Appl Biochem Biotechnol (2009) 157:498–506
DOI 10.1007/s12010-008-8313-1

Characterization of Thermo-stable Endoinulinase from a New Strain *Bacillus Smithii* T7

Wei Gao · Yongming Bao · Yang Liu · Xiuli Zhang · Jingyun Wang · Lijia An

Received: 18 March 2008 / Accepted: 1 July 2008 /
Published online: 29 July 2008

Abstract A new thermophilic inulinase-producing strain, which grows optimally at 60 °C, was isolated from soil samples with medium containing inulin as a sole carbon source. It was identified as a *Bacillus smithii* by analysis of 16s rDNA. Maximum inulinase yield of 135.2 IU/ml was achieved with medium pH7.0, containing inulin 2.0%, $(NH_4)H_2PO_4$ 0.5%, yeast extract 0.5%, at 50 °C 200 rpm shaker for 72-h incubation. The purified inulinase from the extracellular extract of *B. smithii* T7 shows endoinulinolytic activity. The optimum pH for this endoinulinase is 4.5 and stable at pH range of 4.0–8.0. The optimum temperature for enzyme activity was 70 °C, the half life of the endoinulinase is 9 h and 2.5 h at 70 °C and 80 °C respectively. Comparatively lower Michaelis–Menten constant (4.17 mM) and higher maximum reaction velocity (833 IU/mg protein) demonstrate the endoinulinase's greater affinity for inulin substrate. These findings are significant for its potential industrial application.

Keywords Inulin · Inulinase · *Bacillus smithii* · Enzyme characterization · Thermo-stable enzyme · Optimization

Introduction

Inulin is a linear β-(2, 1)-linked fructose polymer that occurs as a reserve carbohydrate in many plant families such as Jerusalem artichoke, dahlia tubers, or chicory root [1]. Inulinase has received much more attention recently as it can be widely applied to hydrolyze inulin for the production of fuel ethanol [2], fructose, and fructo-oligosaccharides, both of which are important ingredients in food and pharmaceutical industry [3]. Fructo-oligosaccharides were found to have good functional and nutritional properties such as low calorie diet,

W. Gao · Y. Bao · Y. Liu · X. Zhang · J. Wang (✉) · L. An
Department of Bioscience and Biotechnology, Dalian University of Technology, Dalian 116024, People's Republic of China
e-mail: wangjingyun@dl.cn

bifidus stimulating factor, and source of dietary fiber in food preparations. These oligosaccharides, therefore, are now widely used to replace sugars in many food applications such as in confectionery, chocolate, and dairy products [4]. Besides, fructo-oligosaccharides were suggested to have growth inhibition effect of tumors [5]. One step enzymatic hydrolysis of inulin with inulinase is the best way to yield fructose and fructo-oligosaccharides. Inulinase can be produced by many microorganisms, such as yeast, fungi, and bacteria [6]. Higher temperature optimum of inulinases is an extremely important factor for the application of these enzymes in commercial production of fructose or fructo-oligosaccharides from inulin, since high temperatures (60 °C or higher) ensure proper solubility of inulin and also prevent microbial contamination [1]. Higher thermo-stability of the industrially important enzymes also brings down the cost of production because lower amount of enzyme is required to produce the desired product. But up to now, only a few of enzymes have an optimum temperature of 60 °C or higher, as required for industrial applications [7–9]. Although the production levels of inulinases in bacteria are not comparable to those of yeast and fungi, due to the ability of many bacteria to survive at high temperatures, attempts have been made to isolate bacterial strains which can produce high quantities of thermo-stable inulinase. But so far, a limited number of thermophiles with inulinase activity have been described [9–12]. Reports about endoinulinase [13, 14] are few, especially heat-stable ones from bacteria.

After screening over 50 bacteria strains which can grow on medium with inulin as a sole carbon source at 45~60 °C from different places of China, one thermophilic strain (*Bacillus smithii* T7) from Tianjin was found to produce large amount of extracellular inulinase. In this paper, optimization of the conditions for inulinase production by *B. smithii* T7, purification of the endoinulinase from the extracellular extract and characterization of the inulinase were reported.

