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Assessing the Performance of Bacterial Cellulases: the Use of *Bacillus* and *Paenibacillus* Strains as Enzyme Sources for Lignocellulose Saccharification

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Abstract Plant biomass offers a renewable and environmentally favorable source of sugars that can be converted to different chemicals, second-generation ethanol, and other liquid fuels. Cellulose makes up approximately 45 % of the dry weight of lignocellulosic biomass. Prior to the enzymatic hydrolysis of cellulose, lignin and hemicellulose must be structurally altered or removed, at least in part, by chemical and/or physical pretreatments. However, the high cost and low efficiency of the enzymatic hydrolysis prevent the process from being economically competitive. For this reason, it is necessary to find enzymes suitable for this type of process, with higher specific activities and greater efficiency. Members of the *Bacillus* and *Paenibacillus* genera have been traditionally used for the production of many enzymes for industrial applications. Cellulases produced by both genera have shown activity on soluble and crystalline cellulose and high thermostability and/or activity over a wide pH spectrum. In this review, the most recent information about the characterization of cellulolytic enzymes obtained from new strains of the *Bacillus* and *Paenibacillus* genera are reviewed. We focused on the variety of isoenzymes produced by these cellulolytic strains, their optimal production and reaction conditions, and their kinetic parameters and biotechnological potential.

Keywords *Bacillus* · Cellulases · Cellulosomes · Lignocellulose · *Paenibacillus*

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Introduction

There is a need to diversify energy sources that are sustainable and less environmentally harmful than fossil fuels. One of the most viable options to replace or complement the demand for gasoline is ethanol [1], which is also an attractive product in the chemical and medical-pharmaceutical industries. Ethanol is non-toxic or carcinogenic when used as a fuel. Vehicles can use it without requiring major modifications in engines. Furthermore, ethanol is easy to store, transport, and distribute within the existing infrastructure [2, 3].

There are biotechnological alternatives to producing biofuels from renewable raw materials, e.g., agro-industrial wastes. These are abundant and of low cost (or none) and are not used for human consumption [4, 5]. A few examples include stover, cobs of corn, sorghum stover, barley straw, wheat, sugar cane bagasse, agave leaves, rice bran, and oats, among others. These residues are abundant and have marginal use [4]. The annual worldwide production of lignocellulosic residues from cereals is estimated to be 2802 million Mg year^{-1} [6]. Lignocellulosic biomass from this agricultural waste constitutes approximately 90 % of the dry weight of plants and is mainly composed of cellulose, hemicellulose, and lignin. The compositions of the different residues are shown in Table 1 [3].

In Mexico, renewable energy sources have a specific legal framework, i.e., the Law on the Use of Renewable Energy and Energy Transition Financing, published in the Official Diary on 28 November 2008. This regulation establishes the obligation of the Secretary of Energy to develop a special program for the exploitation of renewable energy, a national strategy for energy transition, and a sustainable use of energy. In this context, there are several available renewable energy alternatives based on solar, wind, hydraulics, geothermal, and biological sources [7, 8].

Table 1 Compositions of various types of lignocellulosic biomasses

Residue	Cellulose	Hemicellulose	Lignin	Reference
Sugarcane bagasse	38.1	26.9	18.4	[116]
Corn stover	36.4	22.6	16.6	[117]
Corn stover	39.0	19.1	15.1	[116]
Corn stover	29.9	23.0	21.7	[15]
Corn cob	45.0	35.0	15.0	[114]
Sorghum stover	29.7	15.4	25.9	Unpublished data
Wheat straw	38.2	24.7	23.4	[117]
Wheat straw	30.0	50.0	15.0	[114]
Rice straw	34.2	24.5	23.4	[117]
Rice straw	41.0	21.5	9.9	[116]
Rice husks	36.1	19.7	19.4	[116]
Cotton	80–95	5–20	0	[114]
Paper	85–99	0	0–15	[114]
Newspaper paper	64.4	21.7	21.0	[116]
Newspaper paper	40–55	25–40	18–30	[114]
Soft wood stalks	45–50	25–35	25–35	[114]
Wood hard stalks	40–55	24–40	18–25	[114]

Although Mexico has only started to commercialize first-generation ethanol as a biofuel [9], there is an insufficient production level of corn, other grains, sugar cane, and beet that will generate enough ethanol to complement the national gasoline demand. These products have other purposes, such as human consumption and livestock feed, and should not be considered for use as raw materials for the manufacture of biofuels. However, residues of these crops can be used for the production of second-generation biofuels.

