Journal of Genetic Engineering and Biotechnology (2015) **13**, 65–69

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

Characterization of two thermostable inulinases from *Rhizopus oligosporus* NRRL 2710

Saleh A. Mohamed [a,b,*], Hala A. Salah [b,c], Maysa E. Moharam [d], M.S. Foda [d], Afaf S. Fahmy [b]

[a] *Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia*
[b] *Molecular Biology Department, National Research Centre, Dokki, Cairo, Egypt*
[c] *Chemistry Department, Faculty of Science, King Khalid University, Abha, Saudi Arabia*
[d] *Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt*

Received 3 October 2014; revised 30 November 2014; accepted 6 December 2014
Available online 30 December 2014

KEYWORDS

Rhizopus oligosporus;
Inulinase;
Purification;
Characterization

Abstract Two inulinases (Inu2 and Inu3) were purified from *Rhizopus oligosporus* NRRL 2710 by chromatography on DEAE-Sepharose and Sephacryl S-200 columns. The molecular weight of Inu2 and Inu3 were determined to be 76 and 30 kDa, respectively. Inu2 and Inu3 had the same pH optimum at 5.0, temperature optimum at 50 and 60 °C, and thermal stability up to 60 and 70 °C for 1 h, respectively. Inu2 and Inu3 had low km values (0.93 and 0.70 mM, respectively) indicating the high affinity toward inulin. Mg^{2+}, Ca^{2+}, Zn^{2+} and EDTA did not significantly influence the enzyme activity. Ni^{2+}, Cu^{2+}, Fe^{2+} and Co^{2+} showed a partial inhibitory effect, and Hg^{2+} had a strong inhibitory effect. *p*-Chloromercuribenzoate had a partial inhibitory effect on Inu2. From these findings, *R. oligosporus* inulinases can be beneficial enzymes for industrial enzymatic production of high fructose syrup.

1. Introduction

Inulin is a widespread polyfructan in the roots and tubers of Jerusalem artichoke, chicory, dandelion, burdock and dahlia. It consists of linear chains of β-2,1-linked D-fructofuranose residues terminated by a glucose residue through a sucrose-type linkage at the reducing end [19]. Inulin has received great interest as an inexpensive and abundant material for the production of fructose and fructooligosaccharides, which is used in food, drink and pharmaceutical industries [19].

Fructose can be obtained by the acid hydrolysis of inulin. Acid hydrolysis of inulin, however, is not an appropriate method for fructose production because it results in the formation of difructose anhydrides, which do not have a sweetening capacity but cause undesirable color [19]. Thus, the use of microbial inulinases has been proposed as the most promising approach for obtaining fructose or fructooligosaccharides from inulin.

Inulin-hydrolyzing enzymes are encountered in plants, filamentous fungi, yeasts, and bacteria. Among the filamentous fungi, *Aspergillus* and *Penicillium* species are common

* Corresponding author at: Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah 21589, Saudi Arabia.
E-mail address: saleh38@hotmail.com (S.A. Mohamed).

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2014.12.001

inulinase-producers [21,15,6,10,2]. Previous studies have revealed that several microorganisms produce endoinulinase as well as exoinulinase, e.g. *Aspergillus niger* [15], *Aspergillus ficuum* [21], *Chrysosporium pannorum* [22], *Penicillium purpurogenum* [12] and *A. ficuum* JNSP5-06 [18], in which three exo-type inulinases and two endo-type inulinases were purified and characterized [13]. Extracellular inulinase was also purified from a filamentous fungus, *Rhizopus* sp. strain TN-96 [14]. Previously, we investigated some of the physiological aspects of *Rhizopus oligosporus* NRRL 2710 with special reference to those dealing with the formation of thermostable inulinase using artichoke leaves as a potential substrate [3]. Therefore, the objective of this study was to purify and characterize two thermostable inulinases from *R. oligosporus* NRRL 2710.

