exhibited broad temperature and pH optima and good temperature stabilities, suggesting their potential use in animal feed applications.

Often where a mixture of enzymes, having different pH optima, is used in a single-stage process, operating pH is adjusted to a suboptimal point between the two pH optima. For example, a combination of glucoamylase (pH optimum 4.0) and pullulanase (pH optimum 5.0) are used in starch saccharification. Protein engineering programs are in progress to converge the pH optima of the two enzymes.

Gene technology, involving the use of the genus *Bacillus* as host or a source of DNA, has been widely used to develop new commercial enzyme-producing hosts. Genetic strategies have been used to improve and (or) optimize the quantity and properties of the desired enzyme product and to delete genes encoding for undesired enzyme activities. The overexpression of poly(3-hydroxybutyrate)depolymerase from *Paucimonas lemoignei* was found to be more efficient in *B. subtilis* than in *E. coli* (Braaz et al. 2002).

Strategies for the development of recombinant *Bacillus* strains for commercial enzyme production

The discovery of Spizizen in 1958 of *B. subtilis* transformation was a significant milestone in the development of modern molecular biology. Engineered plasmids, derived from natural plasmids of the same or closely related organisms, could be introduced into a host strain by competent cell transformation, protoplast fusion, and electroporation (Ferrari and Hoch 1989). Plasmids could be replicated, inherited, and transcribed into host strains. However, because of recombination occurring over many generation cycles during fermentation processes with *Bacillus* species, segregation and replication events of modified plasmids were problematic with the frequent deletion of plasmid sequences. As a result, mutant strains lacking the relevant recombination enzymes were developed, and this dramatically improved recombinant strain stability (Rao et al. 1998).

Few systems have been developed so far for controlled high level expression of *B. subtilis* genes (Harwood et al. 2002). Native promoters, such as that for the α-amylase gene, capable of directing synthesis of around 20 g/L of extracellular proteins, are most often used in industrial microbiology.

The insertion of DNA directly into the chromosome, by design of constructs flanked by sequences homologous to the chromosomal genes, produced more stable clones (Mountain 1989). Now with the thorough knowledge of genomic nucleotide sequence of *Bacillus* species, further modifications are possible, producing strains with specific desired traits.

While cloning of genes, by encoding novel enzymes on plasmids into *B. subtilis* hosts using antibiotic resistance approaches to plasmid maintenance, is effective for laboratory studies, incorporating antibiotics into large-scale production fermentations is not acceptable. Diderichsen (1986) used a different approach that utilized the *dal* gene as a selectable

Table 3. Alkaliphilic enzymes from *Bacillus* spp.

Bacillus strain	pH optimum	Stability/other	Other comments	Reference
Protease				
B. clausii				
221	11.5	Retained 75% activity at pH 12	Temp. optimum 60 °C	Horikoshi 1971
AB42	9–12		Temp. optimum 60 °C	Aunstrup et al. 1972
PB12	9–12		Temp. optimum 50 °C	Aunstrup et al. 1972
YaB	11		High elastase activity	Tsai et al. 1983, 1986
NKS21	10–12			Tsuchida et al. 1986
B21	10–12		Used in recovery of silver from X-ray films	Fujiwara and Yamamoto 1987; Fujiwara et al. 1991; Ishikawa et al. 1993
AH101, closely related to *B. halodurans*	12–13	10 min at 60 °C in pH range 5–12	High elastase and keratinolytic activity; digested human hair and nail at pH 11–13	Takami et al. 1992*a*, 1992*b*, 1999
KSM-K16	10.6		Detergent	Kobayashi et al. 1995
Starch-degrading enzyme				
B. pseudofirmus				
A-40-2 (α-amylase)	10–10.5	More stable in presence of EDTA than are *B. subtilis* and *B. licheniformis* enzymes	Saccharifies 70% of starch to glucose, maltose, and maltotriose	Horikoshi 1971
NRRL B-3881 (α-amylase)	9.2	More stable in presence of EDTA than are corresponding *B. subtilis* and *B. licheniformis* enzymes	Produces maltose, maltotriose, small amount of glucose and maltotetraose	Boyer and Ingle 1972; Boyer et al. 1973
38-2 (CGTase)	8.5	Cyclomaltodextrin glucanotransferase	Converts starch to cyclodextrins	Nakamura and Horikoshi 1976
17-1 (CGTase)			Along with 38-2 are best producers of CGTase among 1000 strains	Nakamura et al. 1991
3-22 (psychrophilic) (CGTase)			Produces predominantly β-cyclodextrin (yield from amylose 85%–90%, from potato starch 70%–80%); cyclodextrin product used in foods, chemicals, and pharmaceuticals	Georganta et al. 1993
Pullulanase				
B. halodurans				
202-1	8.5–9.0	Stable at pH 6.5–11 at 4 °C for 24, at 50 °C for 15 min; optimum temp. 55 °C		Nakamura et al. 1991
KSM 1876	10–10.5		Highest pullulanase, used in detergents	Ara et al. 1992
Xylanase				
B. halodurans				
c-125 (Xylanase A)	6–10	Some activity at pH 12	Food processing	Honda et al. 1985
W2 and W4	6–7	Stable pH 4.5–10.5 for 1 h at 45 °C; optimum temp. 70 °C	Degree of xylan hydrolysis 70% after 24 h	Okazaki et al. 1984
KCIM 59				Dey et al. 1992

