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Bacillus Subtilis: An Industrially Important Microbe for Enzymes Production

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Summary

Bacillus subtilis is a Gram positive, rod shaped bacteria. It is commonly found in soil. It is an endospore forming bacteria that it allows withstanding to inappropriate extreme temperatures as well as dry environments. *B. subtilis* is considered and obligate aerobe, but can also function anaerobically if the presence of only nitrates or glucose. *Bacillus subtilis* is not considered pathogenic or toxic and is not a disease causing agent. *B. subtilis* has high motility due to its flagellum. *B. subtilis* produces enzymes such as proteases and amylase. *B. subtilis* was widely used as a broad spectrum antibiotic. It can also convert dangerous explosives into non-toxic forms to nitrogen, carbon dioxide and water. The proton binding properties of the surface of this bacterium plays a role in the degradation of radioactive waste. *B. subtilis* has an unique property as a soil inoculant. Moreover, commercial applications of *B. subtilis* include cleaning agents in detergents, in de-haring and batting in the leather industry are known well, in the production of special Japanese and Korean cuisine, starch modification, the de-sizing of textiles, and other specialized chemicals this bacteria is commonly used. *B. subtilis* also produces some fungicidal compounds, which are being investigated as control agents of fungal pathogens. It is currently being used as a fungicide for plant and ornamental seeds as well as various agricultural seeds. Besides its many uses and applications, *B. subtilis* has become the model agent in laboratory research because of its easy genetic manipulation. It has been applied to studies of *Escherichia coli*, because they house many similarities. In this review we highlight whole of these properties of *Bacillus subtilis* considering update knowledge.

Keywords: *Bacillus subtilis; Escherichia coli; Proteases; Amylase*

Introduction

Numerous chemical transformation processes are implemented and are essential in various industrial applications. However, these processes involve various commercial and environmental drawbacks. While the commercial factors includes poor product yields, sophisticated equipment that can control and monitor a wide, variable range of pH, temperatures and pressures, processing and disposal of the toxic by-products, the environmental factors could be related to various health hazards. Therefore, to circumvent these problems, in most of the cases nowadays, the chemical processes are substituted by the more efficient, environmental friendly biological transformation system which engages a wide range of biological enzymes derived from diverse microorganisms.

Enzymes are basically metabolic catalysts that speed up the rate of biological reactions. In addition to their key role in biological systems, most of the basic enzymes such as amylase, protease, cellulase, lipase and some other enzymes are produced and used in different industries, for instance, in detergent, food, agriculture, starch, dairy, textile, brewing, baking, pulp and paper industries [1,2]. The main reasons for preferring enzymes in industrial areas involve capability of enzymes to work under mild conditions with a very high reaction rates. They also capable to work under a wide range of different pHs, at high temperatures and pressures. They carry out the industrial

process in satisfactorily minimum amounts even on large scales, decreasing energy, chemical and water consumption. Therefore it is being environment-friendly approachment [3]. In accordance to the frequent use of enzymes in industry, there has been developed a big industrial enzyme market in the world. According to BCC Research's report (Report Code: BIO030G), in 2010, the global industrial enzyme market was valued at $3.6 billion and the estimated growth of this market at a compounded annual growth rate of 9.1% to reach $6 billion by 2016, pointing out a fast-growing market.

Bacteria and fungi are most frequently used microorganisms for industrial enzyme production [3]. Since producing enzymes from microbial sources are cheaper, and their enzyme production and secretion systems are well-known and more controlled, they are preferred over enzymes from animal or plant sources [4]. Among enzyme producing bacteria, *Bacillus subtilis* is the primary working horse for a number of reasons. *B. subtilis* is a rhizobacterium that produces more than two dozen of biologically active molecules including antibiotics and different types of enzymes, generating a high potential for biotechnological and biopharmaceutical applications [5]. On the basis of its success in industrial production, there is its high adaptability to changing environmental conditions [6,7]. Besides this worthwhile characteristic feature, *B. subtilis* is regarded as a safe (GRAS-Generally recognized as safe organism) microorganism by the Food and Drug Administration of USA (FDA) with total lack of toxic by-products. It also has a very high enzyme production yield together with its extracellular secretion feature which enables direct secretion of high amounts of enzymes into fermentation medium.