2. Materials and methods

2.1. Microorganism

R. oligosporus NRRL 2710 was obtained from the culture collection of the Northern Regional Research Laboratory, USDA, Peoria, IL, USA.

2.2. Medium and growth conditions

The organism was grown on 5% finely ground dry artichoke leaves in water as a complete growth medium (pH 7.0) with a particle size of less than 300 micrometers. Incubation was carried out at 30 °C for 48 h in a rotary shaker operated at 200 rpm. The fermented broth was filtered through double-layered Whatman paper. After centrifugation of the filtrate at 4000 rpm for 20 min, the supernatant was collected as the crude extract for further purification [3].

2.3. Inulinase purification

Crude extract was dialysed against 20 mM sodium acetate buffer pH 5.5 and applied to a DEAE-Sepharose column (10 × 1.6 cm i.d.) pre-equilibrated with 20 mM sodium acetate buffer pH 5.5. The column was eluted with a stepwise gradient of NaCl from 0.0 to 0.3 M in the same buffer at a flow rate of 60 ml/h and 3 ml fractions. Absorbance was monitored at 280 nm. The elution profile revealed four major peaks with inulinase activities, which were designated as Inu1, Inu2, Inu3 and Inu4 by the order of elution. The Inu2 and Inu3 fractions with the highest activity were pooled, concentrated by dialysis against solid sucrose, and loaded onto a Sephacryl S-200 CL-6B column (93 × 1.6 cm i.d.) equilibrated with 20 mM sodium acetate buffer pH 5.5. The column was eluted with the 20 mM sodium acetate buffer pH 5.5 at a flow rate of 20 ml/h and 3 ml fractions.

2.4. Molecular weight determination

Molecular weight was determined by gel filtration technique using Sephacryl S-200. The column (93 × 1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS–polyacrylamide gel electrophoresis [4]. SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for calibration.

2.5. Inulinase assay

Enzyme activities were assayed by measuring the concentration of reducing sugars released from inulin. The reaction mixture (0.5 ml) containing 2 mM inulin, 0.05 M sodium acetate buffer, pH 5.0 and 0.1 unit of inulinase was incubated at 40 °C for 1 h. The increase in reducing sugar was estimated by the 3,5-dinitrosalicylic acid method [11]. Absorbance was read at 560 nm. One unit of enzyme was defined as the amount of enzyme which liberated 1 μmol fructose per h.

2.6. Protein determination

Protein content was determined as described by Bradford [8] using bovine serum albumin as standard. Protein in the column effluents was monitored by measuring the absorbance at 280 nm.

2.7. Effect of pH and temperature on inulinase activity and stability

The effect of pH on inulinase activity was determined in the pH range from 4.5 to 7.0 using 0.05 M sodium acetate buffer (pH 4.5–5.5) and sodium phosphate buffer (pH 6.0–7.0). The optimal temperature for inulinase activity was determined by incubating the enzyme–substrate mixtures at various temperatures (10–80 °C) in 0.05 M sodium acetate buffer, pH 5.0 and the liberated reducing sugars were measured. Thermal stability of inulinase was measured in terms of residual activity after the incubation of inulinase at different temperatures (10–80 °C) for 1 h prior to substrate addition. Activity at zero time was taken as 100% activity.

2.8. km

The km values were determined from Lineweaver–Burk plots by using inulin concentrations from 0.2 to 1.0 mM.

2.9. Effects of metal ions and other chemicals on inulinase activity

The effects of metal cations on inulinase activity were investigated by preincubating the enzyme with 2 mM Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Hg^{2+}, Ba^{+} and Ni^{2+} for 15 min prior to substrate addition. Activity in the absence of metal cations was taken as 100% activity. The effects of 2 mM EDTA and *p*-chloromercuribenzoate on inulinase activity were determined under the same conditions as mentioned above.

3. Results and discussion

The purification of *R. oligosporus* inulinase was summarized in Table 1. From the elution profile of the chromatography on DEAE-Sepharose column (10 × 1.6 cm i.d.) four inulinases

Table 1 Purification scheme for *Rhizopus oligosporus* inulinase.