Table 3 (*concluded*).

Bacillus strain	pH optimum	Stability/other	Other comments	Reference
*G. stearothermophilus**				
T-6				Khasin et al. 1993
TAR-1				Nakamura et al. 1994
Cellulase				
B. subtilis				
N–4	E1,E2 pH 10	2 CMCases, active over pH range 5–10, stability Enzyme 1 to 60 °C; Enzyme 2 to 80 °C		Horikoshi et al. 1984
1139	9	Stable at pH 6–11, at 4 °C for 24 h, and at 40 °C for 10 min		Fukumori et al. 1985; Crickmore et al. 1998
B. subtilis (chimeric cellulases)				
N-4			pH optimum depended on sequence at C-terminal	Nakamura et al. 1991
NK1	6–10.5		Substrate-dependent pH optimum	Takami and Horokoshi 2000
KSM-635		Metal ions, chelate, protease surfactants compatible	Enzyme constitutively produced, laundry detergent enzyme	Joyet et al. 1986; Itoh et al. 1991
Variant of KSM-635			Enzyme production 20–25 g/L	Declerck et al. 1988; Itoh 1997
Pectinase				
B. halodurans C-125	9	Optimum temp. 50 °C		Takami and Horokoshi 2000

Note: CGTase, cyclomaltodextrin glucanotransferase; CMCase, carboxymethyl cellulase.
*Formerly *B. stearothermophilus*.

marker for plasmid maintenance. The *dal*-bearing plasmid was introduced into a dal$^-$ mutant, which cannot produce the D,L-alanine racemase. The D,L racemase is required to produces the D-alanine component of the cell wall, and hence, the genetic constructs in these hosts keep selective pressure on maintenance of the plasmid. Chromosomal integration of genes expressing novel enzymes can also be used to generate stable high-yielding *B. subtilis* production strains. Strategies involving amplification of gene copy number have resulted in high enzyme-producing strains sufficiently stable to use in large-scale fermentations (Gupta et al. 2002).

In contrast to *B. subtilis*, effective introduction of new DNA, effective manipulation of chromosome, and achievement of high transformation frequencies have proven much more challenging in other *Bacillus* hosts, such as *B. licheniformis* and *B. clausii*. Consequently, alternative procedures, such as electroporation, transformation, protoplast regeneration, and conjugation, are used for these strains (Jeong et al. 1998; Harwood et al. 2002). The hosts, *B. licheniformis* and *B. clausii*, are extremely important for commercial processes, as they frequently exhibit higher enzyme production yields than *B. subtilis*. In these strains, high enzyme production often requires a combination of well-functioning expression systems from classical high producing strains and multiple copies of the gene of interest.

It has been argued (Outtrup and Jorgensen 2002) that the ideal production strains should contain one genetically stable, chromosomally integrated gene with very high levels of expression and product secretion. Strategies towards achieving this goal have shown success in *B. subtilis* and *B. licheniformis* (Jorgensen and Diderichsen 1997; Jorgensen 1999).

Production of heterologous proteins

Bacillus species are great candidates as hosts for the production of heterologous proteins because of their high capacity to secrete proteins into the extracellular medium. In contrast, Gram-negative bacteria accumulate proteins in the protoplasm or periplasmic space, and accumulating products may be toxic to the organism. Intracellular accumulations can lead to problems related to the formation of insoluble protein inclusion bodies, incorrect protein folding, and inefficient disulfide bond formation. Gram-negative bacterial hosts are also often producers of endotoxins, which are toxic to humans, and can be problematic in the production of recombinant proteins as biopharmaceuticals. Intracellular protein production adds to protein recovery and purification costs.

Wang and Doi (1989) described the various genetic signals, required for effective transcription and translation of foreign genes in *B. subtilis*, and the requirements to overcome barriers to gene expression. Compared with *E. coli*, expression of heterologous genes in *B. subtilis* was not as effective because of the lack of some strong, controllable, and well-characterized promoters. The utilization of foreign promoters by the *B. subtilis* RNA-polymerase holoenzyme was found to be generally inefficient, and it was important to replace the foreign gene promoter with naturally expressed

Table 4. Selected heterologous *Bacillus* spp. and human hormone proteins expressed in *Bacillus subtilis*.