Materials and Methods

Screening Bacteria for Producing Inulinase

Thermophilic bacteria enriched culture from 1 g different soil samples (collected locally in China) were carried out respectively with selecting medium 50 ml (inulin, 20 g/L, yeast extract, 5.0 g/L, $(NH_4)_2SO_4$, 5.0 g/L, pH 7.0) in 250-ml flask at 55 °C incubator for 48 h. Single colony picked after enriched bacteria culture spread and cultivated on selecting agar medium (selecting medium with 2% agar) at 55 °C for 48 h, was used for further isolation, identification, and inulinase activity assay.

Identification of the Thermophilic Strain for Producing Inulinase

Among 50 isolates tested, one strain was found to be a potent inulinase producer and it was designated as T7. The 16S rDNA gene was amplified by polymerase chain reaction (PCR) with TaKaRa 16S rDNA bacterial identification PCR kit. The target fragment, separated by 1% agarose electrophoresis and recovered using TaKaRa agarose gel DNA purification kit, was sequenced with forward primer 5′-GAGCGGATAACAATTTCACA CAGG-3′, reverse primer 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′, and Internal primer 5′-CAGCA GCCGCGGTAATAC-3′ in TaKaRa Biotechnology Co., Ltd. The sequences obtained were compiled and compared in the GenBank databases using BLAST program.

Optimization of Medium Ingredients and Process Parameters for Producing Inulinase by Bacteria Strain

Fermentation was carried out with selecting medium for optimization of carbon sources, nitrogen sources and pH, temperature, rotating rate effects on producing inulinase.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [15] on 10% polyacrylamide. Proteins were detected by Coomassie brilliant blue R staining.

Enzyme Purification

The supernatant collected from bacteria culture by centrifuge (SORVAL Rc5C Plus SLA-3000 rotor, 6,000 rpm, 25 min) at 4 °C, after addition of 40% ammonium sulfate, was centrifuged (6,000 rpm, 15 min) again at 4 °C. The supernatant fraction was precipitated with 80% ammonium sulfate and centrifuged (6,000 rpm, 15 min) at 4 °C. The precipitate collected was dissolved and dialyzed against 20 mM Tris HCl buffer (pH 8.0) at 4 °C, and then applied on a DEAE-Sepharose column 20 ml (Pharmacia XK16×20) equilibrated with 50 mM Tris HCl buffer (pH 8.0). The column was washed with 5 volumes of 500 mM NaCl and eluted with 3 volumes 600 mM NaCl in the same buffer at a flow rate of 1.0 ml/min.

Thin Layer Chromatography for Characterization of Products Hydrolyzed by Inulinase

Precoated silica thin layer chromatography (TLC) plates (Merck, Germany), after activated, were spotted with samples, developed twice with the solvent systems using *n*-butanol, isopropanol, acetic acid, and water (7:5:2:4, volume ratio) as an irrigating solvent and the sugars were visualized by heating the plates at 105 °C for 30 min after spraying with 3% urea in butanol, ethanol, water, orthophosphoric acid (80:8:5:7, volume ratio) [16].

Effects of pH and Temperature on Enzyme Activity and Stability

The effects of pH on the enzyme activity of purified enzyme were investigated in the pH range 4.0–9.5 at 60 °C (0.1M sodium acetate buffer for pH 4.0–5.5, citrate phosphate buffer for pH 6.0–6.5, sodium phosphate buffer for pH 7.0–8.0, and Tris–HCl buffer for pH 8.5–9.5). The stability of the purified enzyme at different pH values was determined by estimating the residual activity after the enzyme was incubated at 40 °C for 2 h at pH 4.0–9.5. The optimal temperature for inulinase activity of the enzyme was measured by incubating the enzyme–substrate mixtures for 10 min at different temperature (40–80 °C) at pH 4.5 and the released reducing sugars were measured. Thermal stability of the enzyme was measured in terms of residual activity after the incubation of enzyme in 0.1M sodium acetate buffer (pH 4.5) at 70 °C and 80 °C for up to 12 h.

Kinetic Parameters

The Michaelis–Menten constant (Km) and maximum reaction velocity (Vmax) for inulin (0.1–6.4 mM), sucrose (2.5–160 mM) and raffinose (0.5–32 mM) were determined from simple Lineweaver–Burk plots.

