# Degumming Crude Enzyme Produced by *Bacillus cereus* HDYM-02 and its Application in Flax Retting

Dan Zhao,[a,b] Chao Pan,[a,b] Wenxiang Ping,[a,b] and Jingping Ge [a,b,*]

A cellulase-free, degumming crude enzyme was produced by *Bacillus cereus* HDYM-02, using konjaku flour as an inexpensive substrate. After 48 h fermentation, this crude enzyme consisted of pectinase and mannanase, whose maximum activity was 756.7 U/mL and 2967.3 U/mL, respectively. This crude enzyme exhibited considerable stability under the conditions resembling industrial flax retting. After 120 h incubation, more than 50% of the maximum activity of both pectinase and mannanase was retained at pH value 4.0 to 7.0, and at least 70% of the maximum activity was detected at 25 °C to 40 °C. The degumming liquid retted by this crude enzyme contained more galacturonic acid and reducing sugar than those in the degumming liquid retted by commercial pectinase. The application of *B. cereus* HDYM-02 crude enzyme resulted in higher weight loss of flax stems, better properties, higher productivities, and smoother surfaces of flax fibres. This study showed promise for the use of *B. cereus* HDYM-02 crude enzyme for flax retting in the textile industry.

*Keywords: Flax; Retting; Degumming; Pectinase; Mannanase; Bacillus cereus*

*Contact information: a: Laboratory of Microbiology, College of Life Science, Heilongjiang University, Harbin, Heilongjiang, 150080 P. R. China; b: Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education, Harbin, Heilongjiang, 150080 P. R. China;*
**Corresponding authors:gejingping@126.com; zhaodan4u@163.com*

## INTRODUCTION

Flax (*Linum usitatissimum*) is an annual bast fibre plant. Flax fibre is known as the "fibre queen". Flax fibre is widely used in the textile, material, and medical fields, due to its desirable properties such as being antimicrobial, moisture retentive, and air permeable (Kulma *et al.* 2015; Wang *et al.* 2015; Bonizzoni *et al.* 2016). Flax stems contain cellulose, pectin, hemicellulose, lignin, waxy substance, aromatic compounds, ash, *etc*. The content of each component varies depending on flax varieties and analytical methods (Yan *et al.* 2014). The process to obtain pure flax fibre is called degumming, during which the flax stem is retted in water or dew. In the course of degumming, gummy substances in the stem are removed by enzymes, which are either produced by microbial strains or added directly. Many degumming enzyme-produced microbial genera, including *Bacillus*, *Aspergillus*, and *Clostridium*, have been used in microbial degumming (Di Candilo *et al.* 2010; Tian *et al.* 2014; Zhao *et al.* 2016). Though microbial degumming shortens the retting duration and improves fibre properties, the microbial strains themselves probably introduce contamination to the environment. However, purified commercial enzyme costs too much. Degumming by means of crude enzyme, *i.e.* microbial fermentation supernatant, is gaining increased interest to degum in industrial retting because it costs less and is more eco-friendly (Sharma *et al.* 2011; Guo *et al.* 2013).

Among various gummy substances existing in flax stems, pectin is the most predominant component (Akin 2010). Therefore, most studies have focused on the degradation of pectin by pectinolytic enzymes (Das *et al.* 2012; Chiliveri *et al.* 2016). Besides pectin, hemicellulose, mainly represented by mannan, also takes up a large proportion of the flax gummy substances (Akin 2010).Mannan needs to be degraded by mannanase. Therefore, the thorough removal of gummy substances needs the synergistic action of more than one specific degumming enzyme. Pectinase and mannanase are both essential. In recent years, several degumming crude enzymes fermented by various microbial strains with inexpensive substrates have been used in retting experiments and have been shown to enhance degumming efficiencies, such as accelerating gum removal and increasing fibre productivity (Evans *et al.* 2002; Guo *et al.* 2013; Tian *et al.* 2014; Zhao *et al.* 2017a). Nevertheless, since flax fibre is popular and short in demand, and crude enzymes that contain more specific degumming enzymes with high activities are still needed in the textile industry.

*B. cereus* HDYM-02 was isolated from flax degumming liquid in a previous study (Ge *et al.* 2008). This bacterial strain has been applied in flax retting in the form of direct inoculation (Ge *et al.* 2006; Zhao *et al.* 2016), indicating its degumming potential. In this research, the composition of crude enzyme produced by *B. cereus* HDYM-02 was investigated with polysaccharides as substrates. The activities of pectinase and mannanase were measured periodically, and the stability of the pH value and temperature under conditions similar to industrial retting was evaluated. The flax retting experiment was conducted with the addition of *B. cereus* HDYM-02 crude enzyme, while commercial pectinase retting and natural retting were set as the positive and negative control, respectively. The removal extent of gummy substances together with the properties and productivities of flax fibres were detected to estimate the application potential of this crude enzyme for flax degumming in the textile industry.

## EXPERIMENTAL

### Materials

*Microbial strains and media*

*B. cereus* HDYM-02, used for degumming crude enzyme production in this research, was isolated from the flax degumming liquid (Ge *et al.* 2008), stored, and sub-cultured every two months at 4 °C on Baird-Parker(BP) agar slants until its use.

The BP medium (w/v) consisted of peptone 1%, beef extract 0.3%, NaCl 1%, and had a pH value of 7.0 to 7.2. *B. cereus* HDYM-02 was cultivated in the BP medium, and the flasks were shaken at 37 °C at 120 r/min for 12 h. Cells were collected, washed twice with 0.85% NaCl solution, and adjusted to $10^8$ cell/mL as the seed inoculums.