Step	Total protein (mg)	Total activity (units)*	S.A. (units/mg protein)	Fold purification	Recovery %
Crude extract	16.4	2806	171	1.0	100
Chromatography on DEAE-Sepharose					
0.0 M NaCl (Inu1)	7.8	250	32	0.18	8.9
0.1 M NaCl (Inu2)	0.26	406	1561	9.12	14.4
0.2 M NaCl (Inu3)	0.172	630	3662	21.4	22.4
0.3 M NaCl (Inu4)	0.92	200	217	1.26	7.1
Gel filtration on Sephacryl S-200					
Inulinase (Inu2)	0.089	290	3258	19.0	10.3
Inulinase (Inu3)	0.067	485	7238	42.3	17.2

* One unit of inulinase activity was defined as the amount of enzyme producing 1 μmol fructose per h under standard assay conditions.

Inu1, Inu2, Inu3 and Inu4 were separated (Fig. 1). Due to the low activity level of Inu1 and Inu4, further purification was restricted to Inu2 and Inu3, which contained the highest activity. A Sephacryl S-200 column was used to obtain Inu2 and Inu3 (Fig. 2) with the highest possible specific activity 3258 and 7238 units/mg protein, which represented 20 and 40-fold higher than that of inulinases from GS115/*inu*A1[5] and *Streptomyces* sp. [20], respectively.

The homogeneity of the purified *R. oligosporus* Inu2 and Inu3 was demonstrated by the presence of one single protein band on SDS–polyacrylamide gel (Fig. 3).The molecular weight of *R. oligosporus* Inu2 and Inu3 was estimated to be 76 and 30 kDa by gel filtration, respectively. These molecular weights were confirmed by SDS–PAGE (Fig. 3), and estimated to be 76 and 30 kDa as single subunit. Different molecular weights has been reported for inulinases from *A. ficuum* (31–70 kDa) [13], *Streptomyces* sp. (45 kDa) [20], *Rhizopus* sp. (83 kDa) [14] and *Aspergillus candidus* (54 kDa) [17].

The effect of pH on the activity of *R. oligosporus* Inu2 and Inu3 was examined. As shown in Fig. 4A, the enzyme showed hydrolase activity from exhibited pH 4.5–6.0. The pH optima were 5.0 in 50 mM sodium acetate buffer for the two enzymes. The two enzymes were stable for 2 h in the same pHs (data not shown). Similar pH optimum was reported for inulinases from *A. ficuum* (pH 4.5–5.0) [13], *Streptomyces* sp. (pH 6.0) [20], GS115/*inu*A1 (pH 4.5) [5], *Aspergillus awamori* (pH 4.5) [1] and *Penicillium janczewski* (pH 4.8–5.0) [9].

R. oligosporus Inu2 and Inu3 were found to have temperature optima at 50 and 60 °C, respectively (Fig. 4B). The activities of enzymes were gradually decreased with increasing the temperature. Similarly, temperature optima for inulinase from *A. ficuum* [13], GS115/*inu*A1[5] and *Rhizopus* sp. [14] were around 40 and 55 °C. The high temperature optimum was reported for inulinase from *streptomyces* sp. (70 °C) [20]. The effect of temperature on the thermal stability of *R. oligosporus*

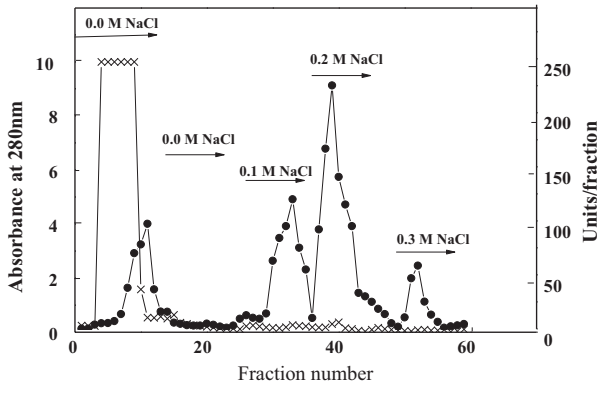

Figure 1 A typical elution profile for the chromatography of *R. oligosporus* inulinase on DEAE-Sepharose column (10 × 1.6 cm i.d.) previously equilibrated with 20 mM sodium acetate buffer, pH 5.5 at a flow rate of 60 ml/h and 3 ml fractions. X——X absorbance at 280 nm, ●——● units/fraction.