Inulinase Activity Assay

The hydrolyzed reaction of inulin in 1 ml volume (containing 20 mg inulin, 20 ul enzyme sample and 800 ul of 0.1M, pH 4.5 acetate buffer) was incubated at 60 °C or 70 °C for 10 min, and stopped immediately by keeping the reaction mixture at 100 °C for 10 min. The amount of reducing saccharide released was detected by the method of Nelson–Somogyi [17], and enzyme sample was replaced by the inactivated one (heated at 100 °C for 10 min), and used as the blank control. One inulinase unit (IU) was defined as the amount of enzyme that produces one micromole of reducing sugar per minute under the tested conditions.

Results and Discussions

Identification of the Screened Strain T7

From the analysis of the 16S rDNA gene sequence, The T7 strain was found to be very similar to *B. smithii* and the similarity is 99% based on 16S rDNA. Combined with physiological, biochemical characteristics (data not shown), the strain T7 was identified as a strain of *B. smithii,* and named *B. smithii* T7 (accession number in Genbank is EU628681).

Optimization of Medium Composing

It has been reported that different carbon sources have significant influences on inulinase production by microorganism [18]. Inulinase produced by *B. Smithii* T7 was also influenced greatly by different carbon sources in the medium (data not shown). The order of enzyme production from different carbon sources was inulin>lactose>fructose>mannitol>glucose>sucrose. Inulin is the best carbon source for *B. smithii* T7 to produce inulinase. This result indicates that the inulinase produced by *B. smithii* T7 is an inducible enzyme. The optimal carbon source is the same as *Kluyveromyces marxianus* [19] and *Cryptococcus aureus* G7a [20]. In order to determine the optimum concentration of inulin for inulinase production, different concentrations (1.0–4.0%, *w*/*v*) were tested, and a progressive increase in enzyme production was observed with the increase of inulin concentration up to 2.0% (*w*/*v*) and thereafter, a decline in enzyme activity was observed. Maximum enzyme production was 69.5 IU/ml at 2.0% (*w*/*v*) inulin concentration (data not shown).

Different nitrogen sources were supplemented in the production medium containing 2.0% inulin. The order of production of inulinase under the influence of nitrogen sources was $(NH_4)H_2PO_4$>ammonium sulphate>peptone>yeast extract>ammonium chloride>beef extract>urea (data not shown). Maximum inulinase production, 103.5 IU/ml, was shown by $(NH_4)H_2PO_4$. There was an increase in inulinase production with an increase in the concentration of $(NH_4)H_2PO_4$ up to 0.5% (*w*/*v*) and thereafter, a decline in this function was recorded. Enzyme production with 0.5% $(NH_4)H_2PO_4$ was 104.3 IU/ml. The higher concentration of $(NH_4)H_2PO_4$ was inhibitory for inulinase synthesis; this indicates that appropriate concentration of nitrogen source in medium for inulinase production should be remarkable.

Effect of different initial pH in the medium on inulinase production for *B. Smithii* T7 revealed that the optimal initial pH in medium for inulinase production was 7.0, and maximum enzyme production was 85.7 IU/ml (data not shown). Inulinase production can be more in neutral environment as reported [7, 10].