The fermentation medium (w/v), which was used for degumming crude enzyme production, contained 2% of one of the following three poly-saccharide substrates, *i.e.* pectin (PEC), konjaku flour (KON), or carboxymethyl cellulose (CMC); and also consisted of 0.5% yeast, 0.5% sodium nitrate ($NaNO_3$), 0.5% dipotassium phosphate ($K_2HPO_4$),0.02% magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and the pH value ranged between 7.0 to 7.2. Fermentation experiments were conducted at 37 °C at 120 r/min with 2% seed inoculation (v/v). The culture supernatants without cells were used as the degumming crude enzyme.

*Chemicals*

Pectin (CAS 9000-69-5) and konjaku flour were purchased from Sigma Aldrich (Shanghai, China) and Jinxing Konjaku Flour Factory (Chengdu, China), respectively. Petinase (CAS9032-75-1) was purchased from Uteam-BIOTECH (Shanghai, China).All other chemicals were analytical reagents.

## Methods

*Enzyme activity determination*

The pectinase activity was measured using the method of Sharma *et al*. (2011). One unit of enzyme activity was defined as the amount of enzyme that released 1 μg of galacturonic acid per min under certain conditions. The determination of mannanase activity was in accordance with the method of Akino *et al*. (1988). One unit of activity was defined as the amount of enzyme that caused the production of 1μmol of mannose per min.

*Flax retting experiment and relative parameters assay*

Flax seeds were purchased from Heilongjiang Academy of Agriculture Sciences (Harbin, China)and planted in fields (Harbin, China). Flax stems were harvested after approximately 110 days of growth and dried for retting. The flax retting experiment was conducted by following the authors' previous report (Zhao *et al.* 2017a) at 35 °C for 120 h. Three flax retting groups were set as follows: CE indicated adding crude enzyme obtained in this research, CP indicated adding commercial pectinase with the same dose as CE, and NR indicated natural retting. Each retting group was conducted three times independently.

The residual activity of pectinase and mannanase was measured after the crude enzyme was incubated for 120 h, at pH 4.0 to 7.0 and 25 °C to 40 °C, respectively. In the degumming liquid, the content of reducing sugar was measured *via* the DNS (dinitrosalicylic acid) method (Miller 1959), while galacturonic acid was assayed using the method of Dietz and Rouse (1953). Bacterial growth was measured on BP agar plate with the unit of cfu/mL (colony forming unit per milliliter). Flax fibres were obtained by manual decortication. The strength, ratio variation of strength, long fiber ratio, fibre yield, residual gum and fineness were measured *via* the China national standard GB/T 17345-31 (2008).The micrographic morphology of flax stems and flax fibres was obtained using scanning electron microscopy (SEM; Hitachi S4800,Tokyo, Japan).

*Statistical analysis*

All of the data in this research were obtained from three independent experiments, and are presented as the mean value ± standard deviation. An analysis of variance (ANOVA) and Tukey's test were adopted to compare the differences among data. A *P*-value less than 0.05 indicated that the differences were significant. The software JMP 9.0.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

# RESULTS AND DISCSUSSION

## Production of Degumming Crude Enzyme

Four different media were used in this study to cultivate *B. cereus* HDYM-02 for investigating the production of degumming crude enzyme. The BP medium was used as a

negative control. Pectin and konjaku flour resembled pectin gum and hemicellulose present in flax stems, respectively. Carboxymethyl cellulose represented flax fibre. The activities of two specific degumming enzymes, pectinase and mannanase, were assayed as shown in Fig. 1. Both activities of pectinase and mannanase in PEC and KON media were much higher than those in BP medium, which indicated that these two enzymes were extra-cellular induced by poly-saccharide substrates. In terms of pectinase, the activity peak emerged at 48 h and was 833.3 U/mL in the PEC medium and 756.7 U/mL in KON medium (Fig. 1a). In terms of mannanase, the activity peak also emerged at 48 h, 828.0 U/mL and 2967.3 U/mL in PEC medium and KON medium, respectively (Fig. 1b).Considering that konjaku flour is much more available and cheaper than pectin, konjaku flour is more ideal than pectin in preparation of degumming crude enzyme. The fermentation time to collect crude enzyme was set at 48 h in this study.

It could be clearly deduced from Fig. 1 that CMC medium was not effective in inducing the production of pectinase and mannanase, whose activities were constantly lower than 200 U/mL. Moreover, what needed to be explained was that the activity of cellulase was barely detected in the above-mentioned four media (data not shown). This result suggested that the crude enzyme would not damage the flax fibres during retting application.

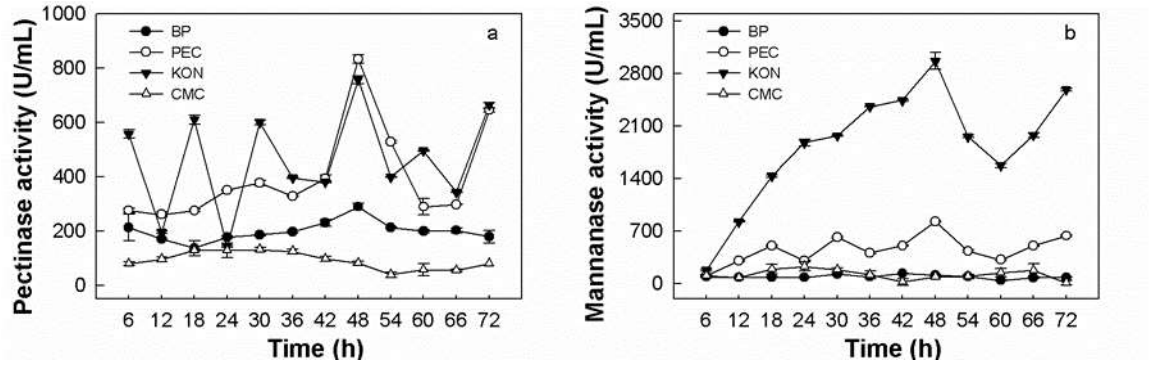


**Fig. 1.** a: Pectinase and b: mannanase activity of degumming crude enzyme produced by *B. cereus* HDYM-02 in different media; BP: Baird-Parker medium, abbreviations indicated different poly-saccharide substrate media, PEC: pectin, KON: konjaku flour, CMC: carboxymethylcellulose