This high production of enzymes by *B. subtilis* has a few bottlenecks related to the protein secretory pathway of this microorganism. For instance, poor translocation of the secreted proteins, degradation of these proteins by the high level of secretory proteases from these microorganisms and protein misfolding, immensely interferes with the production of enzymes and complicate the use of *B. subtilis* as an industrial enzyme source. However, mapping of the full genome of this microorganism making it accessible for various genetic manipulations, indeed evade these difficulties to a certain extent [8].

B. subtilis for industrial enzyme production

B. subtilis is especially prefered for α-amylase, protease, cellulase, lipase, pullulanase and xylanase enzymes, the production of which represents about 60% of the commercially available industrial enzymes [9,10].

α-Amylase

α-Amylase (E.C.3.2.1.1) is one of the most important enzymes that comprises 25% of the industrial enzyme market which is generally used in starch liquefaction, baking, brewing, detergents, textile, pharmaceutical industries [11]. α-Amylase acts on glycogen, starches and oligosaccharides in a random fashion, catalyzing the endo-hydrolysis of α-1,4-D-gylcosidic linkages in polysaccharides containing 3 or more α-1,4-linked glucose units [2]. Especially, *B. subtilis* is one of the champion α-Amylase producing bacteria because of its capability of overproducing α-Amylase that is stable even under high temperatures. There are several identified *B. subtilis* strains producing α-Amylase with different characteristics such as strain 168, strain KIBGE HAS etc. In a recent study, a novel α-amylase from *B. subtilis* KIBGE HAS has been purified and enzyme has been shown to be very stable with various surfactants and detergents [12]. Together with its stability, the enzyme has been found to be highly thermostable, since it retained about 62% of its activity at 70°C for 15 min. This α-Amylase also has been shown to be highly stable at −18°C in 124 days according to storage stability study [12]. Although many *B. subtilis* strains produce α-Amylase enzyme natively, continuously increasing demand due to its increased application areas created need for more thermotolerant and pH tolerant α-Amylases together with higher production yields at low costs. For all these purposes, the most applicable and successful ways are genetic modification of *B. subtilis* strains, site-directed or random mutations to produce enzymes with desired properties. Recently, there is increasing number of studies for a better α-Amylase production. For instance, in a previous study, the mutant B. subtilis GCBUCM-25 strain was found to be the best for biosynthesis of α-amylase among different *Bacillus* species such as *B. subtilis*, *B. licheniformis* and *B. sterothermophilis* [13]. Furthermore, another research group showed that there were 3 randomly-mutated α-Amylase genes from *B. subtilis* 168 strain which had higher enzymatic activity compared to the wild type [14]. Identification of the sequences on the

mutated sites of the gene will give insights about the essential regions of the genes to be manipulated for greater enzyme activity in future analysis. In addition to these mutation studies, using *B. subtilis* as a host system for expression of α-Amylase belonging to other bacterial species is also a way of effective enzyme production. *B. subtilis* MTCC2423 strain carrying the plasmid pC194 containing a thermostable α-amylase gene from *Bacillus stearothermophilus* has been shown to significantly enhance α-amylase production with the increase in specific growth rate, biomass and yield [15].

Protease

Protease is another enzyme that is used in industry as frequently as amylase. Basically, protease is the enzyme catalyzing protein hydrolysis. For industrial applications, bacterial proteases are preferred over the enzymes produced by animal or plant sources, as they have most of all the characteristics required for diverse biotechnological applications [16]. *B. subtilis* is the dominant one among all bacteria for production and secretion of extracellular proteases. These proteases have a broad range of application primarily in food and detergent industry [17], and also in pharmaceutical, leather, and waste processing industries [18-20].

There are a number of different *B. subtilis* strains with good protease producing activities partially in a substrate-dependent manner. For instance, *B. subtilis* NCIM 2724 strain has been found to produce very high yields of protease from papaya peel as substrate compared to other *Bacillus* species such as *Bacillus licheniformis* [21]. However, another *B. subtilis* strain has shown its superior protease producing activity when utilized *Parkia biglobossa* (Africa Locust Beans) shell as its substrate Production of Cellulase and Protease from Microorganisms Isolated from gut of *Archachatina marginata* (giant African snail). Among all proteases, alkaline proteases are especially used as laundry and dishwashing detergent additives [22]. Their primary function in detergent industry is to degrade proteinaceous stains on fabrics. For this purpose, one of the most promising enzymes is *B. subtilis* RSKK96 protease because of its ability to function in a broad range of temperature which makes this enzyme a strong candidate for laundry detergent applications and also in food and pharmaceutical industries [23]. In addition, there are other *B. subtilis* proteases with some unique properties such as organic solvent-stable alkaline serine protease (Bsubap-I), purified from *B. subtilis* DM-04 strain [24]. Bsubap-I has been found to be thermostable and also stable against many surfactants, organic solvents and detergents. All these characteristics, together with its dehairing activity, make Bsubap-I a powerful candidate for applications especially in laundry detergent formulations and in leather industry.