Product	Product origin	*B. subtilis* host strain	Construct configuration	Mode of cloning	Cellular location	Reference
α-Amylase	*B. amyloliquefaciens*	BRB1	D	CI	SE	Kallio et al. 1987
α-Amylase	*B. licheniformis*	SO103	D	PL	SE	Ortlepp et al. 1983
α-Amylase	*B. licheniformis*	GP208	F	PL	SE	Sloma et al. 1988
α-Amylase	*B. licheniformis*	QB1098	F	CI	SE	Joyet et al. 1986; Decerck et al. 1988
α-Amylase	*G. stearothermophilus**	TN106	D	PL	SE	Aiba et al. 1983
Alkaline protease	*B. amyloliquefaciens*	BR151	D	PL	SE	Vasantha et al. 1984
Alkaline protease	*B. amyloliquefaciens*	168	D	PL	SE	Wells et al. 1983
Neutral protease	*B. amyloliquefaciens*	BR151	D	PL	SE	Vasantha et al. 1984
Neutral protease (nprT)	*G. stearothermophilus**	MT-2	D	PL	SE	Fugii et al. 1983
Neutral protease (nprM)	*G. stearothermophilus**	MT-2	D	PL	SE	Kubo and Imanaka 1988
Keratinase	*B. licheniformis* PWD-1	FDB-29	D	PL	SE	Wang and Shih 1999
Endoxylanse	*Bacillus* sp.	DB104	D	PL	SE	Jeong et al. 1998
Penicillin G acylase	*B. megaterium*	WB-600	D	PL	SE	Yang et al. 2001
Growth hormone	Human	MT500	F	PL	SE	Wells et al. 1983; Paddon and Hartley 1987
Growth hormone	Human	BG2035	D	PL	IE	Hartley 1988
Interferon-alpha2	Human	IH6140	F	PL	SE	Tsukagoshi et al. 1984; Kreft et al. 1983
Interferon-alpha2	Human	I168	D	PL	IE	Wang and Doi 1989
Interferon-beta	Human	MT400	F	PL	SE	Wells et al. 1983; Paddon and Hartley 1987
Interferon-beta	Human	I168	D	PL	IE	Ortlepp et al. 1983
Interferon-beta	Human	1S53	D	PL	IE	Sloma et al. 1988
Interleukin-1beta	Human	BR151	F	PL	SE	Joyet et al. 1986
Proinsulin	Human	1S53	F	PL	SE	Sloma et al. 1986
Tissue plasminogen activator	Human	DB104	D	PL	IE	Duvall et al. 1983

Note: D, direct; F, fused; CI, chromosomal integration; PL, plasmid vector; IE, intracellular expression; SE, secreted.
**Geobacillus stearothermophilus*, formerly *B. stearothermophilus*.

B. subtilis promoters. In addition, hybrid promoters have been constructed in a manner that enables expression to be controlled in response to defined physiological conditions. Structurally stable expression vectors have also been developed in *B. subtilis* (Janniere et al. 1990; Haima et al. 1990). By introducing regulatory genes on a stepwise basis into the host, productivity can be substantially increased (Yoneda 1980). Standard methods are available for chromosomal integration of foreign DNA and gene amplification and for the production of genetically stable strains.

In general, the main modes of expression that can be achieved in *B. subtilis* can be characterized with respect to the configuration of the gene construct as direct (D) or fused (F), can be characterized with respect to the mode of cloning as plasmid vector (PL) or chromosomal integration (CI), and can be characterized, according to gene-product cellular location as intracellular expression (IE) or secreted (SE). Some examples of enzymes of industrial interest from other *Bacillus* species and some human hormones that have been cloned into *B. subtilis* are illustrated in Table 4.

A major problem in the production of heterologous eukaryotic proteins in *B. subtilis* (and in other commercial enzyme producers, such as *Aspergillus*) is the large number of extracellular proteases, which attack secreted cloned foreign proteins (Doi et al. 1984; Wang et al. 1988). Consequently efforts have been made to produce protease-deficient mutants. A variety of protease-deficient mutants have been successfully constructed, resulting in effective extracellular production of a number of intact heterologous proteins. Concerns regarding the use of extracellular-protease-deficient mutants (i.e., that these strains would not grow and sporulate normally) were not upheld in laboratory tests (Sloma et al. 1988; Wang et al. 1989), but there were reports that their growth was more limited in protein-rich industrial media (Nyberg 1984; Reid et al. 1986).