## Stability of Degumming Crude Enzyme

The stability of degumming crude enzyme produced by *B. cereus* HDYM-02 in the konjaku flour medium after 48 h fermentation was investigated to assess its potential in retting application. Table 1 shows that the activity of crude enzyme significantly ($P<0.05$) declined along with the incubation time. However, in general, the crude enzyme showed a wide stability range of pH value, from 4.0 to 7.0. More than 60% and 50% of its maximum activity was retained after 72 h and 120 h incubation, respectively. Both pectinase and mannanase were more stable from pH 4.0 to 5.0 than from 6.0 to 7.0.

The temperature stability of degumming crude enzyme produced by *B. cereus* HDYM-02 was shown in Table 2. Both pectinase and mannanase were considerably stable from 25 °C to 40 °C. The residual activity was more than 80% and 70% of the initial activity after 72 h and 120 h incubation, respectively. However, at 25 °C, at least more than 85% of its maximum activity was retained from 30 °C to 40 °C after 120 h incubation. Taking cost and temperature stability into consideration, flax retting experiments were conducted at 35 °C in this study.

**Table 1.** The pH Stability of Degumming Crude Enzyme Produced by *B. cereus* HDYM-02 in Konjaku Flour Medium

| Time (h) | Residual Activity of Pectinase (%) | | | | Residual Activity of Mannanase (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 |
| 24 | 95.6 ± 2.5$^{a}$ | 95.4 ± 2.5$^{a}$ | 78.6 ± 2.1$^{a}$ | 75.8 ± 1.9$^{a}$ | 92.1 ± 2.4$^{a}$ | 88.1 ± 2.3$^{a}$ | 78.4 ± 2.1$^{a}$ | 74.9 ± 2.1$^{a}$ |
| 48 | 93.1 ± 2.4$^{ab}$ | 86.1 ± 2.1$^{b}$ | 74.4 ± 2.0$^{a}$ | 70.3 ± 1.4$^{a}$ | 89.7 ± 2.4$^{a}$ | 81.0 ± 2.0$^{b}$ | 69.3 ± 2.1$^{b}$ | 65.4 ± 1.3$^{b}$ |
| 72 | 90.2 ± 2.2$^{bc}$ | 82.8 ± 2.1$^{bc}$ | 60.5 ± 2.3$^{b}$ | 62.4 ±2.0$^{b}$ | 88.0 ± 2.0$^{a}$ | 74.1 ± 1.9$^{c}$ | 64.7 ± 1.8$^{c}$ | 61.7 ± 2.0$^{bc}$ |
| 96 | 85.4 ± 2.0$^{c}$ | 78.4 ± 2.2$^{c}$ | 56.8 ± 1.9$^{bc}$ | 58.7 ± 1.7$^{b}$ | 75.2 ± 1.5$^{b}$ | 63.7 ± 1.8$^{d}$ | 57.7 ± 1.6$^{d}$ | 56.8 ± 1.6$^{c}$ |
| 120 | 72.3 ± 1.7$^{d}$ | 77.7 ± 1.5$^{c}$ | 52.4 ± 1.6$^{c}$ | 57.3 ± 1.1$^{b}$ | 66.4 ± 1.4$^{c}$ | 56.6 ± 1.7$^{e}$ | 52.2 ± 1.6$^{e}$ | 50.3 ± 1.3$^{d}$ |
| Different letters indicate significant (P<0.05) differences among relative activities within the same column | | | | | | | | |

**Table 2.**Temperature Stability of Degumming Crude Enzyme Produced by *B. cereus* HDYM-02 in Konjaku Flour Medium

| Time (h) | Residual Activity of Pectinase (%) | | | | Residual Activity of Mannanase (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | 25 °C | 30 °C | 35 °C | 40 °C | 25 °C | 30 °C | 35 °C | 40 °C |
| 24 | 92.4 ± 2.1$^{a}$ | 93.4 ± 1.5$^{a}$ | 95.4 ± 2.5$^{a}$ | 94.1 ±2.0$^{a}$ | 93.4 ± 2.1$^{a}$ | 92.4 ± 1.7$^{a}$ | 95.4 ± 2.1$^{a}$ | 96.2 ± 1.7$^{a}$ |
| 48 | 88.2 ± 2.0$^{b}$ | 90.1 ± 2.0$^{ab}$ | 92.6 ± 2.0$^{ab}$ | 92.1 ± 2.1$^{a}$ | 90.2 ±2 .0$^{ab}$ | 90.6 ± 2.1$^{ab}$ | 93.1 ± 2.0$^{ab}$ | 95.3 ± 2.0$^{a}$ |
| 72 | 80.8 ± 1.1$^{c}$ | 88.7 ± 1.8$^{b}$ | 91.4 ± 1.6$^{ab}$ | 90.8 ± 2.0$^{a}$ | 86.8 ± 1.4$^{b}$ | 88.4 ± 1.8$^{ab}$ | 93.2 ± 1.7$^{ab}$ | 93.5 ±1.9$^{ab}$ |
| 96 | 77.3 ± 2.0$^{cd}$ | 88.0 ± 2.0$^{b}$ | 90.4 ± 1.5$^{b}$ | 90.6 ± 1.8$^{a}$ | 78.3 ± 2.0$^{c}$ | 87.0 ± 2.1$^{b}$ | 90.4 ± 1.5$^{b}$ | 91.6 ± 1.9$^{b}$ |
| 120 | 76.5 ± 1.4$^{d}$ | 87.3 ± 1.7$^{b}$ | 90.0 ± 1.7$^{b}$ | 90.7 ± 1.7$^{a}$ | 70.5 ± 1.6$^{d}$ | 87.7 ± 1.6$^{b}$ | 90.8 ± 1.5$^{b}$ | 91.4 ± 1.6$^{b}$ |
| Different letters indicate significant (P<0.05) differences among relative activities within the same column | | | | | | | | |