The useful components of agroindustrial residues, i.e., cellulose, hemicellulose and lignin polymers, are strongly intermeshed and chemically bonded by non-covalent forces and cross-linking bonds [10]. Cellulose is approximately 45 % of the dry weight of lignocellulosic material. This linear homopolysaccharide is composed of D-glucose subunits that form cellobiose and is linked by β-1,4-glycosidic bonds. Cellulose is primarily present in crystalline form (i.e., organized chains linked by hydrogen bonds and van der Waals forces and highly resistant to enzymatic hydrolysis) and, to a lesser extent, as amorphous cellulose (i.e., unorganized chains more susceptible to enzymatic degradation) [10, 11].

Prior to the enzymatic hydrolysis of cellulose, lignin and hemicellulose must be structurally altered or removed, at least in part, by chemical and/or physical pretreatments [12–15]. These treatments also disrupt the crystalline structure of cellulose and increase enzyme accessibility [16, 17]. The enzymatic hydrolysis of agroindustrial waste requires a consortium of enzymes called cellulases (e.g., endocellulases, exocellulases, and β-glucosidase), ligninases (e.g., laccases, oxidases, and peroxidases), and hemicellulases (e.g., xylanase, xylosidase, arabinofuranosidase, feruloyl esterase, acetyl xylan esterase, galactosidase, and glucuronidase) [10, 18, 19]. The degradation of lignocellulosic residues is affected by factors including the source of substrate, enzymatic activity, and reaction conditions (e.g., temperature, pH, and reactant concentrations). Enzymatic treatments can be applied before or after the traditional physicochemical pretreatments of plant biomass to reduce the severity and eventually replace thermochemical processes by simplifying the processing of the raw material [13].

Cellulases consist of the following three types of enzymes: (I) endo-β-1,4-glucanases (EC 3.2.1.4), also called carboxymethylcellulases (named after the substrate used for their detection), randomly attack internal sites in amorphous cellulose to produce binding sites for subsequent attack by cellobiohydrolases; (II) exo-β-1,4-glucanases or cellobiohydrolases (EC 3.2.1.91) hydrolyze crystalline cellulose by removing monomers and dimers from the end of the glucan chain; and (III) β-glucosidases (EC 3.2.1.21) hydrolyze glucose dimers and, in some cases, cellulose oligosaccharides to glucose [18].

In addition to the biofuel industry, cellulases can also be applied in waste water treatment, starch processing, animal food production, grain alcohol fermentation, malting and brewing, the production of lactic acid and single cell proteins, the extraction of fruits and vegetables, the pulp and paper industry, and the textile industry [20, 21].

Cellulose enzymatic treatments tend to be expensive, slow, and relatively inefficient [22–26]. A few of the technical challenges in the use of cellulose at the large-scale for biofuels production include low specific enzymatic activity and high costs of cellulase production. Furthermore, cellulose is

resistant to direct enzymatic attack due in part to strong protection by lignin and hemicellulose. Cellulose molecules in crystalline form are compactly packed and are impermeable to enzymes and water [27]. The hydrolysis of crystalline cellulose is considered a limiting step in the conversion of lignocellulosic biomass to ethanol. For this reason, it is necessary to find new microorganisms producing enzymes suitable for this type of process and with higher specific activities and greater efficiency [28]. The characterization of these enzymes could help to accomplish cellulosic biomass breakdown at the industrial scale.

Endoglucanase activity is the first stage in the enzymatic breakdown of cellulose. A few cellulolytic microorganisms appear to secrete distinct variants of endoglucanases [29–35]. Some of these bacterial endoglucanases, mainly from extremophilic microorganisms, have been cloned, purified, and studied for their catalytic properties (e.g., optimum reaction conditions, thermal stability, variety of isoenzymes, and sometimes kinetic parameters) [20, 28, 36–44].

Because the enzymatic breakdown of cellulose is the result of a set of synergistic activities, the knowledge of the catalytic properties of individual cellulases is insufficient to determine the potential applications of an enzyme complex produced by a particular microorganism [23]. To this degree, studies have characterized cellulolytic enzyme complexes mainly of fungal origin. These works have focused on finding optimal reaction conditions or proper balances of the three cellulolytic activities [45–51]. Other studies have evaluated the behavior of enzyme complex mixtures from different fungi [25, 52–55]. However, scarce information exists on the kinetic behavior of cellulolytic enzyme complexes [35, 46, 56–59], and because enzymes can be inhibited by their own reaction products, this effect will largely determine their potential applicability [17, 22, 23, 35, 60–66].