There is a distinct group of proteases, named aminopeptidases (APs; EC 3.4.11) which catalyse the cleavage of the N-terminal amino acid residues from peptides and proteins. Aminopeptidases are mainly used in food industry to produce protein hydrolysates for production of food additives in medicine and sport. Recent studies for the production of aminopeptidases commonly focus on the *Escherichia coli* expression system which is very limiting for food industry due to its pathogenity and low secretion efficiency. Alternatively, an aminopeptidase from *B. subtilis* Zj016 strain (BSAP) was cloned, identified and then over expressed in *B. subtilis* expression system using PMA5 expression vector to construct PMA5-BSAP. Safety and yield analysis evidently showed that the production of aminopeptidase in recombinant *B. subtilis* WB600 is better and higher than that in recombinant *E. coli* [25]. Moreover, high expression and secretion efficiency of *B. subtilis* are likely to reduce the large-scale industrial production costs.

On the other hand, there is strong evidence indicating the advantages of plasmidless, engineered *Bacillus* strains over plasmid-carrier variants in terms of their stability and potential ecological safety in industrial applications. In the related study, a plasmidless mutant *B. subtilis* JE852 strain (*nprE, aprE*) which has low levels of extracellular protease activity was used for expression and secretion of glutamyl-specific protease (GSP) from *Bacillus amyloliquefaciens*. In order to achieve this, they ectopically inserted ten copies of the GSP gene cassette into random and designated points in the *B. subtilis* chromosome. This constructed plasmidless *B. subtilis* strain was analysed for its GSP production performance and results revealed that plasmidless strain had the same level of GSP production as the recombinant plasmid-carrying strain [26]. This novel ectopic multi-copy integration strategy may be a great guide for the construction of plasmidless and markerless, recombinant Bacillus strains for industrial enzyme production.

Cellulase

Cellulase, the catalyst of cellulolysis, is another crucial enzyme for industrial processes. Cellulase is frequently used in a number of industrial applications such as biological laundry detergent production and also in textile industry for rejuvenation and biopolishing of fabrics, in animal feed additives for better nutritional quality, waste water treatment, wine-making and brewing [27,28]. In addition, applicable cellulase is also a worldwide emerging requirement to hydrolyze the lignocellulosic biomass (plant biomass) for bioethanol production in order to prevent waste pollution that is caused by petroleum fuel shortages, increased greenhouse gases and incomplete combustion of fossil fuel [29,30]. However, the main obstacle in bioethanol production from cellulosic biomass is the high cost of cellulase production [31]. Although filamentous fungi are the major source of cellulase, production of this enzyme from fungi is a very expensive procedure. Thus, solution of the problem solely depends on the identification of novel cellulase producing bacterial strains or genetically modifying them in order to obtain high yields of cellulase. Among bacteria, especially *B. subtilis* is the most studied bacterial species due to its ability to produce and secrete large amounts of extracellular cellulose [32,33]. Recently, *B. subtilis* LFS3 strain has been found to secrete both acidophilic and thermophilic cellulase which are very desirable properties for biomass conversion of lignocellulosic waste and also for different industries such as fruit juice and animal feed additives [34].

Another study indicated the existence of a thermostable cellulase, produced by *B. subtilis* DR strain that was isolated from a hot spring. It was found to retain its activity at 75°C after incubation for 30 minutes. Owing to its extreme heat tolerance, this *B. subtilis* DR cellulase is likely to be more important for lignocellulosic biomass conversion [35].

Besides screening for novel cellulase producing strains, there are also efforts to investigate new ways of low-cost cellulase production by searching for natural, low-cost growing media materials. According to the results of a recent study, *B. subtilis* KO strain has shown high cellulase productivity when grown on very low-cost molasses media [36].

On the other hand, genetical engineering of *B. subtilis* for higher amounts of cellulase production is another attractive and effective way of lowering costs. One of the most frequently seen genetic manipulations is using *B. subtilis* as an expression system for high level expression of cellulase genes belonging to other bacterial species. For this purpose, insertion into an expression vector or direct chromosome insertion methods can be used with some advantages and disadvantages. Although transcription and translation elements are need to be optimized to obtain high expression levels, using chromosomal insertion is a better choice when compared with plasmid insertion method bearing plasmid stability problems and the potential risk of spreading the antibiotic resistance gene encountered during large-scale biocommodity production [37].

