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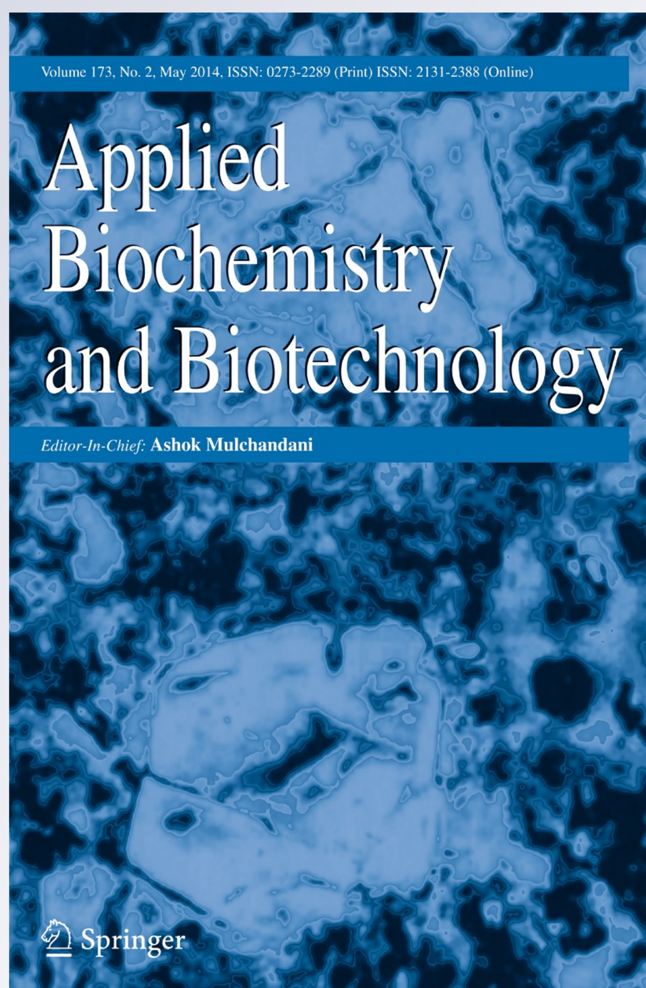

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Cloning of LicB from *Clostridium thermocellum* and its efficient secretive expression of thermostable β-1,3-1,4-glucanase

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Abstract β-1,3-1,4-glucanase is a widely used enzyme in brewing and in animal feed processing. To produce the bacterial enzyme at an industrial scale, the enzyme should be able to be secreted from microbial cells into fermentation broth and be stable in different conditions. In this study, the *LicB* gene encoding β-1,3-1,4-glucanase (lichenase) from *Clostridium thermocellum* was secretively expressed in a secretive strain, *Bacillus subtilis* WB800, with eight extracellular protease deletion which made LicB expressed obviously and reached 1.18 U/g cell mass. The secreted β-1,3-1,4-glucanase was found to be active from 40 °C to 80 °C and achieved the optimal activity at 80 °C. The enzyme also has a wide pH range (pH 4–11). The most common metal ions and chemicals were found to be inert on its activity. The property of *LicB*-encoded β-1,3-1,4-glucanase and its efficient secretive expression makes it a potential enzyme for industrial production and application.

Keywords β-1,3-1,4-glucanase · *LicB* · *Clostridium thermocellum* · Secretive expression · Thermostability

Introduction

β-glucan is a natural occurring polysaccharide in bacterial, fungi, and plant cell wall, such as barley, rye, rice, and wheat [1]. In the cell wall of cereal crops, β-glucan exists in a form of β-1,3-1,4-glucan linked by β-1,3 and β-1,4 D-glucopyranosyl units [2]. In the brewing processes, β-glucan may cause turbid or gel formation problems in purification and storage. To solve the problems, β-glucanase is commonly used to degrade β-glucan during milling and in fermentation processes [3, 4]. β-glucanase is also be used to improve the digestibility of barley-based feeds and diets [5].