Cellulolytic Bacteria

A wide variety of bacteria and fungi produce enzymes that catalyze the hydrolysis of cellulose. Bacterial enzymes that degrade vegetal biomass primarily include anaerobes, facultative anaerobes, and extremophiles (e.g., thermophiles, alkaliphiles, and halophiles). These bacterial cellulases have relatively high activity on crystalline cellulose, such as Avicel or cotton fibers [67]. However, the enzymatic degradation of cellulose remains an expensive, slow, and inefficient task. Interest in the application of these enzymes for the degradation of lignocellulose has motivated the search and isolation of these cellulolytic bacteria in different environments [22]. A few of the most studied environments have been soil samples obtained from mesophilic and extremophilic environments, where species of the order Bacillales thrive. For example, *Bacillus* sp. and *Paenibacillus* sp. have been found to produce enzymes (either as cellulosomes or secreted proteins) that hydrolyze lignocellulose to assimilate released sugars, i.e., hexoses and pentoses, as carbon sources [22, 28, 35, 42–44, 46, 68–71].

As catalysts for commercial applications, high tolerance and high temperature stability are desirable properties for enzymes. Compared with fungal cellulases, bacterial cellulases typically have better thermostability. Additionally, bacteria have shorter generation times and can easily grow to reach high cell densities using inexpensive sources of carbon and nitrogen. This provides the ability to efficiently produce great amounts of enzymes. Additionally, the expression systems and bacteria cultures are easier to handle. As such, high expression levels of endogenous cellulases in bacteria are more easily achieved than with fungal cellulases [40]. The *Bacillus* and *Paenibacillus* genera currently have a large number of applications as producers of different types of enzymes and metabolites at the industrial level [20, 72, 73]. Their commercial applications as producers of cellulases and hemicellulases need to be seriously explored. Herein, we discussed the most recent studies on the cellulolytic strains of these bacterial genera.

Bacillus and *Paenibacillus* Genera as Sources of Novel Cellulases

Cellulosome Producer Strains

Cellulases and hemicellulases can be secreted by cells as free enzymes or as extracellular cellulosomes. The collective action of multi-enzymatic systems can be more efficient than the activity of individual enzymes [3, 74]. Cellulosomes are supramolecular extracellular machines produced by anaerobic microorganisms belonging mainly to class Clostridia [22] and are able to degrade crystalline cellulose and other polysaccharides of the plant cell wall. Cellulosomes have also been identified in the mesophilic anaerobic species of *Acetivibrio*, *Bacteroides*, *Butyrivibrio*, and *Ruminococcus* [75–77]. However, there is evidence of the existence of cellulosomes in a facultative anaerobic bacteria of the genus *Bacillus* [69, 78–80] and in fungi, such as *Neocallimastix*, *Piromyces*, and *Orpinomyces* [81, 82]. Other cellulolytic bacteria are also speculated to produce cellulosomes [39].

Cellulosomes were first identified in 1983 by Lamed et al. [83] in *Clostridium thermocellum*, an anaerobic, thermophilic, and spore-forming microorganism. Cellulosomes have high activity for crystalline cellulose [67] and have also been reported in *Clostridium cellulolyticum* and *Clostridium cellulovorans* [22].

Each cellulosome contains many different and complementary types of enzymes that act on carbohydrates, including cellulases, hemicellulases, and carbohydrate esterases.

Furthermore, carbohydrate-binding module (CBM) domains bind tightly to cellulose. These enzymes are held together by carrier proteins (scaffoldins) to form a multi-enzyme complex. This cellulosome arrangement improves plant cell wall degradation; the close interaction of the different enzymes exploit enzymatically accessible regions of cellulose. Intermediary products of enzymatic subunits can be quickly transferred to other subunits for their subsequent hydrolysis [74, 76, 84–87]. One advantage from a biotechnological point of view is that cellulosomes do not need to be attached to cells to function. Furthermore, cellulosomes can function in aerobic and anaerobic conditions [3]. The existence of the cellulosomes reduces energy waste in microorganisms that continuously produce and release cellulases.