In Gram-positive bacteria, mature proteins are released into the surrounding medium, following translocation across the membrane and passage through the cell wall. However, heterologous proteins are vulnerable to wall-associated proteases during the slow protein-folding process (Braun et al. 1999). Thus, in addition to trying to eliminate these cell-wall-associated extracellular proteases, efforts are focusing on the engineering of extracellular "foldases" to promote better secretion of heterologous proteins by *Bacillus* species.

In contrast to *B. subtilis*, *B. brevis* produces very low extracellular protease and has exhibited high productivity of

heterologous proteins (Udaka and Yamagata 1993; Kajino et al. 1997). Bacterial and human α-amylase, human epidermal growth factor, and human growth hormone (hGH) have all been produced in this host (Takagi et al. 1989*a*; Yamagata et al. 1989; Konishi et al. 1990). The use of mutants expressing low protease activity, the incorporation of EDTA in the medium, and the manipulation of the signal peptide elevated yields of hGH to 240 mg/L medium (Kajino et al. 1997).

Bacillus insecticides

Mosquito species, like *Culex*, *Aedes*, or *Anopheles*, and blackflies are involved in the dissemination of disease-causing microorganisms. For example, mosquito-dispersed diseases, like malaria or flavivirus-borne encephalitis (dengue fever, yellow fever, etc.), threatens 3 billion people in tropical and subtropical regions (Priest 1992). Mosquito-borne diseases are also becoming more prevalent in moderate climates, since the introduction of West Nile Virus to hibernating mosquitoes of western hemisphere countries, including the U.S. and France since 1999. In 2002, 3475 cases and 201 deaths have been reported in the U.S.A., an increase of over 200% in deadly cases compared with the previous year.

The first discovery of *Bacillus* species with insecticidal potential was made in Japan in 1901 and led to the characterization of *B. thuringiensis* by Berliner in 1911. *Bacillus thuringiensis* can be further divided into over 60 different subspecies like *B. thuringiensis* subsp. *israelenis*, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *darmstadiensis* (Schnepf et al. 1998). Furthermore, variations occur in the δ-endotoxin crystal structure, and more than 260 endotoxin protein sequences are listed on the Cry Nomenclature Web Page (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/index.html).

The global market of bioinsecticides accounts for 100 million US dollars (only 2% of total insecticides sales), but this percentage could increase to 10%–15% (Bishop 2002). The reasons for this are the shorter registration periods, the lack of side effects on humans, and the low incidence of side effects on other organisms. In addition, there is no environmental accumulation as is observed for chemical pesticides (Bishop 2002).

Entamopathogenic *Bacillus* species with current or potential applications come from four species: *B. thuringiensis*, *B. sphaericus*, *B. popilliae*, and *B. lentimorbus* (Phelps and McKillip 2002; Schirmer et al. 2002). The high profile and widely used commercial insecticides are produced by *B. thuringiensis*. The mother cell of *B. thuringiensis* contains large parasporal bodies or crystals (δ-endotoxin or insecticidal crystal proteins) adjacent to the spore (Bishop 2002). All related forms of δ-endotoxins become activated by larval ingestion. *Bacillus thuringiensis* is used as pesticide-like chemical control agents, and because the endotoxin and spores that comprise the insecticide are easily biodegradable, repeated applications are required (Ward 1989). *Bacillus popilliae* is introduced into environments where it is not present to prevent ongoing severe infection by its target insect (Deacon 1983). The advantages of microbial insecticides are their relative safeness compared with chemical agents, their slight ecological impact, and their compatibility with other agents.

Bacillus thuringiensis is a biopesticide that is highly adaptable and its genes have also been cloned into transgenic plants (Sharma and Ortiz 2002; Tabashnik et al. 2002). There is also a potential to use genetic methods to modify protein composition and (or) structure to alter insecticidal host specificity and selective toxicity (Ward 1989; Khanna and Raina 2002). δ-Endotoxins of *B. thuringiensis* are produced as the bacterium enters the sporulation phase and can amount to as much as 30% of the dry weight of the mother cell (Pearson and Ward 1988*a*, 1988*b*; Baum et al. 1999). In addition to having Lepidoptera, Coleoptera, and Diptera as target insects, organisms susceptible to δ-endotoxins are found among protozoa, nematodes, flatworms, and mites (Feitelson et al. 1992).

The δ-endotoxins generally have a molecular mass of 130–140 kDa and contain three functional domains (Groschulski et al. 1995; Ellar 1997; Schnepf et al. 1998). Domain I represents a membrane insertion region forming pores in the epithelia of guts of target organisms. Domain II has receptor binding (initial and (or) reversible) properties and determines target host specificity, while domain III has various functions, including receptor binding (irreversible). δ-Endotoxins are protoxins requiring alkaline solubilization in the insect midgut and proteolytic activation (Carroll et al. 1997).