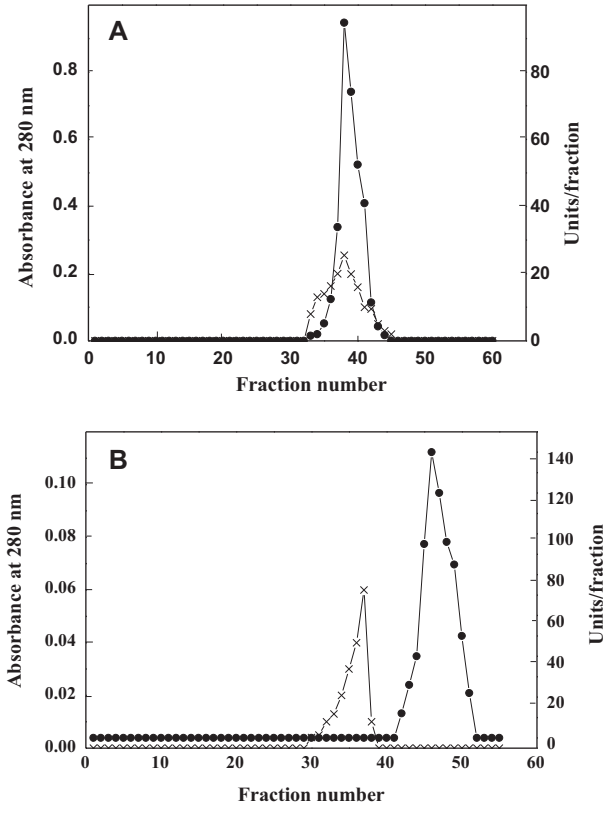

Figure 2 A typical elution profile for the chromatography of *R. oligosporus* Inu2 (A) and Inu3 (B) DEAE-Sepharose fractions on Sephacryl S-200 column (93 × 1.6 cm i.d.) previously equilibrated with 20 mM sodium acetate buffer, pH 5.5 at a flow rate of 20 ml/h and 3 ml fractions. X——X absorbance at 280 nm, ●——● units/fraction.

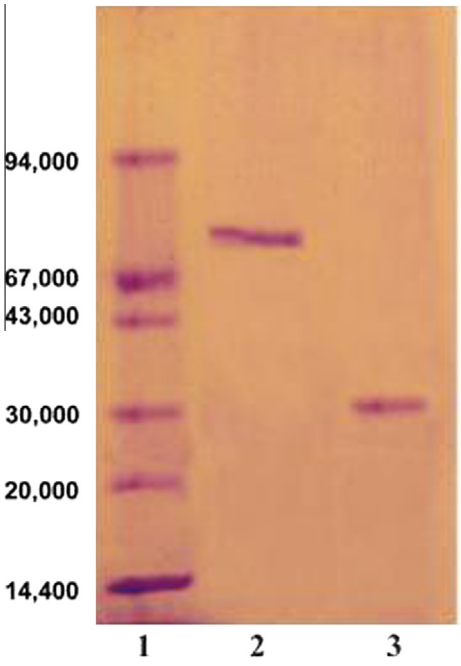

Figure 3 SDS–PAGE for molecular weight determination of *R. oligosporus* inulinase. (1) Protein markers, (2) Sephacryl S-200 Inu2; (3) Sephacryl S-200 Inu3.