## Application of Degumming Crude Enzyme in Flax Retting

Pectinase and mannanase catalyzed the degradation of gummy substances that were present in flax stems. The products of enzymatic reactions were galacturonic acid and reducing sugar. The higher contents of galacturonic acid and reducing sugar were, the more thoroughly degradation of gummy substances were. Three groups of flax retting experiments were conducted, *i.e.*, adding the crude enzyme produced by *B. cereus* HDYM-02, adding same-dose commercial pectinase, and natural retting as a negative control. Dynamic changes in galacturonic acid and reducing sugar during the 120 h flax retting process were shown in Figs. 2a and 2b, respectively. In general, all through the retting process, the contents of both galacturonic acid and reducing sugar in the crude enzyme retted samples were significantly (P<0.05) higher than those in commercial pectinase retted and natural retted samples. The highest values of galacturonic acid and reducing sugar in the crude enzyme retted sample were 897.2 μg/mL (72 h) and 78.5μg/mL (96 h), respectively. The results revealed that the degumming crude enzyme in this study was efficient in degrading gummy substances. Moreover, the changing trends of galacturonic acid and reducing sugar exhibited a similar "wave pattern,"*i.e.*, increase-decrease recycle pattern. This pattern indicated the release of galacturonic acid and reducing sugar and their subsequent utilization by microbes was an alternant ongoing process during flax retting. During the whole retting duration, the cfu values representing bacterial growth in the crude enzyme retting group were consistently higher than those in either commercial pectinase retting or natural retting group. The crude enzyme preparation was the culture supernatant of *B. cereus* HDYM-02. So the supernatant contained the media nutrients. These nutrients were utilized by the bacteria in the retting solution and resulted in higher cfu values.

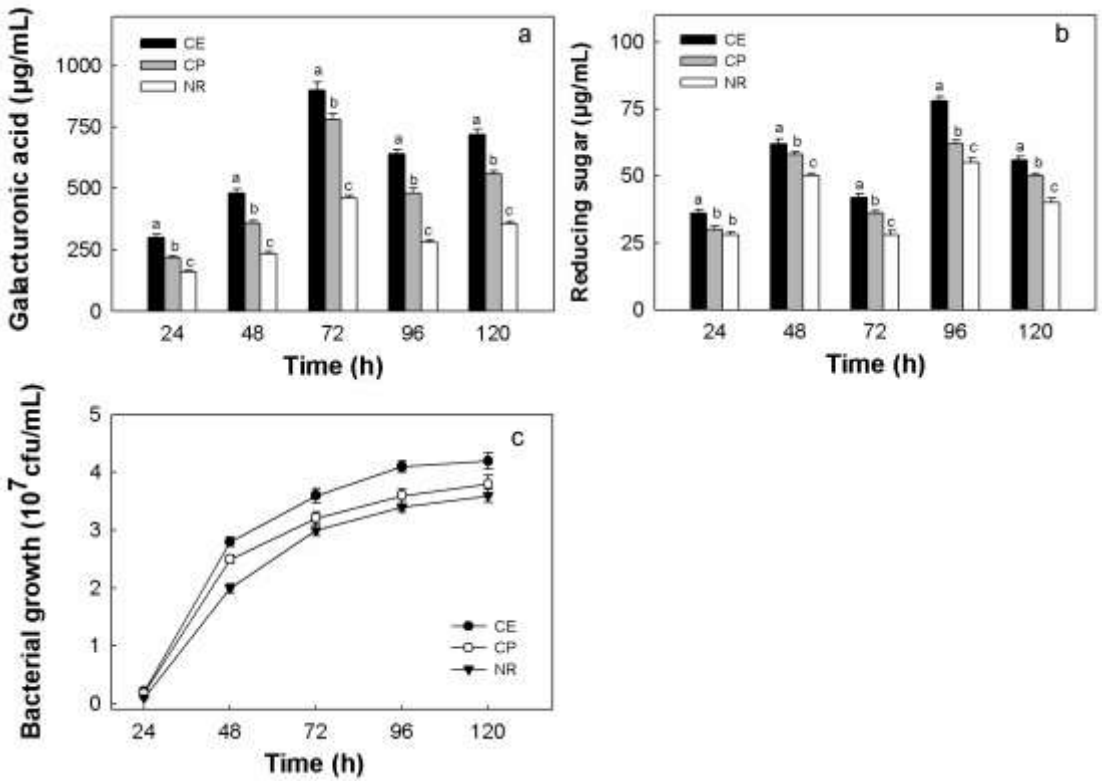


**Fig. 2.**Changes in: a: reducing sugar, b: galacturonic acid and c: bacterial growth during flax retting process; CE: crude enzyme retting, CP: commercial pectinase retting, NR: natural retting; different letters indicate significant (P<0.05) differences among values at the same time

Weight loss was another indicator of degumming extent. The data in Table 3 showed that after 108 h retting, the percentage decrease in the weight loss of the crude enzyme retted stems was 13.3%, which was significantly (P<0.05) higher than those of either commercial pectinase retted stems (11.6%) or natural retted stems (9.5%). The data were reasonably in accordance with the results of degumming extent revealed by galacturonic acid and reducing sugar (Fig. 1).