Available information about mesophilic, facultative anaerobic, and cellulosome producers remains insufficient. *B. megaterium* has been reported to produce celluloxylanosomes (with cellulase and xylanase activities) [69]. Similarly, *Paenibacillus curdlanolyticus* B-6 produces two multi-enzyme cellulosome complexes (400 and 1450 kDa) with cellulolytic and xylanolytic activities. Although this bacterium was isolated from an anaerobic environment, the multi-enzyme production occurs during aerobic and mesophilic conditions [88, 89]. The facultative anaerobe *Bacillus licheniformis* SVD1 also produces a 2000-kDa multi-enzyme complex under aerobic mesophilic conditions [80]. For thermophilic aerobic bacteria, there are few reports about the presence of cellulosomes. The thermophilic strain belonging to the family Paenibacillaceae, *Brevibacillus* sp. JXL (closely related to the *Bacillus* and *Paenibacillus* genera), is one of the few examples observed by scanning electron microscopy showing protuberances, which indicated cellulosome production on the cell surface [39]. More recently, transmission electron microscopy revealed the presence of cellulosome structures in *Paenibacillus polymyxa* EG2 and EG14 [90, 91].

Secreted Cellulase Producer Strains

Members of the *Paenibacillus* and *Bacillus* genera are facultative anaerobes and can produce many different enzymes for industrial applications [20, 72, 73]. Table 2 shows general characteristics of cellulolytic enzymes reported for these bacterial genera. In cellulase production, a few members of *Bacillus* sp. have been reported to produce up to 0.26 U/mL of secreted endoglucanase in liquid culture [40], which represents a 19 times higher production with shorter culture times (between 0.03- and 0.016-fold) compared with those of fungi traditionally used for commercial production [92]. Cellulases from these two genera have shown activity on soluble and crystalline cellulose, high thermostability, and/or high activity over a wide pH spectrum [22, 35].

Table 2 General characteristics of cellulolytic enzymes reported for the *Bacillus* and *Paenibacillus* genera

Characteristics	Value/intervals	References
Production in submerged culture (U/mL)		
Endoglucanase	0.2–2.8	[35, 40, 59, 96, 102, 113]
Avicelase	0.1–0.34	[96]
β-Glucosidase	1.2	[96]
Xylanase	2–12	[96, 102]
Number of isoenzymes	1–14	[28, 35, 37, 38, 40–44, 80, 88, 91, 109, 110]
pH range with high activity		
Activity ≥70 %	2–9	[35]
Activity ≥80 %	4–6	[59]
	4.5–9.5	[37]
	5–7.5	[107]
	6–8	[41]
	7–8	[38]
Optimum pH		
	3.4	[35]
	5.0	[59]
	5.5	[113]
	6.0	[20, 42, 112]
	6.5	[38, 40]
	7.0	[28, 41, 107, 111]
	7.5	[37]
	8.5	[108]
Temperature range with high activity (°C)		
Activity ≥70 %	40–60	[35]
Activity ≥80 %	40–60	[59]
	40–70	[107]
Optimum temperature (°C)		
	37	[37]
	40	[112]
	48	[35]
	50	[28, 40, 59, 107, 113]
	55	[108]
	60	[38, 41, 111]
	65	[20, 42]
Hydrolysis in long term reactions	40 % hydrolysis in 12 h	[35]
Kinetic constants		
Michaelis-Menten constant (K_m) (% CMC)	0.05–0.87	[20, 35, 41, 59, 107, 110]
Maximum reaction rate (V_{max}) (U/mg)	0.056–33.3	[35, 59]
Inhibition constants (K_{ic}, K_{iu}) (mM)	0.03; 0.35	[35]

In 2000, Chu et al. [93] identified five extracellular proteins of *Bacillus subtilis* K-1, which are specifically induced when