Inu2 and Inu3 was investigated by incubation of the enzyme for 1 h in 50 mM sodium acetate buffer, pH 5.0 at different temperatures ranging from 10 to 80 °C prior to substrate addition (Fig. 4C). Inu2 and Inu3 were approximately stable up to 60 and 70 °C. The activities of enzymes were gradually decreased with increasing the temperature. Inu2 and Inu3 retained 20% and 40% of their activities at 80 °C, respectively. Similar results were obtained by Sharma and Gill [20], where *Streptomyces* inulinase was thermally stable up to 70 °C. Nakamura et al. [13] reported that the five *A. ficuum* inulinases retained 80% relative activity after 1 h (pre-heated at 50 °C), but only 40% relative activity was observed after 1 h (pre-heated at 60 °C). In contrast, inulinase from *Rhizopus* sp. remained stable up to 30 °C and was completely inactivated at 60 °C [14].

The affinity of the *R. oligosporus* Inu2 and Inu3 for inulin was determined by the Lineweaver–Burk plot (Fig. 5). The km values were 0.93 and 0.70 mM, respectively. These low km values indicated the high affinity of enzymes toward inulin. Moderate km values were demonstrated for inulinases from GS115/*inu*A1 (km 2.57 mM) [5] and *Streptomyces* sp. (km 1.63 mM) [20]. High km values had been reported for inulinases from *Debaryomyces cantarelli* (15 mM) [7], *Candida salmenticensis* (17 mM) [16] and *A. ficuum* (10–15 mM) [21].

The effect of different metal cations at the concentration of 2 mM on *R. oligosporus* Inu2 and Inu3 assay system is shown in Table 2. Mg^{2+}, Ca^{2+} and Zn^{2+} did not significantly influence the enzyme activity. Ni^{2+}, Cu^{2+}, Fe^{2+} and Co^{2+} showed partial inhibitory effect and Hg^{2+} had a strong inhibitory effect. The strong inhibitory effect observed with Hg^{2+} suggested that some –SH-groups in the protein might be essential for the activity. This has been observed for other microbial inulinases [21,5,20,17]. Several articles studied the effect of metal cations on inulinases. Ohta et al. [14] reported that Ni^{2+}, Mg^{2+} and Zn^{2+} had no significant effect on *Rhizopus* sp. inulinase. However, Co^{2+} positively modulated inulinase activity in the *Streptomyces* sp. [20]