There are 26 *B. thuringiensis*-based products registered by the United States Environmental Protection Agency (USEPA), including 15 containing naturally occurring species and three strains prepared by conjugative plasmid recombination (Copping and Menn 2000). The most widely used strain is *B. thuringiensis* subsp. *kurstaki* HD-1, with effective δ-endotoxins against more than 100 Lepidopteran species, which contributes to its effectiveness and is therefore of high economical value (Priest 1992). In particular, *B. thuringiensis* subsp. *israelensis* seems to become more and more important because of its toxicity on mosquitoes and blackflies.

Examples of strategies used to prepare recombinant insecticides include the development of improved lepidopteran activity by the transfer of a self-transmissible *cry1A*-containing plasmid from *B. thuringiensis* spp. and the development of dual lepidopteran and (or) coleopteran specificity by the transfer of a *cry3A*-encoding plasmid from subspecies *morrisoni* to subspecies *kurstaki* (Bishop 2002). Production of a *cry1C–cry1Ab* chimeric gene in an asporogenic BT strain has lead to development of an insecticide of improved toxicity towards two lepidopteran insects, *Spodoptera littoralis* and *S. exigua*, that are insensitive to many δ-endotoxins (Sanchis et al. 1999). Yield improvements of *cry3A* by *tenebrioniz*, a biovar of subspecies *morrisoni* arising from the development of abnormally large crystals have been achieved by γ-radiation mutagenesis (Baum and Malvar 1995).

Bacillus antibiotics

The genus *Bacillus* produces various classes of antibiotics, as outlined in Table 5. While several bacitracins have been characterized, bacitracin A is the dominant commercial product. *Bacillus* antibiotics are generally produced at the

Table 5. *Bacillus* spp. antibiotics.

Class	Example	Function	*Bacillus* source	Reference
Cyclic oligopeptides	Bacitracin	Inhibit cell wall synthesis	*B. licheniformis* (Bacitracin A)	Katz and Demain 1977
Linear or cyclic oligopeptides	Gramicidins and tyrocidines	Interfere with membrane function	*Brevibacillus brevis**	Priest 1992
	Polymixins, colistins, circulins		*B. polymyxa*, *B. colistinus*, *B. circulans*	
Basic peptides	Edeines	Inhibit formation of the initiation complex on the small ribosome subunit	*Brevibacillus brevis**	Priest 1992
Aminoglycoside antibiotics	Butirosin complex	Affect ribosome function	*B. circulans*	DeFuria and Claridge 1996

*Formerly *Bacillus brevis*.

early stages of sporulation. Recently Eppelmann et al. (2001) demonstrated the transfer of the bacitracin biosynthetic gene cluster from *B. licheniformis* to the engineered host *B. subtilis* and the biosynthesis of bacitracin in high levels. *Bacillus brevis* produces two peptide antibiotics, tyrocidine and gramicidine, at the onset of the stationary growth phase. The pathways for the synthesis of both peptide antibiotics are well characterized. *Bacillus subtilis* also produces several other antibiotics: subtilin, a 32-residue peptide; bacilysin, a dipeptide; subsporins A–C, lipooligopeptides; and rhizocticins A–D, phosphooligopeptides (Priest 1992; DeFuria and Claridge 1996).

The lipopeptide antibiotic surfactins are synthesized by species of *Bacillus*. *Bacillus subtilis* and *B. licheniformis* are the potential producers of surfactin (Peypoux et al. 1999; Fox and Bala 2000; Moran et al. 2000). Surfactin biosynthesis is a nonribosomal process, catalysed by a four component multienzyme complex, surfactin synthetase. Regulation of surfactin biosynthesis includes quorum-sensing control mechanisms and control of fermentation medium components, such as glucose and glutamine. Peptidic variants of surfactin, lichenysin and pumilacidin, have also been isolated from *B. licheniformis* and *B. pumilus*, respectively. While glucose, sucrose, and fructose are efficient carbon sources for surfactin production by *Bacillus* spp. fermentation, for continuous culture operation, the stimulatory effect of iron and manganese has been identified (Peypoux et al. 1999; Wei and Chu 2002). A fermentation yield of surfactin up to 7 g/L has been reported using *B. subtilis* C9 (Kim et al. 1997). Surfactin has exceptional surfactant activity and emulsification properties, thereby giving it potential applications in bioremediation (Mulligan et al. 2001). In contrast to other biosurfactants, surfactin biosynthesis does not follow stimulation by a petroleum fraction. This lipopetide also has potential as an antitumoral, antiviral, antibacterial, and hypocholesterolemic agent (Fox and Bala 2000).