**Table 3.** Properties and Productivities of Flax Fibres

| Retting Method and Duration | Weight Loss (%) | Strength (N) | Residual Gum (%) | Fineness (airflow) | Ratio Variation of Strength (%) | Long Fibre Ratio (%) | Fibre Yield (%) |
|---|---|---|---|---|---|---|---|
| Crude Enzyme Retting (120 h) | 13.6 ± 0.5$^{a}$ | 183.2 ± 8.1$^{a}$ | 6.3 ± 0.2$^{c}$ | 6.2 ± 02$^{c}$ | 17.2 ± 0.5$^{b}$ | 17.6 ± 0.6$^{a}$ | 11.2 ± 0.3$^{a}$ |
| Crude Enzyme Retting (108 h) | 13.3 ± 0.3$^{a}$ | 184.5 ± 6.2$^{a}$ | 6.5 ± 0.2$^{c}$ | 6.4 ± 0.2$^{c}$ | 17.0 ± 0.3$^{b}$ | 17.7 ± 0.5$^{a}$ | 10.9 ± 0.3$^{a}$ |
| Commercial Pectinase Retting (120 h) | 11.6 ± 0.4$^{b}$ | 180.4 ± 5.3$^{a}$ | 9.2 ± 0.4$^{b}$ | 7.4 ± 0.3$^{b}$ | 19.2 ± 0.6$^{ab}$ | 15.6 ± 0.6$^{b}$ | 9.6 ± 0.4$^{b}$ |
| Natural Retting (120 h) | 9.5 ± 0.2$^{c}$ | 154.3 ± 4.2$^{b}$ | 12.5 ± 0.5$^{a}$ | 8.2 ± 0.3$^{a}$ | 21.4 ± 1.0$^{a}$ | 11.8 ± 0.3$^{c}$ | 7.7 ± 0.2$^{c}$ |
| Different letters indicate significant (P<0.05) difference among relative activity within the same column | | | | | | | |

Meanwhile, the addition of the crude enzyme produced by *B. cereus* HDYM-02 brought significantly (P<0.05) better properties and higher productivities of flax fibres than either commercial pectinase retting or natural retting, in larger strength (184.5 N),

less residual gum (6.5%), smaller fineness (6.4 in airflow), smaller rate variation of strength (17.0%), higher long fibre rate (17.7%), and higher fibre yield (10.9%). It could be concluded that the application of *B. cereus* HDYM-02 crude enzyme resulted in more thorough degradation of gummy substances. When the retting duration got longer to 120 h, *i.e.* it equaled to commercial pectinase retting and natural retting groups, these parameters didn't change significantly. However, the values of strength and long fibre rate decreased a little bit. Considering economic cost, crude enzyme retting should have stopped at 108 h. It meant that crude enzyme retting shortened retting duration than commercial pectinase retting and natural retting.

After 120 h retting, the cross-sections of flax stems and surfaces of flax fibres from three retting groups showed clearly different appearances (Fig. 3). Adding enzyme improved flax retting, which resulted in a greater separation among cuticles, fibre bundles, and shives (Figs. 3a and 3b) than natural retting (Fig. 3c). When retting with degumming crude enzyme produced by *B. cereus* HDYM-02, fibre bundles were thoroughly separated from both cuticles and shives. Because the specimen for electron microscopy was very thin, barely the bast region and only a few single fibres were left on the shive surface (Fig. 3a). When retting with commercial pectinase, fibre bundles significantly separated from the cuticles and fibres were loosened (Fig. 3b). While retted with water, Fig. 3c illustrated that fibre bundles completed separation from cuticles. Nevertheless, fibre bundles initially separated from shives, and the connection among fibres was relatively tight. The surfaces of fibres retted by either *B. cereus* HDYM-02 crude enzyme (Fig. 3d) or commercial pectinase (Fig. 3e) were much smoother than those retted by natural process (Fig. 3f). This result was in accordance with the data of residual gum in Table 3.

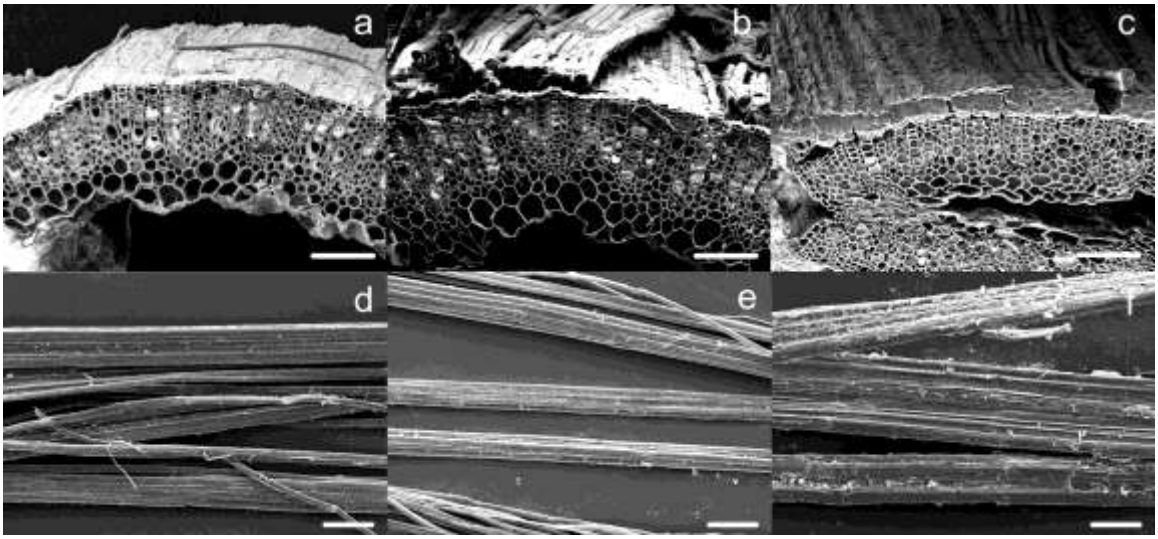


**Fig. 3.** SEM cross-sections of a: crude enzyme, b: commercial pectinase, and c: natural retted flax stems. SEM surfaces of d: crude enzyme, e: commercial pectinase and f: natural retted flax fibres. Scale bars equal 100 μm.