Z. Luo · Q. Gao · J. Bao (✉)
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
e-mail: jbao@ecust.edu.cn

X. Li
Youtell Biotech Co, 526 Ruiqing Road, Pudong District, Shanghai 201201, China

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β-1,3-1,4-glucanase is an endo-β-glucanase and selectively hydrolyze the combined β-1,3- and β-1,4-linked glucans, but does not hydrolyze a single β-1,4-linked glucan in carboxymethyl cellulose or β-1,3-linked glucan [6]. β-1,3-1,4-glucanase is produced by *Bacillus* strains including *Bacillus subtilis* [7], *Bacillus amyloliquefaciens* [8], *Bacillus sphaericus* [9], *Bacillus licheniformis* [10], and *Bacillus circulans* [11, 12]; but, the major disadvantage of β-1,3-1,4-glucanases from *Bacillus* strains is its weak thermal stability (less than 50 °C). β-1,3-1,4-glucanases from *Chaetomium thermophilum* [13] and *Paecilomyces thermophila* [14] are also not stable at high temperature (50–70 °C); besides, its narrow pH availability (pH 4–7). β-1,3-1,4-glucanase (LicB) from *Clostridium thermocellum* may possess thermostability because of its high thermo- and pH tolerance [1]. The molecular mass of LicB gene translation product is 37.9 kDa. The protein consists of an N-terminal signal peptide, a catalytic region, a segment rich in Pro and The residues, and a C-terminal reiterated domain. The catalytic region shows close similarity to lichenases of bacilli (52–58 % identity) and *Fibrobacter succinogenes* (35 % identity) [15]. But, the enzyme is retained as intracellular protein and is not suitable for an industrial-scale application.

The secretive property of protein is an important factor in the application of a large-scale production. *B. subtilis* is frequently used for the secretive expression of various heterogeneous proteins for its excellent protein secretive capacity, utilization of hexose and pentose, fast growth rate, and low nutrient need. In this study, a β-1,3-1,4-glucanase gene *LicB* was cloned from *C. thermocellum* DSM 1237 and secretively expressed in *B. subtilis* WB800 [16]. The extracellular enzyme was collected and used to hydrolyze β-1,3-1,4-glucan in a wide range of temperature and pH. The result shows that the β-1,3-1,4-glucanase originated from *C. thermocellum* demonstrated an excellent thermostability up to 80 °C and a wide pH range from 4 to 11. This secretively produced thermostable β-1,3-1,4-glucanase provided a potential in the food and animal feed applications.

Materials and methods

Strains and plasmids

The strains and plasmids used in this study are shown in Table 1. *B. subtilis* WB800 [16] (deficient of eight extracellular protease genes) was provided by Dr. Y.H.P. Zhang, Virginia Polytechnic Institute and State University. The shuttle vector between *Escherichia coli* and *B. subtilis* pP43JM2 was constructed in our previous studies [17]. Both *E. coli* and *B. subtilis*

Table 1 Strains and plasmids

Strains/plasmids	Characteristics	Sources
Strains		
E. coli DH5α		[13]
B. subtilis WB800	*nprE*, *aprE*, *epr*, *bpr*, *mpr::ble*, *nprB::bsr*, *Δvpr*, *wprA::hyg*	[13]
C. thermocellum DSM1237	Wild type	DSMZ
Plasmids		
pP43JM2	K_m^r *E. coli–B. subtilis* shuttle vector	[13]
pP43JM2-*LicB*	K_m^r *E. coli–B. subtilis* shuttle vector	In this study

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were cultured at 37 °C in LB medium. The transformation of *B. subtilis* WB800 with plasmids was carried out by chemical competence method [18].

Reagents and chemicals

β-1,3-1,4-glucan was purchased from Jiangsu Huayu Chemical Co. (Nanjing, China). Restriction enzymes were purchased from Fermentas (Vilnius, Lithuania). T4 DNA ligase and PrimerSTAR HS DNA polymerase used in PCR were purchased from Takara (Dalian, China). Tryptone and yeast extract were from Oxoid (Cambridge, UK). Bacterial DNA kit was from Omega Bio-Tek (Norcross, GA, USA). PCR purification kit, plasmid mini kit, and gel extraction kit were from Sangong Biotech (Shanghai, China). All other chemicals used in this study were purchased from Sinopharm Chemical Reagent (Shanghai, China).