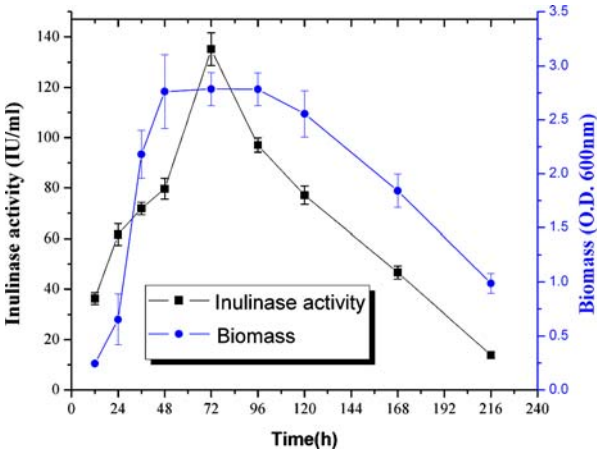

Fig. 1 Enzyme activity and biomass change of *Bacillus smithii* T7 growth

Optimization of Process Parameters

The effect of temperature on inulinase production showed that the maximum inulinase production, 130.8 IU/ml, was observed at 50 °C and the enzyme activity was significantly reduced at higher temperature (data not shown). In general, temperature influences the metabolic activity of cells, for example, *Bacillus stearothermophilus* grows at 41–69 °C and optimally at 54–55 °C [10], The optimal temperature of *Bacillus polymyxa* 29, *B. polymyxa* 722, and *Bacillus subtilis* 68 is 35 °C, while they showed a few inulinase activities at 45 °C [17].

A significant increase of inulinase was observed under agitation state (134.2 IU/ml at 200 rpm) compared to stationary condition (35.8 IU/ml). Rotation rate not only affects the oxygen availability, but also influences on the availability of the nutrients in the medium. Rotation rate of 150 rpm and 180 rpm were reported optimum for the production of inulinase from *K. marxianus* at shake flask level [21, 22].

There was an increase in enzyme production up to 72 h and thereafter it decreased as shown in Fig. 1. Decrease in nutrient availability in the medium, or catabolic repression of enzyme could be the main reason of decline in enzyme activity after 72 h of fermentation.

Purification of Inulinase

The results of purification of inulinase from extracellular extract were shown in Table 1. The crude protein obtained from ammonium sulphate precipitation was loaded on anion–

Table 1 Purification of inulinase from *Bacillus smithii* T7 fermentation supernatant.

Purification steps	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg protein)	Activity yield (%)	Purification fold
CE	1,183.2	4,1676	35.22	100.0	1.00
ASF	775.3	35,951	46.37	86.26	1.32
AECF	12.7	13,674	1076.7	32.8	30.6
SGF	10.3	11,386	1105.4	27.3	31.4

CE Crude extract, *ASF* ammonium sulfate fraction, precipitated between 40% and 90%, *AECF* anion exchange chromatography fraction, using DEAE-Sepharose CL-6B, *SGF* Superdex75 gel filtration

ion-exchange column of DEAE-Sepharose, which resulted in 30-fold purification as compared to the crude extract. The purified inulinase fractions were homogenous, only a single band was observed on SDS-PAGE (Fig. 2), and the subunit molecular weight is about 47 kD.

Characteristics of Inulinase

The purified inulinase from *B. smithii* T7 was incubated with inulin at 60 °C for some time. The released fructose and oligosaccharides were analyzed by TLC. Little fructose and more oligosaccharides were found to be the main products of inulin hydrolysis reaction, which suggests that the inulinase is an endoinulinase (Fig. 3).

Maximum activity was showed at pH 4.5 with sodium acetate buffer in Fig. 4. To determine the effect of pH on stability, the enzyme extract was incubated for 2 h at 40 °C, in different pH buffers. The enzyme also retained almost 60% activity between pH 4.0–7.0, but at pH 7.5 and above, the activity registered a sharp decline.

The optimum temperature of the enzyme was 70 °C (Fig. 5a) at pH 4.5. Compared to inulinases from *Fusarium oxysporum*, *Penicillium janczewskii*, *Polystichum aculeatum*, and *Penicillium digitatum*, which have optimal temperature of 55 °C and 40 °C, respectively [23], the temperature optimum of *B. smithii* T7 is significantly higher. After the enzyme solution was heated at 70 °C and 80 °C for up to 12 h, the residual activities were measured at 70 °C (Fig. 5b). As compared to 80 °C, the enzyme did not show any significant loss of activity after 6 h and it retained about 51% activity after 9 h at 70 °C (Fig. 5b). However, prolonged incubation at 70 °C resulted in a gradual loss of activity of it, but it still retained