Among the degumming studies of bast fibre plants, several researchers have reported that biological degumming not only accelerates the retting process, but also improves fibre properties (Mukhopadhyay *et al.* 2013; Duan *et al.* 2016). Considering two commonly used biological degumming methods, enzyme degumming has several

advantages over microbial degumming, such as higher degumming efficiency and less environmental contamination. A degumming enzyme is usually applied in two forms, either crude enzyme produced by microbial strains or as a commercial purified product. Crude enzyme, fermented by waste peels or wheat bran, is preferred due to the lower cost (Mukhopadhyay *et al.* 2013; Chiliveri *et al.* 2016). In this study, *B. cereus* HDYM-02 produced degumming crude enzyme with konjaku flour as the substrate, which was also easily available and low-cost (Fig. 1).

Pectin is the most abundant gummy substance that exists in the stems of bast fibre plants. Pectin in kenaf and hemp could be effectively degraded by pectinolytic enzyme produced by *B. tequilensis* SV11-UV3 (Chiliveri *et al.* 2016). Except for pectin, gumming substances also contain hemicellulose, mainly represented by mannan. Mannanase produced by genetically recombined *Pichia pastoris* and *B. licheniformis* have been applied in the retting of ramie and flax, respectively (Wang *et al.* 2017; Zhao *et al.* 2017a). In this study, desirably, *B. cereus* HDYM-02 crude enzyme exhibited relatively high activities of both pectinase (756.7 U/mL at 48 h, Fig. 1a) and mannanase (2967.3 U/mL at 48 h, Fig. 1b), which was assumed to simultaneously degrade two gummy substances, pectin and hemicellulose, promising its potential application in retting practice.

In addition to compositions of the degumming enzyme, the stability of degumming enzyme should also be taken into consideration in retting practice. The majority of reported pectinases produced by *Bacillus* were alkaline, and they were stable and exhibited maximum activities at pH 7.0 to 11.0. For example, Guo *et al.* (2013) demonstrated that crude enzyme produced by *Bacillus* sp. Y1 contained pectate lyase, which played an important role in efficiently degumming pectin during ramie retting. This pectate lyase activity of *Bacillus* sp. Y1 was highest at a range of pH 7.0 to 10.5, and at least 81% of its maximum activity was retained within 8 h. However, the retting process usually lasts from 5 days to 6 days (Ruan *et al.* 2015) and the pH value fluctuated over a wide range. The authors' previous research showed that during flax retting, the pH value varied from approximately 6.5 down to 4.5 (Zhao *et al.* 2017b). In this study, more than 50% activity of both pectinase and mannanse was retained at pH values between 4.0 to 7.0 after 120 h incubation (Table 1), indicating its suitability in flax retting. Another factor that remarkably determines the exertion of enzyme activity is temperature. *B. cereus* HDYM-02 crude enzyme was not sensitive to temperatures ranging from 25 °C to 40 °C, at which more than 70% activity remained after 120 h incubation (Table 2). The industrial degumming is usually conducted at ambient or a slightly heated up temperature. The wide range of temperature would enable easier use of this crude in industrial retting.

The gummy substances that exist in stems of bast fibre plants were degraded by the degumming enzyme, which resulted in the release of simpler molecules, such as galacturonic acid and reducing sugar. These two molecules were measured to substantiate the degumming efficiency (Kapoor *et al.* 2001; Evans *et al.* 2002; Wang *et al.* 2009).The authors' previous research showed that compared with natural degumming liquid, the direct addition of *B. cereus* HDYM-02 led to higher contents of both galacturonic acid and reducing sugar (Zhao *et al.* 2016). When used in the crude enzyme form of *B. cereus* HDYM-02 in this research, the addition of crude enzyme consequently resulted in more galacturonic acid (Fig. 2a) and reducing sugar (Fig. 2b) compared with commercial pectinase retting and natural retting. This result further confirmed the degumming ability of strain *B. cereus* HDYM-02 and expanded its application form. Moreover, because there is a good correlation between the gum loss and the released galacturonic acid (Guo

*et al.* 2013), weight loss is also used to indicate the degradation extent of gummy substances. Similar results were reported such as pectate lyase produced by *B. megaterium* AK2 (Mukhopadhyay *et al.* 2013) and *B. pumilus* DKS1 (Basu *et al.* 2009) degummed ramie fibres. The weight loss significantly ($P<0.05$) increased when the flax stems were retted by crude enzyme (13.6%) compared with commercial pectinase (11.6%) and natural retted stems (9.5%), which demonstrated the advantage of crude enzyme over commercial enzyme.