Semisynthetic antibiotics have become more and more important for the antibiotics industry because of increasing resistance among pathogenic bacteria towards traditional antibiotics, for example, the β-lactams, like penicillin or cephalosporin. By breaking the β-lactam ring, the production of modified antibiotics opens many possibilities to alter the effect on these resistant bacteria. An easy way to cleave this bond is the use of *B. cereus* and *B. megaterium* β-lactamase (Yang et al. 2001).

Purine nucleotides

Purine nucleotides and nucleosides have applications in medicine and as flavor enhancers. The nucleotides guanylic (GMP), inosinic, and xanthanylic acids are directly produced by *Corynebacterium glutamicum*, but an alternative is to use *B. subtilis* to produce the nucleosides and to prepare the nucleotides by subsequent chemical phosphorylation (Demain 1978). Purine ribonucleotide synthesis in *B. subtilis* is subject to complex feedback inhibition and end-product repression, and mutants resistant to these feedback effects have been developed.

Mutants of *B. subtilis* K have been used for the industrial production of inosine, which easily passes through the cell membrane into the extracellular medium. Yields of greater than 20 g/L are produced by adenine auxotrophic mutants (Shiio 1989). The corresponding 5′-nucleotides are prepared in yields of >90% by a simple chemical process (Yoshikawa et al. 1969). Guanosine-producing *B. subtilis* strains, lacking guanylic acid reductase, have been derived by mutation of inosine producers, with reported yields of >8 g/L of guanosine (Momose and Shiio 1969). While the industrial demand for purine nucleosides other than inosine and guanosine is low, mutants of *B. subtilis* have been developed that produce >15 g/L of these products. The xanthosine and adenosine producers are guanine and adenine auxotrophs, respectively (Ishii and Shiio 1973).

A stoichiometric model of *B. subtilis* metabolism, for quantitative analysis of theoretical growth and commercially relevant biochemicals' (inosine, guanosine, riboflavin, and folic acid) production capacity, was developed by Sauer et al. (1998). An estimate for the bioenergetic efficiency generated, by fitting the model to experimentally determined growth yields, showed that the maximum theoretical yields of all products studied are limited by pathway stoichiometry at high bioenergetic efficiencies. The maximum yields that can reasonably be expected with *B. subtilis* on glucose were estimated to be 0.343, 0.160, and 0.161 mol product/mol glucose for purine nucleosides, riboflavin, and folic acid, respectively.

Vitamins

Only a small number of vitamins are produced commercially by fermentation. Recent advances in the cloning of riboflavin, cobalamin, and biotin biosynthesis genes in *Bacillus* species, in combination with the exploitation of genomics and metabolic reconstruction strategies, provide potential for the use of *Bacillus* strains in vitamin production. It was observed that mutations in the *ribO* operator site of the riboflavin operon, which contains six structural genes, resulted in the development of a number of overproducers (Stepanov et al. 1977; Debabov 1982).

Traditional industrial producers included the ascomycetes, *Eremothecium ashbyii* and *Ashbya gossypii* (Debabov 1982). However, advantages were perceived in developing bacterial and yeast fermentations where microorganisms could exhibit high growth rates and use simpler synthetic media. There are currently three microorganisms in commercial use for riboflavin production: *A. gossypii*, a filamentous fungus; *Candida famata*, a yeast; and *B. subtilis*. A number of strains of *Ashbya* and *Candida famata* can produce yields of riboflavin of about 20 g/L in approximately 5-day fermentations. *Corynebacterium ammoniagenes* fermentation is also reported to produce vitamin yields of 20 g/L but in a shorter 3-day fermentation cycle. To obtain riboflavin production from *B. subtilis*, deregulation of purine synthesis and a mutation in a flavokinase-flavin adenine dinucleotide synthetase was required (Stahlmann et al. 2000).

In *B. subtilis*, riboflavin biosynthesis is associated with purine metabolism. Many riboflavin overproducers have modified *ribO* sites. Recombinant DNA techniques have been used together with fermentation strategies to develop commercially attractive levels of riboflavin by *B. subtilis* (Perkins et al. 1999). *Bacillus subtilis* has been recently developed to produce yields of up to 30 g/L in 3-day fermentation and hence is becoming the most competitive microbial riboflavin producer. Metabolic responses to cofeeding of different carbon substrates in carbon-limited chemostat cultures of riboflavin-producing *B. subtilis* indicated that biomass yield was lower but riboflavin yield was significantly higher in acetoin- and gluconate-co-fed cultures as compared with glucose alone (Dauner et al. 2002). Sauer and Bailey (1999) devised a rational metabolic engineering strategy to improve riboflavin production.