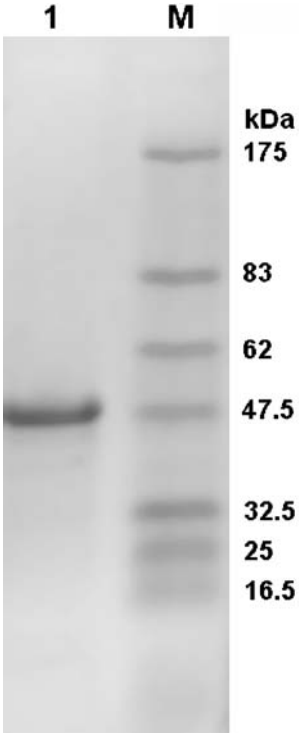

Fig. 2 SDS-PAGE analysis of the purified inulinase. Lane *1*, Inulinase sample. Lane *M*, molecular weight standards

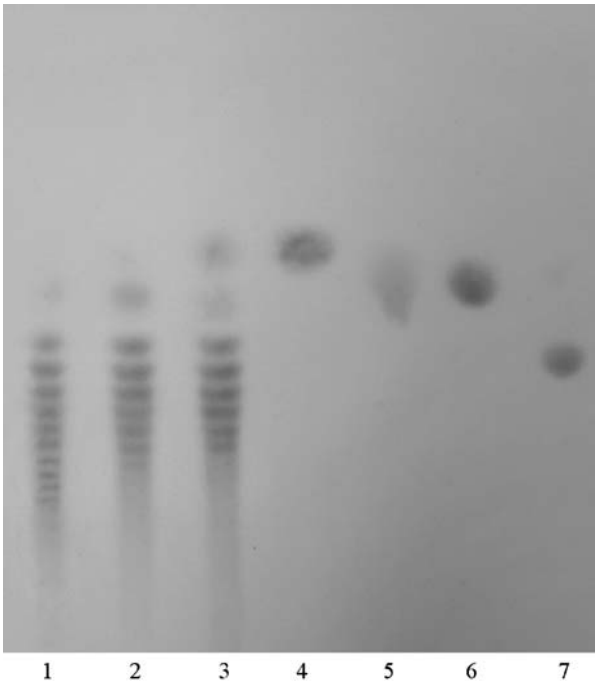

Fig. 3 Thin layer chromatography for characterization of inulin hydrolysis products by inulinase. Lane *1* control, Lane *2* hydrolysis 3 h, Lane *3* hydrolysis 12 h, Lane *4* fructose, Lane *5* glucose, Lane *6* sucrose, Lane *7* raffinose

37% activity even after 12 h. The thermo-stability of the inulinase from *B. smithii* T7 is higher than that of inulinase from *Bacillus sp*. 11 and *B. stearothermophilus* KP1289 which retained only 7% activity at 70 °C and 50% activity at 69 °C after 15 min and 10 min, respectively [8, 24]. Further more, the enzyme incubated at 80 °C for 6 h, retained 40% activity, which has never been reported before. It can be concluded that half-life of the new heat-resistant inulinase at 70 °C and 80 °C are 9 and 2.5 h respectively.

Besides inulin, the inulinase from *B. smithii* T7 also hydrolyzed other sugars, such as raffinose and sucrose, with minimum Km and maximum Kcat being observed for inulin

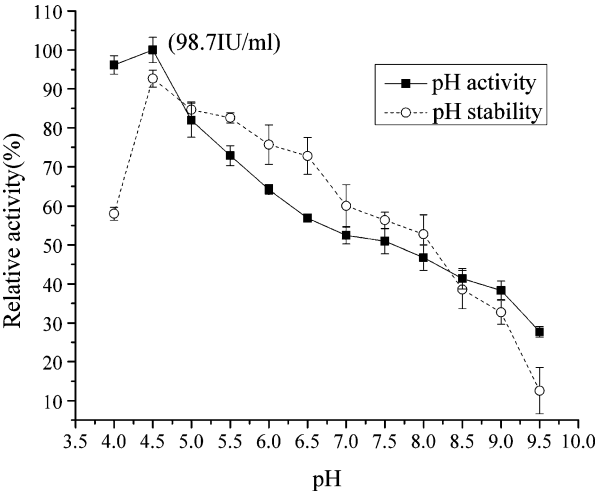

Fig. 4 Effect of pH on activity and stability of the inulinase from *Bacillus smithii* T7. Data expressed as the mean±SEM of three tests. Values in *parenthesis* represent the inulinase activity taken as 100%