cultured in media with xylan, i.e., a polymer of xylose that constitutes most hemicelluloses. Three of these proteins were related to the metabolism of xylan and were identified as xylose isomerase produced from *Clostridium thermosacchrolyticum*, endo-β-1,4-xylanase from *Bacillus* sp. C-125, and endo-β-1,4-xylanase from *Bacillus stearothermophilus*. The other two identified proteins were similar to 3-dehydroquinate dehydratase produced from *B. subtilis* (related to quinic acid catabolism) and GltC, a regulatory protein of *B. subtilis* (related to the synthesis of glutamate). Furthermore, three other proteins were detected; however, these proteins showed no similarities to other proteins in available databases. The isolation of the *Bacillus* strains with cellulolytic activity has also been reported in semi-arid soils of Brazil and in forest soils [94, 95]. Additionally, Kim et al. [96] reported three strains (SL9-9, C5-16, and S52-2) of *Bacillus* isolated from soil and composts of South Korea that produced CMCase (0.2 to 0.9 U/mL), Avicelase (0.1 to 0.34 U/mL), β-glucosidase (up to 1.2 U/mL), and xylanase (2 to 12 U/mL) in submerged culture, which suggested synergic cellulolytic systems in *B. subtilis*. CMCase, Avicelase, and xylanase activities were observed in cell-free culture supernatants, and β-glucosidase activity was detected in cell debris. This suggested that the three enzymes were extracellular, and β-glucosidase was cell membrane bound.

The *Paenibacillus* genus has been reported in recent years as a producer of lignocellulose-degrading enzymes [88, 97–100]. The *Paenibacillus* sp. B39 strain was isolated from compost made with poultry manure. This strain produced a high molecular weight (148-kDa) cellulase having the activities of Avicelase and carboxymethylcellulase, showing the highest activity at 60 °C at pH 6.5 [38]. The *Paenibacillus campinasensis* BL11 strain was isolated from strongly alkaline black liquor resulting from the washing step of the Kraft process during paper pulp treatment. This strain is thermophilic and spore-forming and produces extracellular enzymes, such as xylanases, two cellulases, pectinase, and cyclodextrin glucanotransferase [37]. Most recently, Ghio et al. [101] reported the isolation of *Paenibacillus* sp. VG-4-A-2 and VG-4-A-3 with cellulolytic activity from soil samples of Argentine forests; these strains were closely related to *Paenibacillus alvei* strains. Subsequently, Ghio et al. [102] identified *Paenibacillus* sp. A59, which showed a broad range of hydrolytic activities, being capable of degrading carboxymethylcellulose (endoglucanase 0.45 U/mL), xylan (xylanase 3.6 U/mL), starch, pectin, casein, and chitin. Fathallh et al. [103] reported the isolation and identification of *Paenibacillus woosongensis* SDCB10 and SDCB11, which have the capacity to produce cellulase, xylanase, β-glucanases, and mannanases on Dubose solid medium. Shi et al. [104] reported on *Paenibacillus* sp. E18, which produces a bifunctional xylanase-glucanase. A wide variety of extracellular cellulases was observed in the *P. polymyxa* BEb-40 strain, which was isolated from decomposing sorghum straw on farmland in El Bajio, Mexico. A thermophilic strain *Brevibacillus* sp. JXL (belonging to the Paenibacillaceae family) was isolated from swine manure and can use crystalline cellulose, carboxymethylcellulose, xylan, cellobiose, glucose, and xylose as carbon sources [39].

Basic information on the production of cellulases by these microorganisms is scarce. The few reports that exist have only described isolating enzymes from culture broths. These enzymes have mainly been endoglucanases produced by thermophilic species of the *Bacillus* genus. These reports have described a few enzymatic properties, such as Michaelis-Menten constants (K_m), maximum reaction rates (V_{max}), and stabilities at extreme temperatures and pH values [28, 40–44, 68, 71]. As mentioned earlier, enzymes obtained from these extremophile microorganisms usually show optimum performance under extreme pH or temperature. The maintenance of this type of hydrolytic condition for production scale operations can be costly. Furthermore, these conditions do not facilitate the application of these enzymes in processes such as simultaneous saccharification and fermentation (SSF), where the fermentative microorganism culture is not adapted for extreme conditions (the optimal temperature for enzymatic hydrolysis is typically close to 55 °C, while that of yeast fermentation is typically at 30 °C) [105, 106]. Herein, we show the most recent information on the qualitative and quantitative characterization of cellulolytic enzymes produced by the strains of the *Bacillus* and *Paenibacillus* genera (Table 2).