More recently, *B. subtilis* was engineered to display a multicellulase-containing minicellulosome that makes the bacteria highly cellulolytic [38]. The minicellulosome was designed to contain a covalently attached mini scaffolding protein on the cell wall and three cellulase enzymes derived from *Clostridium cellulolyticum* that are non-covalently attached. The minicellulosome assembly is spontaneous to increase the applicability of the system. Since the minicellulosome contains only three types of enzymes, increasing the number of displayed enzymes may lead to even more powerful cellulase producing bacteria. This study represents an invaluable system for the low-cost and efficient conversion of lignocellulosic biomass. Furthermore, there are some other assistant enzymes, produced by *B. subtilis*, such as expansin which can accelerate the action of cellulase in case of degradation of lignocellulosic material.

Expansin is a plant cell wall-loosening and cellulose disrupting enzyme. It has been shown that, in combination with cellulase, BsEXLX1 expansin from *B. subtilis* has been found to increase the cellulase activity as 5.7 fold compared to activity of cellulase alone [39]. Bacteria have been widely explored for cellulase production owing to their high growth rate, expression of multienzyme complexes, stability at extreme temperature and pH, lesser feedback inhibition, and ability to withstand variety of environmental stress [3]. Among them, Bacillus sp. continues to be dominant bacterial workhorse due to the capacity to produce and secrete large quantities of extracellular enzymes [40,41].

Xylanase

Xylanase is another important enzyme for bioconversion of lignocellulosic material, since xylan is one of the main groups of hemicellulose, which is an important constituent of lignocellulosic material. *B. subtilis* GN156 strain that is well known for its high potential of cellulolytic enzymeproduction [40], has been shown to produce higher levels of xylanase in the presence of xylan (1.5 %, w/v) as the substrate [41]. *Bacillus* species are known to be attractive industrial organisms due to their rapid growth rates leading to a short fermentation cycle and for their capacity to secrete important enzymes and proteins such as xylanase into the extracellular medium. Considering the industrial importance of xylanase, *Bacillus* spp. isolated from different soils and screened are good xylanase producers. They have major role as industrial microorganisms due to their high growth rates affecting short fermentation cycle period and their capacity to secrete proteins into the extra cellular medium. *Bacillus* spp. enzymes make up alone about 50% of the total enzyme market [31]. For the production of any industrial enzyme, an inexpensive substrate and an efficient fermentation process are essential for commercial viability. It has been established that solid-state fermentation has several advantages over submerged fermentation, due to a smaller volume of solvent required for product recovery, resulting in higher productivity per unit volume, lower contamination and foaming problems and better exploitation of various agro-residues as substrates [12,23,38,40,42,43]. Other than its potential use in bioconversion of plant material, xylanases have been widely used for bleaching in pulp and paper industry [44,45], as food additives in food industry, for the improvement of nutritional properties of agricultural silage and grain feed [42,43]. Cellulose producers are *B. subtilis* AS3 [27], and *B. pumilus* [6].

Lipase

Another industrially important enzyme that needs to be mentioned is lipase (EC.3.1.1.3, triacylglycerol acylhydrolases) which can catalyze ester synthesis, ester hydrolysis and transesterification reactions by hydrolyzing triacylglycerols at an oil-water interface. Because of the wide usage of these reactions in a variety of industrial processes, lipases are of great importance in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics [46]. It can also be used for the biodegradation of oil effluents [47].

Although there are various lipase producing bacteria, *B. subtilis* lipases have attracted more attention because of its usability in food and chemistry industries as being GRAS. However, one important obstacle for broad utilization of wild-type *B. subtilis* is its low lipase expression profile. For this reason, recombinant DNA technology has been widely used to obtain increased yield of lipase production [47]. One advantageous feature of lipases from *B. subtilis* is that they are prone to be correct folding in *B. subtilis* host systems, and hence lipases can be over expressed in a *B. subtilis* host using suitable plasmids for higher amounts of lipase production [48].

Recently, there are not too much suitable plasmids for lipase expression in *B. subtilis* host systems. Thus, plasmid design and construction studies can be a savior to solve the low lipase expression problem in *B. subtilis*.