Gene cloning and enzyme purification

The *LicB* gene fragment was amplified from *C. thermocellum* DSM1237 genomic DNA using the primers TCTAGAATGAAAAACAGGGTAATTTC (forward) and AAGCTTTTCAAAGTGACGGAATTG (reverse). The PCR product and the vector pP43JM2 were cleaved by *XbaI* and *HindIII*, gel purified, and ligated into pP43JM2 to give the plasmid pP43JM2-*LicB*. The *LicB* gene fragment was located at the downstream of the promoter P43 and the signal peptide *NprB* (Fig. 1).

pP43JM2-*LicB* was introduced into *B. subtilis* W800 to the recombinant strain *B. subtilis* pP43JM2-*LicB*. The recombinant was cultivated in 20 ml LB medium with 50 μg/ml kanamycin at 37 °C, 220 rpm for 24 h. One-milliliter media (OD_{600nm}=2.0) was collected, and the cells were centrifuged at 10,000×*g* for 5 min at 4 °C; then, the strain was collected. The cell was suspended and washed by 1 ml 50 mM Tris–HCl buffer (pH 7). Then, the cell was crushed by ultrasonication, centrifuged at 16,000×*g* for 5 min at 4 °C, and then, the supernatant was

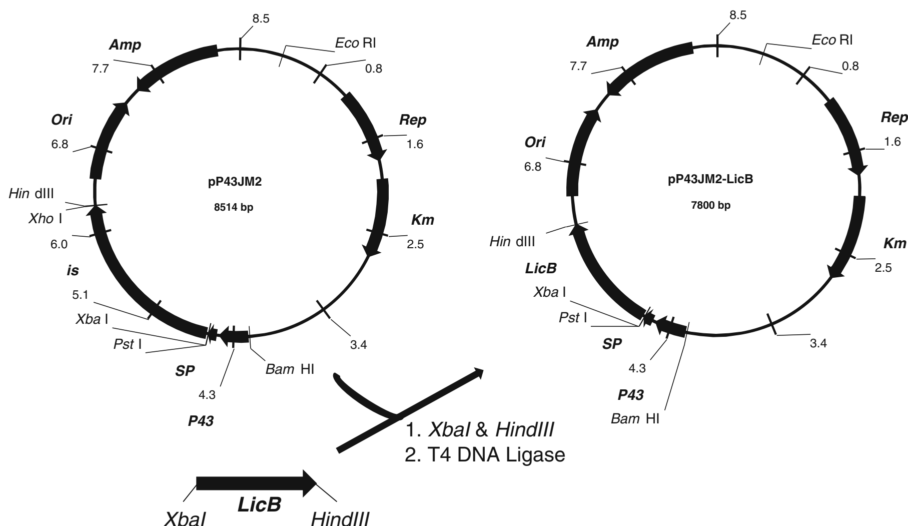

Fig. 1 Shuttle vector pP43JM2 and recombinant plasmid pP43JM2-*LicB*. *LicB* fragment was amplified by PCR. The PCR product and the vector pP43JM2 were cleaved by *XbaI* and *HindIII*, gel purified, and ligated to give the plasmid pP43JM2-*LicB*

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collected to give the intracellular protein sample. A 15-μl sample was added in the SDS-PAGE. To get the extracellular protein sample, 20 ml media (OD_{600nm}=2.0) was collected and centrifuged at 10,000×*g* for 5 min at 4 °C, and then, the supernatant was collected. The proteins in the supernatant were precipitated using ammonium sulfate at 60 % (*w*/*v*) saturation and redissolved in 1 ml 50 mM Tris–HCl buffer (pH 7) to give the crude enzyme solution. A 15-μl sample was added in SDS-PAGE.

β-1,3-1,4-glucanase activity assay

A 1-ml extracellular enzyme solution, dissolved by 50 mM Tris–HCl buffer (pH 7), was mixed with a 1-ml substrate solution containing 1 g/l β-1,3-1,4-glucan. The mixture was incubated for 5 min and denatured by boiling for 3 min. The concentration of β-1,3-1,4-glucan in the solution was measured by congo red spectroscopic method [19]. A 1-ml solution of 1 g/l β-1,3-1,4-glucan was mixed with 1 ml Tris–HCl buffer (pH 7), which was used as a control to ensure that β-1,3-1,4-glucan was stable in the assay condition. One unit (U) of the enzyme was defined as the enzyme amount for hydrolyzing 1 g of β-1,3-1,4-glucan per minute. Sodium acetate buffer (pH 3–5) and Tris–HCl buffer (pH 6–12) were used to detect the enzyme activity. A 10-mM metal ion or chemicals were added to assay the effect of different metal ions on the enzyme activity.