Production of Cellulases in Submerged Culture

The production of bacterial cellulolytic enzymes is preferably performed with submerged cultures due to advantages in scaling, optimization, and control. A prior study [40] reported that a maximum cellulase activity (0.26 U/mL) was reached in cultures of thermophilic *B. subtilis* DR grown during 24 h in a CMC-supplemented Luria broth. Other studies have reported high levels of activity in other cellulolytic bacteria of the same order of magnitude. Another study [59] isolated extremophilic strains, *Bhargavaea cecembensis* IARI-M-75 and *Bacillus* sp. IARI-AN-27, which produced 0.79 and 0.89 U/mL of endoglucanase activity, respectively, in 96 h cultures with carboxymethylcellulose-supplemented Reese's minimal medium. Other strains from the same work produced activity levels between 0.1 and 0.8 U/mL. In contrast, a commercial enzyme complex NS50013 (Novozymes) was reported to have a specific CMCase activity of 3.1 $U/mg_{total\ protein}$ at pH 4.8 [107]. In comparison, purified 58-kDa cellulases from *Paenibacillus* spp. were reported to have activities ranging from 3.2 to 72.1 U/mg, for Avicel and CMC as substrates, respectively [108]. A recombinant endoglucanase (Endo5A) from *Paenibacillus* sp. MTCC 5639 and a purified enzyme from *Paenibacillus barcinonensis* MG7 had specific activities

of 14.6–24.2 and 16.88 U/mg, respectively [20, 100, 107]. The endoglucanase complexes from *P. polymyxa* BEb-40 showed specific activities between 14.3 and 33.3 $U/mg_{total\ protein}$ (depending on reaction conditions) [35]. Interestingly, these bacterial enzymes showed higher specific activity than the fungal enzyme complexes mentioned above (Table 2).

Detection of Secreted Isoenzymes with Endoglucanase Activity

Electrophoretic and zymographic one- and two-dimensional methods were used to detect cellulolytic enzymes in culture broth samples. These methods have enabled the study of a variety of isoenzymes, the characterization of their molecular weights and isoelectric points, and their isolation and identification by mass spectrometry. Van Dyk et al. [80] described (using zymography) the presence of three endoglucanase active bands in crude supernatants obtained from cultures of *B. licheniformis*. Other studies have reported the existence of similar numbers of enzymes having endoglucanase activity in the *Bacillus* genus: one of 24.4 kDa produced by *B. licheniformis* [43], two secreted by *B. subtilis* 168 grown in LB broth, separated by 2D PAGE and identified by N-terminal sequencing [109], one of 54 kDa secreted by *Bacillus amyloliquefaciens* DL-3 [28], one of 55 kDa produced by *B. subtilis* DR [40], one of 65 kDa from *Bacillus* sp. [41], two of 27 and 54 kDa found in *B. subtilis* LN [44], and one of 52 kDa produced by *B. subtilis* I15 [42]. Ko et al. [37] reported the presence of three cellulases (42, 57, and 86 kDa) in the culture broths of the thermophilic bacterium *P. campinasensis* BL11. Another strain from the genus *Paenibacillus*, named B39, was observed to secrete a 148-kDa endoglucanase [38]. The strain *P. polymyxa* MTCC10056 was observed to produce two endoglucanases of 26.5 and 34 kDa [110]. More recently, zymograms obtained after the electrophoretic separation of crude enzymes (SDS-PAGE) enabled the detection of the activity of three cellulolytic enzymes with approximate molecular masses of 220, 200, and 130 kDa in *P. polymyxa* EG2 and EG14 strains; these strains were identified as cellulosome producers [91].

Although the variety of isoenzymes detected by zymography has generally been low (between 1 and 3 isoenzymes per strain), a number of endoglucanases have been described for *Paenibacillus* strains. Nine non-secreted endoglucanases over a wide range of molecular weights (from 63 to 216 kDa) were detected in *P. curdlanolyticus* B-6 [88]. Furthermore, at least 14 secreted endoglucanases with molecular weights between 38 and 220 kDa were detected in *P. polymyxa* BEb-40 [35] (Table 2).

Further improvements to zymographic detection techniques will surely help in detecting a growing variety of cellulolytic and hemicellulolytic isoenzymes. The environmental conditions to which cellulolytic microorganisms are subjected have fostered the evolution of isozyme complexes suitable for a wide range of environments. These different environments are favorable for the development of enzymes suitable for industrial applications.