There has been a plasmid constructed for lipase expression, named pBSR2, integrating a strong lipase promoter and a terminator into the original pBD64 plasmid [49]. Lipase gene from *B. subtilis* IFFI10210 strain was cloned into the plasmid pBSR2 and transformed into host system *B. subtilis* A.S.1.1655, thereby generating an overexpression strain, called BSL2. Success of the system was approved by showing that lipase yield was 100-fold higher in BSL2 system than that in original *B. subtilis* IFFI10210 strain.

There are some other studies that use other organisms' lipases possessing different characteristics, to be expressed in *B. subtilis*. Especially for detergent and leather industries, non-position specific alkaline lipases are of great importance due to their high performance in hydrolysis and transesterification reactions [50]. Recently, a non-position specific lipase has been isolated from *Proteus vulgaris* to overexpress it in a heterologous host, *B. subtilis* WB800 [51]. For production and secretion of this lipase in *B. subtilis* WB800, different vectors such as pMM1525 (xylose-inducible), pMMP43 (constitutive vector, derivative of pMM1525), and pHPQ (sucrose-inducible, constructed based on pHB201), were used. Results showed that pHPQ-PVL plasmid harbouring *B. subtilis* WB800 cells expressed and secreted 12-fold

higher amount of PVL having identical characteristics compared to the native lipase in *Proteus vulgaris* [51].

Even though most of the studies are focused on searching suitable host systems and developing novel plasmids to express recombinant lipase enzyme with increased activity and yield, there are a few researches aiming optimization of culture conditions to enhance the lipase activity. Very recently, a study has been carried out to optimize culture conditions for enhanced lipase production from *B. subtilis* by using Statistical experimental design method instead of the "one-factor-at-a-time" (OFAT) method which is time consuming, less effective and laborious [52,53]. Statistical experimental design can overcome these problems of OFAT, since it is applicable to any experimental design to evaluate the effects of different factors and searching for optimum conditions of factors for desired responses [54]. Plackett–Burman design and steepest ascend method were applied to define critical medium compositions by evaluating different medium contents. Then, response surface methodology was employed to optimize the concentration of components in order to maximize lipase activity. As a result, the optimal medium compositions were determined as 8 g L^{-1} glycerol, 45 g L^{-1} NH4Cl, 20.4 g L^{-1} $(NH_4)_2SO_4$, 0.8 g L^{-1} $MgSO_4$, 1.2 g L^{-1} $MnSO_4$, 0.8 g L^{-1} $CaCl_2$, 7.2 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 12.6 g L^{-1} K_2HPO_4, which provides about 5 times higher lipase activity than that using the original medium [52].

Concluding Remarks

As demonstrated in this review, *B. subtilis* is a highly efficient, hence a "sine qua non" source of industrial enzymes such as amylases, cellulases, lipases, proteases. It takes the advantage of being a GRAS which allows genetic modifications, easily adapts to environmental changes, produces enzymes in high yield without toxic by-products and secretes them into extracellular environment. The latest studies on production of much more efficient enzymes from *B. subtilis* mostly involve genetic modifications which is just as important as searching for native strains with high productibility. As a result of these modifications such as cloning, protein engineering and mutations, new *B. subtilis* strains producing enzymes with desired yield, activity, specificity and stability have been developed. A few limitations of *B. subtilis* that interfere with industrial enzyme production, such as its natural sporulating property and high amount of secreted proteases that may degrade produced enzymes, are successfully being overcome again by the help of genetic modifications. Through all these positive characteristics, *B. subtilis* keeps its championship among other enzyme producing organisms.

As well as genetically modified *B. subtilis* strains, there are also various identified and characterized native *B. subtilis* strains which are all good enzyme producers. These native strains may become more efficient just by optimizing their culture conditions as successfully tested in a number of studies. There are also other enzyme producing *B. subtilis* strains which have been discovered during investigation of their beneficial effects as biocontrol agents (biopesticides) in agriculture. A recent study has showed that, a novel soil-isolated *B. subtilis* EU07 strain is much more efficient in biocontrol process than some other commercially available *B. subtilis* strains owing to a number of unique pathogen-inhibitory molecules produced by EU07. Underlying its superior biocontrol ability is the notable efficiency of EU07 in expression of high amounts of endo-1,4-beta-glucanase (cellulase), protease and hydrolase enzymes which contribute to degradation of the pathogen cell wall [55]. From this point of view, EU07 strain may become another precious source for industries due to its properties.

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