As was expected (Table 3), flax stems retted by *B. cereus* HDYM-02 crude enzyme exhibited not only better properties in strength, residual gum, fineness, and ratio variation of strength, but also higher productivities in long fibre ratio and fibre yield. These results coincided with the authors' previous data obtained from degumming composite enzyme produced by *B. licheniformis* HDYM-04 (Zhao *et al.* 2017a). Similarly, pectinolytic enzymes produced by four *Bacillus* strains led to remarkable improvement in jute fibre strength and fineness over a control group (Das *et al.* 2012). The application of pectinolytic enzyme produced by *A. niger* also increased the yield of fine fibres during flax retting (Evans *et al.* 2002).

Scanning electron microscopy is an effective method for observing the cross-section of flax stem, revealing the separation of fibre bundles from both cuticles and shives (Akin *et al.* 2001; Ruan *et al.* 2015; Zhao *et al.* 2017a). In this study, the diagram obtained from SEM clearly illustrated the more thorough degumming extent of crude enzyme retted stem. As shown in Figs. 3a and 3d, after 120 h retting, crude enzyme produced by *B. cereus* HDYM-02 almost completely removed gummy substances both in the flax stem and inside the fibre bundle, which resulted in the production of pure, individualized fibres.

## CONCLUSIONS

1. *B. cereus* HDYM-02 produced a cellulase-free degumming crude enzyme, using konjaku flour as an inexpensive carbon source. This crude enzyme contained high activity of both pectinase (756.7 U/mL) and mannanase (2967.3 U/mL) after 48 h fermentation, which had a synergistic action on the degumming of flax stem.
2. This crude enzyme exhibited considerable stability for 120 h under the specific pH value and temperature condition analogous to flax retting practice. For both pectinase and mannanase, more than 50% activity was retained at pH values 4.0 to7.0, and at least 70% activity was retained at 25 °C to 40 °C.
3. Gummy substance was effectively removed by the *B. cereus* HDYM-02 crude enzyme, which resulted in higher contents of galacturonic acid (897.2 μg/mL at 72 h) and reducing sugar (78.5μg/mL at 96 h) in the degumming liquid.
4. Shorter retting duration, better properties, and higher productivities of flax fibre resulted from crude enzyme retting compared with commercial pectinase and natural retting. Under the condition of 12 h lesser retting duration, the crude enzyme retted fibres exhibited significant higher weight loss (13.3%), higher strength (184.5 N), lower residual gum (6.5%), smaller fineness (6.4 in airflow), lower ratio variation of strength (17.0%), higher long fibre ratio (17.7%), and higher fibre yield (10.9%).

5. This study suggested that *B. cereus* HDYM-02 crude enzyme could be a feasible option for application of biological degumming in the textile industry.

## ACKNOWLEDGMENTS

This research was supported by the National Nature Science Foundation of China (31270534, 31770538), the National Nature Science Youth Foundation of China (31300355), and the Post Doctorate Foundation of Heilongjiang Province (LBH-Z15214).

## REFERENCES CITED

Akin, D. E. (2010). "Flax-Structure, chemistry, retting and processing," in: *Industrial Applications of Natural Fibres:Structure, Properties and Technical Applications*, J. Müssig (ed.), John Wiley & Sons, Ltd., New York, USA. DOI: 10.1002/9780470660324.ch4

Akin, D. E., Foulk, J. A., Dodd, R. B., and McAlister, III, D. D. (2001). "Enzyme-retting of flax and characterization of processed fibres," *J. Biotechnol.* 89(2-3), 193-203. DOI: 10.1016/S0168-1656(01)00298-X

Akino, T., Nakamura, N., and Horikoshi, K. (1988). "Characterization of three BETA-mannanases of an alkalophilic *Bacillus* sp.," *Agr. Biol. Chem.* 52(3), 773-779. DOI: 10.1271/bbb1961.52.773

Basu, S., Saha, M. N., Chattopadhyay, D., and Chakrabarti, K. (2009). "Large-scale degumming of ramie fibre using a newly isolated *Bacillus pumilus* DKS1 with high pectate lyase activity," *J. Ind. Microbiol. Biot.* 36(2), 239-245. DOI: 10.1007/s10295-008-0490-y

Bonizzoni, L., Bruni, S., Fanti, G., Tiberio, P., and Zaffino, C. (2016). "Ageing of flax textiles: Fingerprints in micro-Raman spectra of single fibres," *Microchem. J.* 125, 69-74. DOI: 10.1016/j.microc.2015.11.011

Chiliveri, S. R., Koti, S., and Linga, V. R. (2016). "Retting and degumming of natural fibres by pectinolytic enzymes produced from *Bacillus tequilensis* SV11-UV37 using solid state fermentation," *SpringerPlus* 5(1), 1-17. DOI: 10.1186/s40064-016-2173-x

Das, B., Chakrabarti, K., Ghosh, S., Majumdar, B., Tripathi, S., and Chakraborty, A. (2012). "Effect of efficient pectinolytic bacterial isolates on retting and fibre quality of jute," *Ind. Crop. Prod.* 36(1), 415-419. DOI: 10.1016/j.indcrop.2011.10.003

di Candilo, M., Bonatti, P. M., Guidetti, C., Focher, B., Grippo, C., Tamburini, E., and Mastromei, G. (2010). "Effects of selected pectinolytic bacterial strains on water-retting of hemp and fibre properties," *J. Appl. Microbiol.* 108(1), 194-203. DOI: 10.1111/j.1365-2672.2009.04409.x

Dietz, J. H., and Rouse, A. H. (1953). "A rapid method for estimating pectic substances in citrus juices," *J. Food Sci.* 18(1-6), 169-177. DOI: 10.1111/j.1365-2621.1953.tb17701.x

Duan, S., Feng, X., Cheng, L., Peng, Y., Zheng, K., and Liu, Z. (2016). "Bio-degumming technology of jute bast by *Pectobacterium* sp. DCE-01," *AMB Express* 6(1), 86-91. DOI: 10.1186/s13568-016-0255-3

Evans, J. D., Akin, D. E., and Foulk, J. A. (2002). "Flax-retting by polygalacturonase-containing enzyme mixtures and effects on fibre properties," *J. Biotechnol.* 97(3), 223-231. DOI: 10.1016/S0168-1656(02)00066-4

GB/T 17345-31 (2008). "Flax suctched line,"General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China; Standardization Administration of the People's Republic of China, Beijing, China.