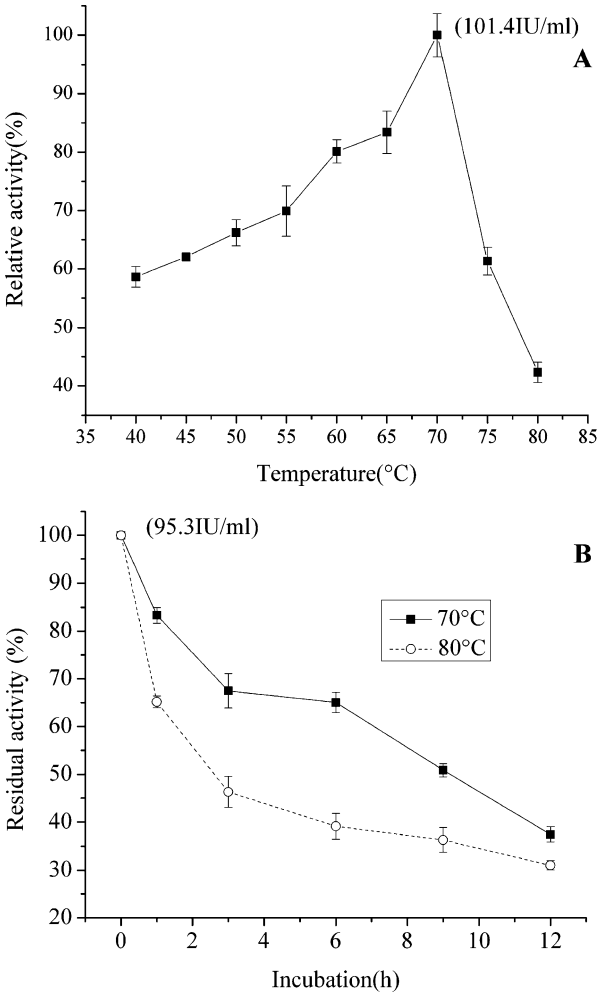

Fig. 5 Temperature effects on activity and stability of the inulinase from *Bacillus smithii* T7. Data expressed as mean±SEM of three tests. Values in *parenthesis* represent the inulinase activity taken as 100%

(Table 2). The purified endoinulinase showed broad substrate specificity, but the comparatively lower Km (4.17 mM) and higher Vmax (833 IU/mg protein) demonstrate its greater affinity for inulin substrate. As compared to the Km values reported for inulinases from other microorganisms, such as *Cryptococcus aureus* G7a (20.06 mg/ml) [20] and *B. subtilis* 430A (8 mM) [25], the lower Km value (4.17 mM) of the purified endoinulinase from *B. smithii* T7 for inulin makes it a better candidate for inulin hydrolysis.

Table 2 Kinetic parameters of the inulinase from *Bacillus smithii* T7.

Substrates	Km (mM)	Vmax (IU/mg)	Kcat (min^{-1})	Kcat/Km(mM^{-1} min^{-1})
Inulin	4.17±0.05	833.3±5.4	200	47.92
Sucrose	32.7±0.25	270.3±2.1	8.26	0.25
Raffinose	5.75±0.36	172.4±1.8	30	5.22

Reaction was performed at pH 4.5 and 70 °C with 0.1–6.4 mM inulin (Mr 5000), 2.5–160 mM sucrose and 0.5–32 mM raffinose. Results represent the average of three experiments

Conclusion

The screened strain of *B. smithii* T7 is a new thermophilic bacterium that can highly produce endoinulinase, which was reported first. The higher temperature optimum and greater thermostability of *B. smithii* T7 are the desirable features for industrial production of inulinase that should be a potential heat-resistant enzyme candidate for commercial use and enzyme functional research.