Congo red spectroscopic assay was carried out by preparing 0.1 mol/l congo red solution (dissolved by pH=8.0 phosphate buffer), and β-1,3-1,4-glucan solution of known concentration was used to give a calibration curve (0–0.1 g/l). The 1 ml diluted sample was mixed with 2 ml congo red solution, and then, the mixture was incubated for 30 min at 20 °C. The optical density at 545 nm (OD_{545}) value of the solution was determined to obtain the concentration of β-1,3-1,4-glucan.

Results and discussion

Expression and characterization of secreted β-1,3-1,4-glucanase

The recombinant *B. subtilis* WB800/pP43JM2-*LicB* was cultured in LB medium for 24 h. The SDS-PAGE of both the intracellular and extracellular proteins was shown in Fig. 2. A single band close to the molecular weight (MW) of *LicB* (37.9 kDa) [15] was found in both the intra- and extracellular expressions of *B. subtilis* WB800/pP43JM2-*LicB*, indicating that *LicB* was efficiently expressed by *B. subtilis* WB800/pP43JM2-*LicB* and was successfully secreted into the extracellular space.

From a preliminary experiment, LicB showed an obvious activity in the condition of 60 °C. The recombinant *B. subtilis* WB800/pP43JM2-*LicB* was cultured in LB medium for 18 h, and then, the extracellular LicB activity was assayed at the condition from pH 3 to 12 at 60 °C for 5 min. The result was shown in Fig. 3, indicating that extracellular LicB exhibited optimal activity at pH 7.0, and it was active within a wide pH range. From pH 6 to 11, LicB could retain more than 80 % of its maximum activity, and in pH 4–5, it still retained more than 60 % of its activity. Compared with the thermal β-1,3-1,4-glucanase from *C. thermophilum* or *P. thermophila* which maintained its 80 % activity within pH 4–6 or pH 6–8 separately [13, 14], LicB showed a wide pH active range, especially at the high pH condition. This is an advantage for LicB, especially since it would be applied in many industrial uses.

When the enzyme is used in industrial processes, it may work in the environment with some metal ions from various sources such as water used for the preparation of a medium. Therefore,

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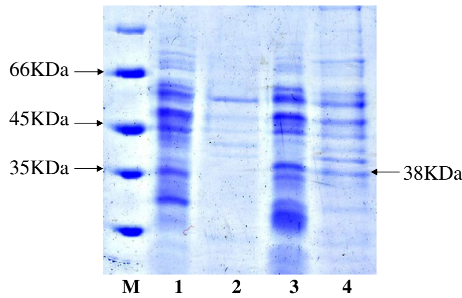

Fig. 2 SDS-PAGE of extracellular protein of *B. subtilis* WB800 and *B. subtilis* pP43JM2-*LicB*. *Lanes 1 B. subtilis* WB800 cells, *2 B. subtilis* WB800 extracellular protein, *3 B. subtilis* pP43JM2-*LicB* cells, and *4 B. subtilis* pP43JM2-*LicB* extracellular protein. Activity was estimated as a percentage of the maximum. The maximum activity was defined as 100 %

the effect of the metal ions on the enzyme activity of the LicB encoding β-1,3-1,4-glucanase was measured. The effect of various metal ions and chemicals at a typical concentration, 10 mM, was assayed at pH 7 and 60 °C, as shown in Table 2. Among these metal ions tested, the enzyme activity decreased seriously in the presence of Cu^{2+} (49.19 %) and Fe^{2+} (63.07 %). Zn^{2+} also decreased about 15 % of the activity. The presence of EDTA (95.56 %), Mn^{2+} (93.41 %), and Co^{2+} (96.59 %) reduced the enzyme activity slightly. Mg^{2+}, K^{+}, Ca^{+}, and Na^{+} almost did not affect the enzyme activity, and the addition of K^{+} (101.47 %) enhanced the activity. LicB was sensitive to Cu^{2+} and Fe^{2+}; but, its activity would not be affected obviously in most conditions with different metal ions.