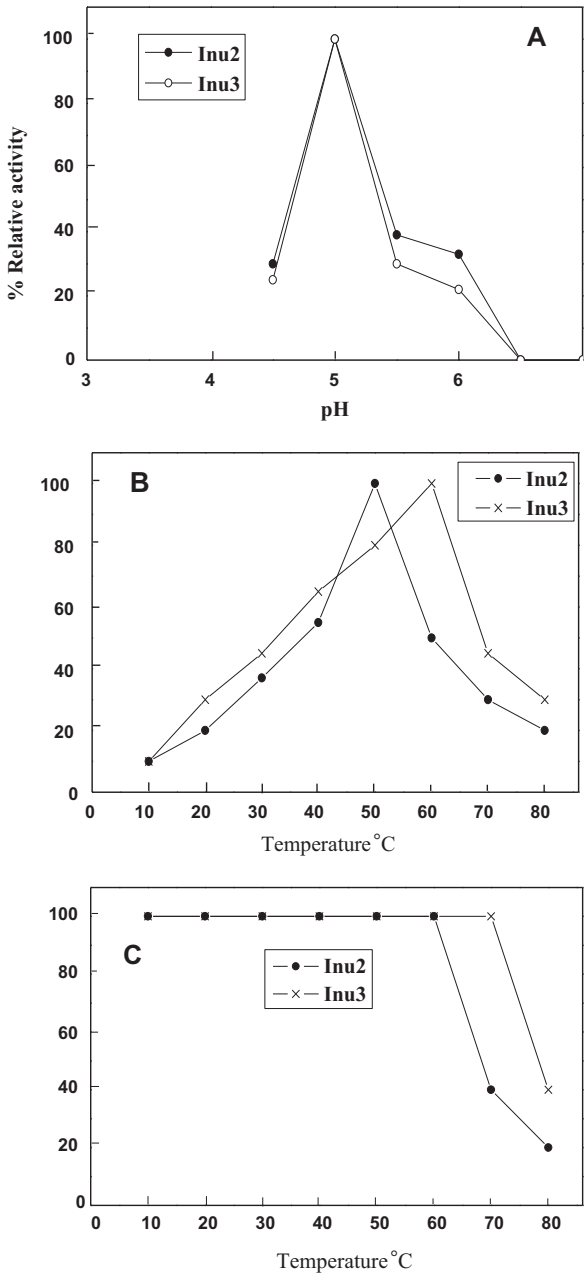

Figure 4 pH optima (A), temperature optima (B) and temperature stability (C) of *R. oligosporus* Inu2 and Inu3. Each point represents the average of three experiments.

In addition, metal chelator EDTA had no effect on *R. oligosporus* Inu2 and Inu3 (Table 2). This was in agreement with a previous study [13]. However, the activity of inulinase from the *Streptomyces* sp. was strongly inhibited by EDTA suggesting the possible involvement of metal for the enzyme [20]. From Table 2, it also appears that *p*-chloromercuribenzoate had a partial inhibitory effect on Inu2 and no effect on Inu3. An appreciable loss of activity of inulinase from *Rhizopus* sp. was observed with *p*-chloromercuribenzoate [14]. The inhibition of Inu2 by *p*-chloromercuribenzoate suggested that the active site of the enzyme contained cysteine residues.

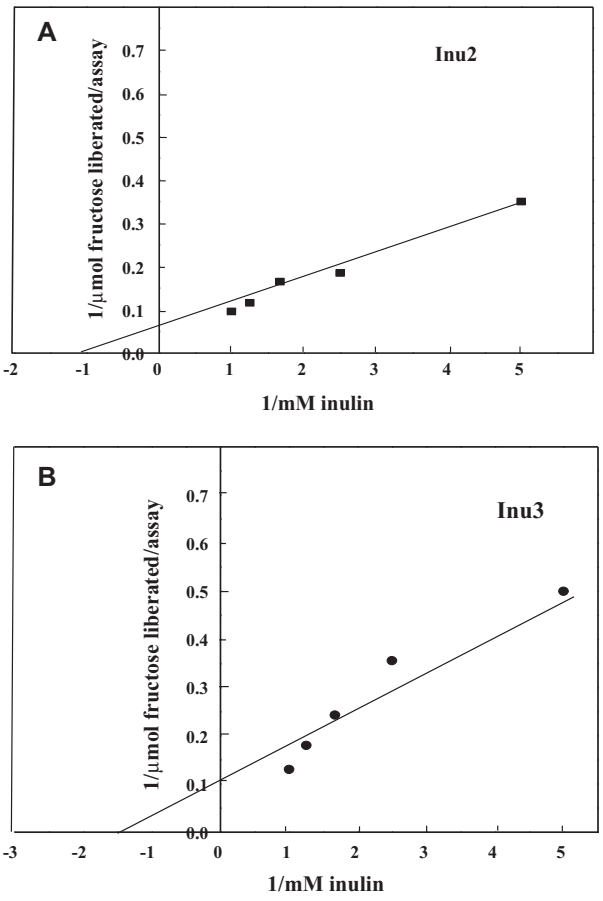

Figure 5 Lineweaver–Burk plots relating *R. oligosporus* Inu2 (A) and Inu3 (B) reaction velocities to inulin as substrate concentrations. The reaction mixture contained in 0.5 ml:0.05 M acetate buffer, pH 5, 0.1 unit of enzyme and concentrations of inulin ranging from 0.2 to 1.0 mM. Each point represents the average of three experiments.