Poly-γ-glutamic acid

γ-PGA is an anionic naturally occurring homopolyamide that is made of D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid groups. γ-PGA is a water-soluble, edible, and biodegradable compound with industrial applications in the food, cosmetics, and medical areas and in wastewater treatment. Specific applications of γ-PGA and its derivatives include uses as thickener, hemectant, cryoprotectant, drug carrier, highly water adsorbant hydrogel, biopolymer flocculent, heavy metal absorber, and animal feed additive (Shih and Van 2001).

Several *Bacillus* species are reported to produce γ-PGA; however, *B. licheniformis* and *B. subtilis* are the most studied *Bacillus* species for γ-PGA production (Ito 1997; Shih and van 2001). The genes required for γ-PGA synthesis in *B. subtilis* were recently cloned, and characterization of the gene product revealed that PGA synthetase is the crucial enzyme in γ-PGA biosynthesis (Ashiuchi and Misono 2002; Urushibata et al. 2002). Using a fed-batch approach and *B. licheniformis* ATCC 9945A in a 2.5-L bioreactor, 35 g/L of γ-PGA with volumetric productivity of 1 $g \cdot L^{-1} \cdot h^{-1}$ has been reported (Yoon et al. 2000). Ogawa et al. (1997) reported pilot-scale production of 35 g/L of γ-PGA by *B. subtilis* (natto) in a 30-L jar fermenter. Commercial production has been achieved by the Meiji Seika Kaisha Company in Japan using *B. subtilis* F-2-01 capable of producing up to 50 g/L of γ-PGA (Tanaka et al. 1997).

D-Ribose

D-Ribose is frequently used as a flavor enhancer in food, pharmaceuticals, cosmetics, health food, and animal feed, as well as for the treatment of myocardiac ischemia and muscular pain. The production of D-ribose by fermentation has received much attention lately (De Wulf and Vandamme 1997). The amount of D-ribose produced world-wide by fermentation is estimated to be around 2000 metric tonnes per year by companies such as BASF and Merck (Germany), Hoffman-La Roche (Switzerland), Pfizer (U.S.A.), and Takeda (Japan).

Microorganisms that secrete D-ribose are deficient in the enzyme transketolase and (or) D-ribulose-5-phosphate 3-epimerase. Several strains of *B. subtilis* and *B. pumilus* and their mutants are reported to produce significant amounts of D-ribose (reviewed by De Wulf and Vandamme 1997). Recent developments in genetic engineering and fermentation technology have contributed to improvements in D-ribose productivity by *Bacillus* fermentations. D-Ribose yields, exceeding 90 g/L from 200 g/L D-glucose, with significant reduction in fermentation time and the concentration of undesirable by-products, have been achieved (Kishimoto et al. 1990; Miyagawa et al. 1992; De Wulf and Vandamme 1997).

Miscellaneous products from *Bacillus* spp.

Bacilli produce a range of products with industrial applications. Some of the products with industrial applications are presented in Table 6.

Thaumatin, an intensely sweet-tasting protein from the African plant *Thaumatococcus daniellii*, is a potential low-calorie substitute for sugar (Edens and van der Wel 1985). With the α-amylase promoter and ribosome-binding site, *B. subtilis* has been used to express thaumatin II cDNA fused in the correct reading frame to the α-amylase leader peptide (Illingworth et al. 1988).

Bacillus megaterium accumulates cell densities of up to 3.4 g/L and high quantities of polyhydroxybutyrate (PHB), up to 50% of the cell in 14 days, when grown in a medium containing 5% (w/v) date syrup or beet molasses. To establish a novel recovery system for polyhydroxyalkanoate, a self-disruptive strain of *B. megatarium* was constructed (Hori et al. 2002). A gene cassette carrying the lysis system of *B. amyloliquefaciens* phage, holin and endolysin, was inserted into the *E. coli – B. subtilis* shuttle vector pX under the xylose-inducible expression system, xylR–xylA. For the

Table 6. Miscellaneous industrial products from *Bacillus* species.

Product	Application	*Bacillus* species	Reference
Endotoxins	Biopesticide, insecticide	*B. thuringiensis*, *B. sphaericus*, *B. popilliae*, *B. lentimorbus*	Bishop 2002; Phelps and MacKillip 2002
Purine nucleotides	Flavor enhancers, medicine	*B. subtilis*	Shiio 1989; Saur et al. 1998
Riboflavin	Vitamin ingredient for health food	*B. subtilis*	Perkins et al. 1999; Stahlman et al. 2000; Dauner et al. 2002
Poly-γ-glutamic acid	Used as thickener, drug carrier, biopolymer, animal feed additive	*B. subtilis*	Ogawa et al. 1997; Yoon et al. 2000
D-Ribose	Flavor enhancer in food, health food, pharmaceuticals, cosmetics	*B. subtilis*, *B. pumilus*	Miyagawa et al. 1992; De Wulf and Ogawa 1997
Thaumatin	Sweet-tasting protein for applications in food and pharmaceutical	*B. subtilis*	Illingworth et al. 1988
Polyhydroxybutyrate	Biodegradable plastics	*B. megaterium*	Hori et al. 2002
Streptavidin	Biotin-binding protein, applications in high density biochips	*B. subtilis*	Wu and Wong 2002
2-Acetyl-1-pyrroline	Popcorn, corn chip aroma, and flavor for food applications	*B. cereus*	Romanczyk et al. 1995