Effect of temperature on enzyme activity and thermostability

The recombinant *B. subtilis* WB800/pP43JM2-*LicB* was cultured in LB medium for 18 h, and the assay of the extracellular β-1,3-1,4-glucanase activity of recombinant at different temperature profiles was shown in Fig. 4(a). The optimal temperature was 80 °C, and the maximum activity was up to 0.587 U/g cell mass. The control strains of two *B. subtilis* strains, *B. subtilis* WB800 and *B. subtilis* WB800/JM2, showed low extracellular activity of 0.049 and 0.105 U/g cell mass at 80 °C, respectively. On the other hand, the expression of LicB in *E. coli* also showed an obvious β-1,3-1,4-glucanase activity, which was reported in the previous

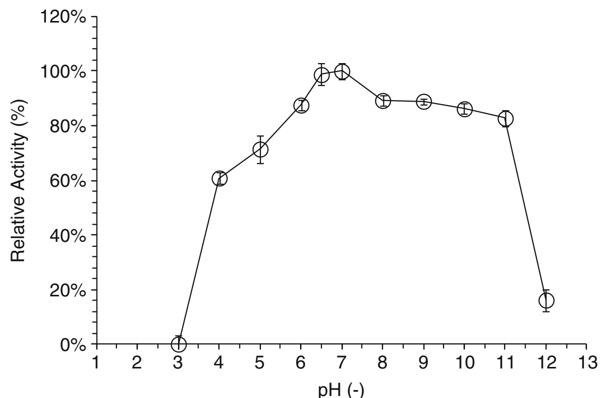

Fig. 3 Extracellular β-1,3-1,4-glucanase activity assay of *B. subtilis* pP43JM2-*LicB* in different pH condition at 80 °C. Sodium acetate buffer (pH 3–5) and Tris–HCl buffer (pH 7–12) were used to apply different pH condition

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Table 2 Effect of metal ions or chemicals on the activity of LicB

Metal ion	Relative activity (%)
Control	100.00
Co^{2+}	96.59±2.12
Fe^{2+}	49.19±3.55
Ca^{2+}	100.03±1.04
K^{+}	101.47±1.83
Mn^{2+}	93.41±2.91
Cu^{2+}	63.07±3.02
Zn^{2+}	85.40±2.66
Na^{+}	99.91±1.09
Mg^{2+}	99.93±0.28
EDTA	95.56±2.47

Enzyme activity was assayed in the presence of a 10 mM concentration of different metal ions at the condition of pH 7 and 60 °C. Activity assayed in the absence of metal ions was taken to be 100 %

publications [1]. So, we ensure that the activity is due to the recombinant protein, especially at high temperature.

The kinetic parameters of the enzyme were measured using β-1,3-1,4-glucan as the substrate at 80 °C, pH 7.0, as shown in Fig. 5. The K_m and V_{max} values were calculated from the Lineweaver–Burk plotting as 1.65±0.13 mg/ml and 0.43±0.03 g/min·mg protein, respectively. Comparing the β-1,3-1,4-glucan enzyme from *B. subtilis* in [20], the K_m value is 2.7 mg/ml at pH 6.0 and 70 °C using β-glucan as the substrate. The lower K_m value may come from the difference of assay conditions (the optimal condition for LicB enzyme in this study is pH 7.0 and 80 °C) and the different substrates used. LicB selectively hydrolyzes the combined β-1,3- and β-1,4-linked glucans, but does not hydrolyze a single β-1,4-linked glucan carboxymethyl cellulose or β-1,3-linked glucan sufficiently. Thus, the use of β-1,3-1,4-glucan as a substrate may lead to the higher affinity to the enzyme and lower value of K_m than that using β-glucan as the substrate.

It is a high optimal temperature when the optimal temperature of common β-1,3-1,4-glucanase would not be higher than 60 °C [7–12, 21]. The β-1,3-1,4-glucanase from the thermal strain *P. thermophila* showed that its optimal temperature is at 70 °C, but it lost nearly 80 % of its activity at 80 °C. LicB showed a maximum act at 80 °C, and approximately 40 % of its maximum activity was maintained at 90 °C. The extracellular β-1,3-1,4-glucanase activity of the control strains, *B. subtilis* WB800 and *B. subtilis* WB800/pP43JM2, appeared obviously only from 40 °C to 60 °C with much lower values (0.217 U/g cell mass and 0.160 U/g cell mass, respectively), and decreased sharply at the elevated temperature of 60 °C. The results showed the thermal extracellular β-1,3-1,4-glucanase activity of *B. subtilis* WB800/pP43JM2-*LicB* at high temperature, which indicated that *LicB* gene from *C. thermocellum* was secretively expressed in the host strain *B. subtilis* WB800 and demonstrated an extraordinary high temperature performance in *B. subtilis.*