References

1. Rocha, J. R., Catana, R., Ferreira, B. S., Cabral, J. M. S., & Fernandes, P. (2006). *Food Chemistry*, *95*, 77–82. doi:10.1016/j.foodchem.2004.12.020.
2. Szambelan, K., Nowak, J., & Czarnecki, Z. (2004). *Biotechnology Letters*, *26*, 245–248.
3. Gill, P. K., Manhas, R. K., & Singh, P. (2006). *Bioresource Technology*, *97*, 355–358. doi:10.1016/j.biortech.2005.02.038.
4. Gill, P. K., Manhas, R. K., & Singh, P. (2006). *Bioresource Technology*, *97*, 894–902. doi:10.1016/j.biortech.2005.04.034.
5. Kang, S., & Kim, S. (1999). *Biotechnology Letters*, *21*, 569–574. doi:10.1023/A:1005514603287.
6. Prabhjeet, S., & Gill, P. K. (2006). *Food Technology*, *44*, 151–162.
7. Kaur, A., Sharma, D., Harchand, R. K., Singh, P., Bhullar, S. S., & Kaur, A. (1999). *Microbiology*, *39*, 99–103.
8. Gill, P. K., Manhas, R. K., Singh, J., & Singh, P. (2004). *Applied Biochemistry and Biotechnology*, *117*, 19–32. doi:10.1385/ABAB:117:1:19.
9. Tsujimoto, Y., Watanabe, A., Nakano, K., Watanabe, K., Matsui, H., Tsuji, K., et al. (2003). *Applied Microbiology and Biotechnology*, *62*, 180–185. doi:10.1007/s00253-003-1261-3.
10. Kato, K., Araki, T., Kitamura, T., Morita, N., Moori, M., & Suzuki, Y. (1999). *Starch*, *51*, 253–258. doi:10.1002/(SICI)1521-379X(199907)51:7<253::AID-STAR253>3.0.CO;2-7.
11. Cho, Y. J., & Yun, J. W. (2002). *Process Biochemistry*, *37*, 1325–1331. doi:10.1016/S0032-9592(02)00018-3.
12. Liebl, W., Brem, D., & Gotschlich, A. (1998). *Applied Microbiology and Biotechnology*, *50*, 55–64. doi:10.1007/s002530051256.
13. Kim, K. Y., Rhee, S., & Kim, S. I. (2005). *Journal of Biochemistry*, *138*(1), 27–33. doi:10.1093/jb/mvi093.
14. Moriyama, S., Akimoto, H., Suetsugu, N., Kawasaki, S., Nakamura, T., & Ohta, K. (2002). *Bioscience, Biotechnology, and Biochemistry*, *66*(9), 1887–1896. doi:10.1271/bbb.66.1887.
15. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.
16. Gill, P. K., Sharma, A. D., Harchand, R. K., & Singh, P. (2003). *Bioresource Technology*, *87*, 359–362. doi:10.1016/S0960-8524(02)00262-6.
17. Spiro, R. G. (1966). *Methods in Enzymology*, *8*, 3–26.
18. Singh, R. S., Sooch, B. S., & Puri, M. (2006). *Bioresource Technology*, *98*, 2518–2525. doi:10.1016/j.biortech.2006.09.011.
19. Silva, F. R., & Santana, C. C. (2000). *Applied Biochemistry and Biotechnology*, *84–86*, 1063–1078. doi:10.1385/ABAB:84-86:1-9:1063.
20. Sheng, J., Chi, Z., Gong, F., & Li, J. (2008). *Applied Biochemistry and Biotechnology*, *144*(2), 111–121. doi:10.1007/s12010-007-8025-y.
21. Zherebtsov, N. A., Shelamova, S. A., & Abramova, I. N. (2002). *Applied Biochemistry and Microbiology*, *38*, 544–548. doi:10.1023/A:1020722510374.
22. Singh, R. S., Dhaliwal, R., & Puri, M. (2007). *Industrial Microbiology and Biotechnology*, *34*, 649–655. doi:10.1007/s10295-007-0237-1.
23. Cazetta, M. L., Martins, P. M. M., Monti, R., & Contiero, J. (2005). *Journal of Food Engineering*, *66*, 301–305. doi:10.1016/j.jfoodeng.2004.03.022.
24. Pessoni, R. A. B., Figueiredo-Ribeiro, R. C. L., & Braga, M. R. (1999). *Journal of Applied Microbiology*, *87*, 141–147. doi:10.1046/j.1365-2672.1999.00805.x.
25. Uzunova, K., Vassileva, A., Kambourova, M., Ivanova, V., Spasova, D., Mandeva, R., et al. (2001). *Zeitschrift für Naturforschung*, *56*, 1022–1028.