Optimization of Enzymatic Hydrolysis

The primary environmental factors that have been studied for eliciting maximum cellulolytic activity are pH and temperature and, to a lesser extent, substrate concentration and reaction times. Herein, we list optimum temperature and pH values that have been reported for recombinant cellulases and also those that have been purified from culture broths. Lee et al. [28] described a purified cellulase (54 kDa) produced by *B. amyloliquefaciens* DL-3 with optimal conditions at 50 °C and pH 7.0. A 55-kDa endoglucanase from *B. subtilis* DR was reported to have maximum activity at 50 °C and pH 6.5 [40]. Another 65-kDa endoglucanase from *Bacillus* sp. achieved maximum activity at 60 °C and pH 7.0 [41]. The recombinant 52-kDa endoglucanase obtained from *B. subtilis* I15 showed optimal behavior at 65 °C and pH 6.0 [45]. Three endoglucanases secreted by *P. campinasensis* BL11 showed maximum activity at pH 7.5 and 37 °C, and a recombinant 38-kDa enzyme from the same strain was optimized at 60 °C and pH 7.0 [37, 111]. A purified 148-kDa endoglucanase secreted by *Paenibacillus* sp. B39 and a 58.6-kDa enzyme obtained from *P. barcinonensis* had optimal conditions at 60 °C and pH 6.5 and 65 °C and pH 6.0, respectively [20, 38]. Ogawa et al. [108] reported *Paenibacillus* spp. strains that expressed endoglucanases (approximately 58 kDa) that hydrolyzed CMC under alkaline conditions (pH 8.5) at approximately 55–60 °C. A recombinant endoglucanase, Endo5A, from *Paenibacillus* sp. MTCC 5639 and an enzyme, Cel5A (64 kDa), from *Paenibacillus xylanilyticus* were reported to have optimal hydrolysis conditions at pH 7.0 and 50 °C and pH 6.0 and 40 °C, respectively [107, 112]. CMCase activity produced by *Paenibacillus terrae* ME27-1 (2.08 U/mL) was optimized at a pH and temperature of 5.5 and 50 °C, respectively. The CMCase produced by this strain was stable from pH 4.0 to 11.0 at more than 60 % activity levels [113]. Interestingly, most of the reported optimal conditions occurred at pH values close to neutrality or slightly alkaline and at thermophilic temperatures. Thus, these endoglucanases have possible applications under alkaline and high-temperature conditions.

P. polymyxa BEb-40 has recently been reported to produce endoglucanases at optimal reaction conditions of 48 °C and pH 3.4. This enzyme complex showed high levels of activity of at least 70 % of the maximum activity for a wide pH range between 2 and 9 from 40 to 60 °C. These wide ranges enable the application of these enzymes in a wide variety of hydrolytic processes, including those wherein biomasses have been subjected to acidic or alkaline pretreatment over mesophilic to

thermophilic temperatures. Furthermore, these enzymes can even be used in SSF processes [35]. Relatively wide pH and temperature ranges have been reported for cellulolytic complexes from other microorganisms from these genera. The enzyme complex produced by the acidophilic bacterium *Bacillus* sp. IARI-AN-27 can reach ≥80 % of its maximum endoglucanase activity from pH 4 to 6 and between 40 and 60 °C (with optimum conditions at pH 5 and 50 °C). The thermophilic bacterium *B. cecembensis* IARI-M-75 was reported to reach the same levels of activity at a pH range of 4.5 to 6.5 and from 50 to 70 °C (with optimum conditions at pH 5 and 60 °C) [59]. The endoglucanase complex produced by *P. campinasensis* BL11 was stable at pH between 4.5 and 9.5 [37]. Other purified endoglucanases have shown narrower ranges of stability, such as those described by Afzal et al. [41] with activities of at least 80 % of respective maximums at pH levels between 6 and 8. A 148-kDa endoglucanase purified from *Paenibacillus* sp. B39 retained at least 80 % of its maximum activity at a pH range from 7.0 to 8.0 [38]. A recombinant endoglucanase Endo5A from *Paenibacillus* sp. MTCC 5639 retained at least 80 % of its maximum activity at a pH range between 5 and 7.5 and from 40 to 70 °C [107]. As can be seen, for these two bacterial genera are enzymes with wide pH and temperature values which show high enzyme activity (Table 2).