Since LicB was secreted by the recombinant strain, then it should be stable and accumulated in culture media at the culture condition. The recombinant was cultured in LB medium for 30 h to the steady stage of the strain, and the extracellular β-1,3-1,4-glucanase activity was assayed as shown in Fig. 4(b). In the exponential phase (culture for 18 h), all the enzyme samples of these three strains were prepared when the strains grew to about $OD_{600\ nm}$=2.5. And in the steady phase (culture for 30 h), *B. subtilis* WB800 grew to $OD_{600\ nm}$=6.0, while *B. subtilis* WB800/pP43JM2 and *B. subtilis* WB800/pP43JM2-*LicB* grew to OD_{600nm}=4.5; *B. subtilis* WB800 without plasmid can grow better in the same culture condition. The results

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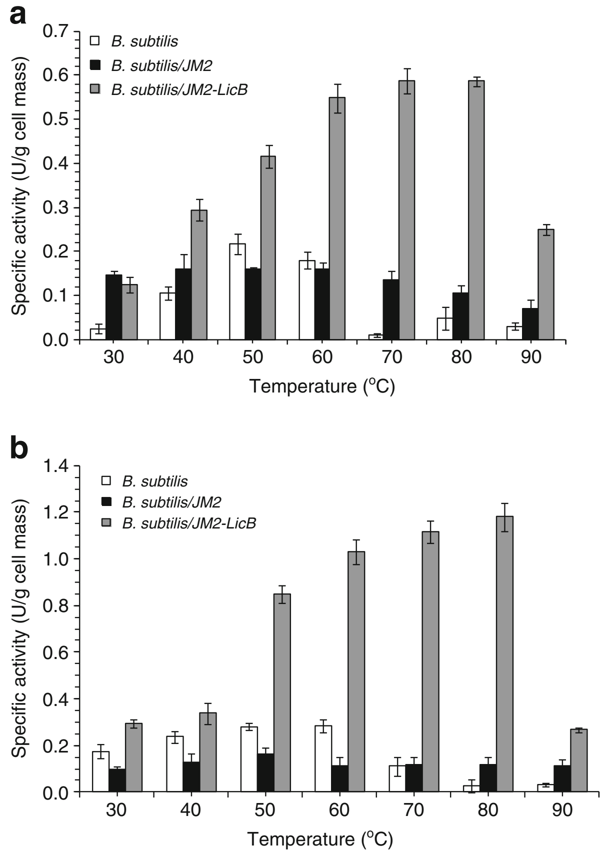

Fig. 4 Extracellular β-1,3-1,4-glucanase activity assay in exponential phase (**a**) and steady phase (**b**). *B. subtilis* WB800 and *B. subtilis* pP43JM2 were used as the control strains. The cell growth was measured by optical density at 600 nm. In exponential phase, all the crude enzyme sample of these three strains were prepared when the strains grew to about OD_{600nm}=2.5. And in steady phase, *B. subtilis* WB800 grew to OD_{600nm}=6.0, while *B. subtilis* WB800/pP43JM2 and *B. subtilis* WB800/pP43JM2-*LicB* grew to OD_{600nm}=4.5 (*B. subtilis* without plasmid can grow better in the same culture condition); 1 U/g corresponds to the 1-g strain hydrolyze 1 g β-1,3-1,4-glucan per minute (The dry cell weight of these three strains has been measured; data not shown)

show that both the two control strains, *B. subtilis* WB800 and *B. subtilis* WB800/pP43JM2, kept the low enzyme activity in the complete culture period; although, there are more than a twofold cell growth: *B. subtilis* WB800 was 0.217 U/g cell mass in growing phase and 0.281 U/g cell mass in steady phase at 50 °C; while for *B. subtilis* WB800/pP43JM2, the activity was 0.160 U/g cell mass in the growing phase and 0.165 U/g cell mass in the steady phase. On the other hand, the extracellular β-1,3-1,4-glucanase activity of *B. subtilis* WB800/pP43JM2-*LicB* increased twice with the growth and accumulation of the cell mass, from 0.587 to 1.1808 U/g cell mass at 80 °C. The extracellular *LicB* protein seems to be stable in the culture media and condition, which may result from its wide pH adaptability and thermostability. Then, LicB could be secretively produced and accumulated in the media with the growth of the recombinant strain; this is suitable for producing and collecting the enzyme in a large scale or conducting fermentation by using the recombinant strain.