production of PHB, the transformant was grown in medium containing glucose as substrate in the presence of xylose. When glucose concentration approached zero, self-disruption was spontaneously induced, releasing intracellularly accumulated PHB into the culture broth.

Recently, the direct fermentation of starch into ethanol by a recombinant *S. cerevisiae* strain YPG-AB, which expresses *B. subtilis* α-amylase and *Aspergillus awamori* glucoamylase as separately secreted polypeptides, was investigated in both batch and fed-batch conditions (Uelgen et al. 2002). Starch fermentation by *S. cerevisiae* YPG-AB was characterized by ethanol yields and (or) productivities higher than those obtained in comparable fermentations on starch reported in the literature.

Streptavidin, a biotin-binding protein, with in vitro and in vivo applications in capturing molecules to detect, locate, and immobilize biotinylated molecules, is an important element in the generation of high-density biochips (Cahill 2001). Because of the ease of protein recovery and availability of protease-deficient strains, the *B. subtilis* expression–secretion (WB800BIO) system has been reported as an attractive system for high yield (35–50 mg/L) streptavidin production (Wu and Wong 2002).

The biotechnological generation of natural flavors and fragrances is rapidly expanding with the production of compounds, like vanillin, benzaldehyde (bitter almond, cherry), and 4-(*R*)-decanolide (fruity-fatty) on a scale of several thousand tonnes per year (Krings and Berger 1998). Flavour components originating from *Bacillus* fermentations are often associated with amino acid and peptide products. Various species of molds, yeasts, and bacteria produce aroma compounds by fermentation or biotransformation reactions. Aroma volatiles, produced in *Bacillus* soya fermentations, contained aroma-active aldehydes, ketones, and acids (Beaumont 2002). Several *B. cereus* strains, isolated from cocoa fermentations, produce 2-acetyl-1-pyrroline as the principal component responsible for a "popcorn, corn chip" aroma (Romanczyk et al. 1995). However, mechanisms and metabolic pathways for the production of *Bacillus* aroma compounds have not been thoroughly explored.

The demand for vaccines against anthrax has increased since September 11th, 2001. One remarkable study investigates the efficient production of one of the three components of the anthrax toxin in an avirulent *B. anthracis* strain. This nontoxic compound, the protective antigen (PA), is used in vaccinations to induce high anti-PA antibody titers to neutralize PA (Ramirez et al. 2002). The possibilities of oral vaccination by antibody induction over mucosal contact with recombinant *Lactobacillus* expressing PA in dairy products have been studied but seem to provide a dose much too low for the desired vaccination. The expression of PA or other valuable proteins in high yield *Bacillus* species, like *B. brevis*, is a very interesting option for future recombinant protein production.

The robustness of the bacterial spores, with simplified genetic manipulation and cost-effective manufacturing, make them attractive vehicles for oral and intranasal vaccination. Spores of *Bacillus* species are produced commercially and are being used as probiotics and competitive exclusion agents (Casula and Cutting 2002). For the first time, Duc et al. (2003) evaluated bacterial spores as a vaccine vehicle. *Bacillus subtilis* spores displaying the tetanus toxin fragment C antigen, have been used for oral and intranasal immunization to demonstrate the generation of mucosal and systemic responses in a murine model.

Concluding remarks

The reports described in this review demonstrate just how effectively *Bacillus* species have defended their position as dominant bacteria in industrial fermentations as advances have been made in recombinant DNA technology. New industrial enzymes produced by *Bacillus* strains have emerged as a result of mutations, cloning, and protein engineering, with desired specificity, activity, and stability properties. Improved expression systems have increased enzyme productivities.

Problems associated with protease degradation of heterologous proteins, expressed and secreted by *Bacillus* strains, are being overcome which will position *Bacillus* species as the pre-eminent hosts, for example, in the production of high value human proteins. *Bacillus* species are becoming more dominant producers of nucleotides, the vitamin riboflavin, the flavour agent ribose, and the food and pharmaceutical supplement γ-PGA. It is clear that the fundamentally positive characteristics of these robust and productive fermentation strains will make them the preferred hosts for the many new and improved products that will emerge as the genomic and proteomic era moves forward.

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