Table 2 Effect of metal cations and other chemicals on the activities of *R. oligoforum* Inu2 and Inu3.

Metal ion	% Relative activity	
	Inu2	Inu3
Non	100	100
Mg^{+2}	98	103
Ca^{+2}	101	95
Zn^{+2}	92	90
Cu^{+2}	65	58
Fe^{+2}	57	40
Co^{+2}	66	77
Ni^{+2}	30	28
Hg^{+2}	12	8
EDTA	94	95
p-Chloromercuribenzoate	25	97

Each value represents the average of three experiments.

4. Conclusion

In conclusion, the purified Inu2 and Inu3 from *R. oligosporus* were characterized by low pH, high thermal stability, and high affinity toward inulin. From these findings, Inu2 and Inu3 from *R. oligosporus* can be considered as efficient as well as beneficial enzymes that can be used for the industrial enzymatic production of high fructose syrup.

References

[1] M. Arand, A.M. Golubev, J.R.B. Neto, I. Polikarpov, R. Wattiez, O.S. Korneeva, et al, Biochem. J. 362 (2002) 131–135.
[2] I. Beluche, J.P. Guiraud, P. Galzy, Folia Microbiol. (Praha) 25 (1980) 32–39.
[3] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[4] H.-Q. Chen, X.-M. Chen, T.-X. Chen, X.-M. Xu, Z.-Y. Jin, Carbohydr. Polym. 86 (2011) 249–254.
[5] H.-Q. Chen, X.-M. Chen, Y. Li, J. Wang, Z.-Y. Jin, X.-M. Xu, J.-W. Zhao, T.-X. Chen, Z.-J. Xie, Food Chem. 115 (2009) 1206–1212.
[6] M. Ettalibi, J.C. Baratti, Appl. Microbiol. Biotechnol. 26 (1987) 13–20.
[7] J.P. Guiraud, C. Viard-Gaudin, P. Galzy, Agric. Biol. Chem. 44 (1980) 245–252.
[8] S.A. Hashem, M.S. Foda, M.A. Amin, S.T. Elsayed, M.E. Moharam, Adv. Food Sci. 29 (2007) 84–89.
[9] A. Kochhar, A.K. Gupta, N. Kaur, J. Sci. Food Agric. 79 (1999) 549–554.
[10] U.K. Laemmli, Nature 227 (1970) 680–685.
[11] G.L. Miller, Anal. Chem. 31 (1959) 426–428.
[12] T. Nakamura, T. Kurokawa, S. Nakatsu, S. Ueda, Nippon Nogeikagaku Kaishi 52 (1978) 159–166.
[13] T. Nakamura, A. Shitara, S. Matsuda, T. Matsuo, M. Suiko, K. Ohta, J. Ferment. Bioeng. 84 (1997) 313–318.
[14] K. Ohta, N. Suetsugu, T. Nakamura, J. Biosci. Bioeng. 94 (2002) 78–80.
[15] S. Onodera, N. Shiomi, Agric. Biol. Chem. 52 (1988) 2569–2576.
[16] S. Onodera, N. Shiomi, Biosci. Biotechnol. Biochem. 56 (1992) 1443–1447.
[17] R.A.B. Pessoni, R. Figueiredo, M.R. Braga, J. Appl. Microbiol. 87 (1999) 141–147.
[18] A.D. Sharma, P.K. Gill, J. Food Eng. 79 (2007) 1172–1178.
[19] E.J. Vandamme, D.G. Derycke, Adv. Appl. Microbiol. 29 (1983) 139–176.
[20] J. Wang, Z.Y. Jin, B. Jiang, A. Adamu, Process Biochem. 39 (2003) 5–11.
[21] R. Xiao, M. Tanida, S. Takao, J. Ferment. Bioeng. 67 (1989) 244–248.
[22] L. Zhang, C. Zhao, D. Zhu, Y. Ohta, Y. Wang, Protein Expr. Purif. 35 (2004) 272–275.