β-1,3-1,4-glucanase should be thermal stable at the high temperature such as in brewing process [22]. The rates of thermal inactivation of LicB were further investigated in the temperature range at 70 °C and 80 °C. *LicB* achieved its maximum activity, but it lost all the activity during less than 1 h at 80 °C. In the condition of 70 °C, it retained more than 50 % activity during the 5 h incubation. The half-lives of β-1,3-1,4-glucanase from *P. thermophila* at 70 °C and 80 °C were 174 and 13 min, respectively (Fig. 6) [14]. Compared with this thermal

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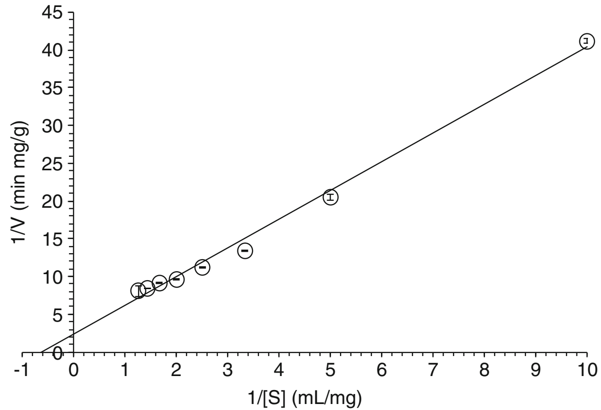

Fig. 5 Lineweaver–Burk plot for determination of K_m and V_{max} values of LicB. The equation was $y=3.814x+2.3086$, $R^2=0.9931$

β-1,3-1,4-glucanase which has the optimal temperature at 70 °C, *LicB* showed the similar inactive trend at 80 °C, but a much higher stability at 70 °C.

From this result, *LicB* showed a good stability at 80 °C, and then, it can be widely used for its thermo-tolerance. But, compared with the expression in *E. coli* [1] which retained 60 % of total activity after a 7 h incubation at both 70 °C and 80 °C, the thermal stability of *LicB* is lower when it was secreted by *B. subtilis*. It is sure that the fold of protein will affect the thermal stability of the enzyme, and the secretive expression may make it become a thermo-labile configuration.

Conclusion

In this study, we introduced *LicB*, which was secreted from *C. thermocellum*, into *B. subtilis* WB800, and made the recombinant strain *B. subtilis* WB800/pP43JM2-*LicB* have the ability

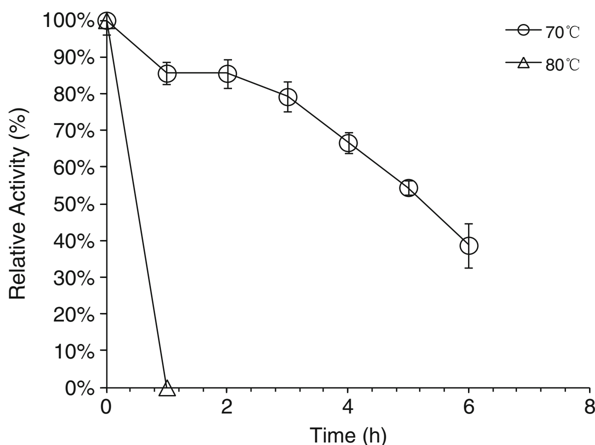

Fig. 6 Thermal stability of extracellular β-1,3-1,4-glucanase of *B. subtilis* pP43JM2-*LicB*. Extracellular enzyme extracts were incubated at 70 °C (*square*) or 80 °C (*diamond*) in the absence of substrate and then assay its activity at 80 °C, pH=7. The activity was estimated as a percentage of the maximum. The original activity was regard as the 100 % activity

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to secrete thermostable β-1,3-1,4-glucanase, which showed the optimal β-1,3-1,4-glucanase activity at 80 °C and pH=7, and it was active within a wide pH range (pH 4–11). The enzyme was inhibited obviously in the presence of Cu^{2+} and Fe^{2+}; but, it kept the activity when other metal ion was added. The secreted protein retained the activity in the extracellular condition and more than 50 % activity when being incubated at 70 °C for 5 h. The thermostability and pH adaptability show that LicB can be widely used in the industrial processing. Now, thermal stable β-1,3-1,4-glucanase *LicB* is able to be secreted in *B. subtilis* WB800; some further biotechnological methods, such as the construction of fusion protein [12, 23] or the codon optimization [2], were also excepted to promote the activity and thermostability of *LicB*.

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