Cellulose Hydrolysis in Long-Term Reactions

Enzymatic cellulose hydrolyses are typically carried out over prolonged reaction times due to low rates of reactions. Substrate levels affect the yield and initial rates of enzymatic reactions, and the extent of this effect is dependent on the ratio of total substrate to total enzyme [114]. For this reason, the performance of newly discovered enzymes should be evaluated under operating conditions that allow for their analysis in potential industrial applications. A number of factors should be varied, including the substrate to total protein ratio (S/TP), the reaction time, and the presence of reaction inhibitors, to determine the maximum yield of fermentable sugars. For example, for fungal purified exoglucanase Cel7A from *Trichoderma reesei*, Bezerra and Dias [115] reported the production of cellobiose using Avicel as a substrate. They found that the maximum cellobiose concentration after 47 h (approximately 8 g/L) was not affected for substrate/enzyme ratios between 37 and 92 (for enzyme concentrations between 600 and 1000 μg/mL and for substrate concentrations between 36,758 and 55,000 μg/mL). Similarly, Gastelum-Arellanez et al. [35], using an endoglucanase complex from *P. polymyxa* BEb-40, recorded CMC hydrolysis data for long-term reactions. After a 10–12-h reaction period, they found that the S/TP ratio increased up to a value of 76 had no significant effects on the CMC hydrolysis level (approximately 40 % of the theoretical maximum). The effects of the S/TP ratio are technically and economically important when considering the potential applications of enzyme complexes because high ratios allow for reduced enzyme loads and therefore, reduced costs. However, few studies have investigated the effects of this ratio for cellulolytic enzymes from *Bacillus* and *Paenibacillus*.

Kinetic Characterization of Cellulase Activity

Cellulolytic enzymes are frequently subjected to competing inhibition effects involving the product of the enzyme reactions, free sugars, and oligosaccharides. The potential applications of an enzyme complex will depend largely on its maximum reaction rate (V_{max}), Michaelis-Menten constant (K_m), and inhibition constants (K_{ic} and K_{iu}). Michaelis-Menten constants were reported for endoglucanases produced by *Bacillus* sp. IARI-AN-27 and *B. cecembensis* IARI-M-75 (0.11 and 0.31 % CMC, respectively) with V_{max} values of 0.635 and 0.056 U/mg [59]. A few purified endoglucanases have shown K_m values comparable with those mentioned above. A 65-kDa endoglucanase produced by *Bacillus* sp. had a K_m value of 0.41 % CMC [41]. Likewise, a 52-kDa endoglucanase from *B. subtilis* I15 presented a K_m of 0.36 % CMC [110]. Recombinant endoglucanases, Endo5a from *Paenibacillus* sp. MTCC 5639 and a 58.6-kDa enzyme from *P. barcinonensis*, were reported to have K_m values of 0.097 and 0.05 % CMC, respectively [20, 107]. Another endoglucanase produced by *P. polymyxa* MTCC10056 showed a K_m value of 0.87 % CMC [113].

An endoglucanase complex of *P. polymyxa* BEb-40 was reported to have a V_{max} = 33.3 U/mg$_{total\ protein}$, which was approximately 52 and 595 times higher than the maximum reaction rates of a few of the above-described enzymes. The K_m value was 0.14 % CMC, and the inhibition constants K_{ic} and K_{iu} were 0.03 and 0.35 mM, respectively, i.e., up to two orders of magnitude lower than the K_m, indicating an important end-product-inhibition effect [35]. The higher maximum specific activities reported for enzymes produced from these two bacterial genera would allow for decreased total enzyme loads (and decreased costs) for cellulose hydrolysis processes. However, for significant inhibitory effects, these enzymes could be used primarily in SSF processes, where the consumption of reducing sugars by the cultured microorganism would reduce the effects of end-product-inhibition. However, data on such processes have not been reported in studies of cellulolytic complexes from *Bacillus* and *Paenibacillus*. Most studies have only focused on determining the apparent values of K_m and V_{max} and have not considered the inhibitory reactions (Table 2).

Conclusion

The *Bacillus* and *Paenibacillus* genera have strains that have produced various commercially applied enzymes and

metabolites. Regarding cellulases, the strains have produced enzymes with very interesting catalytic properties in industrial applications. A few of these strains are capable of producing a wide variety of isoenzymes and have high production yields in submerged cultures. These bacterial enzyme complexes have generally shown high levels of specific activity on lignocellulosic substrates, with competitive hydrolysis yields (and at times, even higher yields) comparable with most cellulolytic fungal enzymes. Furthermore, these complexes have high activity and yields over wide ranges of pH and temperatures, making them attractive and worthwhile to further explore to identify new strains for use in industrial processes for obtaining biofuels and other value-added products. The study and characterization of cellulases present in the secretomes of these cellulolytic strains will help improve the basic understanding of their behavior and generate the information and tools necessary for the development of possible commercial applications.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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