Ge, J. P., Ling, H. Z., Song, G., Feng, Y. G., Wei, W., and Ping, W. X. (2006). "Application of fast degumming with bacteria on retting flax," *Journalof Natural Science of Heilongjiang University*23(3), 307-310.

Ge, J. P., Ping, W. X., Gao, Y., Ling, H. Z., and Song, G. (2008). "Isolation, identification, and phyletic analysis of a pectinase-producing bacterial strain HDYM-02," *J. Microbiol.* 28(6), 44-47.

Guo, F., Zou, M., Li, X., Zhao, J., and Qu, Y. (2013). "An effective degumming enzyme from *Bacillus sp*. Y1 and synergistic action of hydrogen peroxide and protease on enzymatic degumming of ramie fibres," *BioMed Res. Int.* 2013, Article ID 212315. DOI: 10.1155/2013/212315

Kapoor, M., Beg, Q. K., Bhushan, B., Singh, K., Dadhich, K. S., and Hoondal, G. S. (2001). "Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibres," *Process Biochem.* 36(8-9), 803-807. DOI: 10.1016/S0032-9592(00)00282-X

Kulma, A., Skórkowska-Telichowska, K., Kostyn, K., Szatkowski, M., Skała, J., Drulis-Kawa, Z., Preisner, M., Żuk, M., Szperlik, J., Wang, Y. F., *et al.* (2015). "New flax producing bioplastic fibres for medical purposes," *Ind. Crop. Prod.* 68, 80-89. DOI: 10.1016/j.indcrop.2014.09.013

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.* 31(3), 426-428. DOI: 10.1021/ac60147a030

Mukhopadhyay, A., Dutta, N., Chattopadhyay, D., and Chakrabarti, K. (2013). "Degumming of ramie fibre and the production of reducing sugars from waste peels using nanoparticle supplemented pectate lyase," *Bioresource Technol.* 137(11), 202-208. DOI: 10.1016/j.biortech.2013.03.139

Ruan, P., Raghavan, V., Gariepy, Y., and Du, J. (2015). "Characterization of flax water retting of different durations in laboratory condition and evaluation of its fibre properties," *BioResources* 10(2), 3553-3563. DOI: 10.15376/biores.10.2.3553-3563

Sharma, S., Mandhan, R. P., and Sharma, J. (2011). "*Pseudozyma* sp. SPJ: An economic and eco-friendly approach for degumming of flax fibres," *World J. Microb. Biot.* 27(11), 2697-2701. DOI: 10.1007/s11274-011-0743-1

Tian, Y., Liu, X., Zheng, X., and Wang, L. (2014). "Production of efficient enzymes for flax retting by solid state fermentation with *Aspergillus niger*," *Int. J. Cloth. Sci. Tech.* 26(3), 212-221. DOI: 10.1108/IJCST-04-2013-0035

Wang, H., Xian, G., and Li, H. (2015). "Grafting of nano-$TiO_2$ onto flax fibres and the enhancement of the mechanical properties of the flax fibre and flax fibre/epoxy composite," *Compos. Part A- Appl. S.* 76, 172-180. DOI: 10.1016/j.compositesa.2015.05.027

Wang, Y., Wang, Z., Du, G., Hua, Z., Liu, L., Li, J., and Chen, J. (2009). "Enhancement of alkaline polygalacturonate lyase production in recombinant *Pichia pastoris* according to the ratio of methanol to cell concentration," *Bioresource Technol.* 100(3), 1343-1349. DOI: 10.1016/j.biortech.2008.07.049

Wang, Y., Shu, T., Fan, P., Zhang, H., Turunen, O., Xiong, H., and Yu, L. (2017). "Characterization of a recombinant alkaline thermostable *β*-mannanase and its application in eco-friendly ramie degumming," *Process Biochem.* 61, 73-79. DOI: 10.1016/j.procbio.2017.06.008

Yan, L., Chouw, N., and Jayaraman, K. (2014). "Flax fibre and its composite – A review,"*Compos Part B-Eng* 56(1), 296-317. DOI: 10.1016/j.compositesb.2013.08.014

Zhao, D., Liu, P., Pan, C., Du, R., Ping, W., and Ge, J. (2016). "Bacterial succession and metabolite changes during flax (*Linum usitatissimum* L.) retting with *Bacillus cereus* HDYM-02," *Scientific Reports* 6, Article ID 31812. DOI: 10.1038/srep31812

Zhao, D., Liu, P., Pan, C., Du, R., Ping, W., and Ge, J. (2017a). "Flax retting by degumming composite enzyme produced by *Bacillus licheniformis* HDYM-04 and effect on fibre properties," *J. Text. I.* 108(4), 507-510. DOI: 10.1080/00405000.2016.1171482

Zhao, D., Wang, Y., Pan, C., Guo, S., Na, J., and Ge, J. (2017b). "Study on the physicochemical properties and enzyme activity of *Bacillus cereus* HDYM-02 on flax in bio-degumming," *Journal of Natural Science of Heilongjiang University* 34(4), 467-473.

Article submitted: January 24, 2018; Peer review completed: March 25, 2018; Revised version received and accepted: April 7, 2018; Published: May 18, 2018.
DOI: 10.15376/biores.13.3.5213-5224