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Improving stability of a novel dextran-degrading enzyme from marine *Arthrobacter oxydans* KQ11

Delong Wang[a,b], Mingsheng Lu[b,d], Xiaobei Wang[a], Yuliang Jiao[b,c], Yaowei Fang[b,c], Zhaopu Liu[a,*], Shujun Wang[b,c,**]

[a] *Key Laboratory of Marine Biology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China*
[b] *School of Marine Science and Technology, Huaihai Institute of Technology, Lianyungang, Jiangsu 222005, China*
[c] *Jiangsu Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Lianyungang, Jiangsu 222005, China*
[d] *Jiangsu Marine Resources Development Research Institute, Lianyungang, Jiangsu 222005, China*

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ABSTRACT

Dextranases can hydrolyze dextran, so they are used in the sugar industry to mitigate the milling problems associated with dextran contamination. Few studies have been carried out on the storage stability of dextranase, let alone the dextranase of *Arthrobacter oxydans* KQ11 isolated from sea mud samples. This study improved the storage stability of dextranase from marine *A. oxydans* KQ11 by adding enzymatic protective reagents (stabilizer and antiseptic). Initially, the conditions (55 °C and 30 min) for maintaining 50% dextranase activity were obtained. Then, the best stabilizers of dextranase were obtained, namely, glycerol (16%), sodium acetate (18%) and sodium citrate (20%). Results showed that p-hydroxybenzoic acid compound sodium acetate (0.05%), D-sodium isoascorbiate (0.03%), and potassium sorbate (0.05%) were the best antiseptics. Subsequent validation experiment showed that dextranase with enzymatic protective reagents maintained 70.8% and 28.96% activities at the 13th week at 25 and 37 °C, respectively.

1. Introduction

Dextran, a long-chain homoglycan of α-D-glucopyranose (α-1,6 glucosidic bonds), is synthesized from glucose by dextransucrase (EC 2.4.1.5) (Zohra et al., 2013). Dextranases (EC 3.2.1.11) hydrolyze the α-1,6 glucosidic bond contained in dextran (Chen, Zhou, Fan, & Zhang, 2008; Finnegan, Brumbley, O'Shea, Nevalainen, & Bergquist, 2004; Khalikova, Susi, Usanov, & Korpela, 2003; Kim & Kim, 2010; Larsson, Andersson, Stahlberg, Kenne, & Jones, 2003; Wu, Zhang, Huang, & Hu, 2011). These enzymes are usually classified as endo- and exodextranases according to their modes of action. Dextran can be modified by dextranases and used in several biotechnological applications.

Dextranases are found in different types of microorganisms, such as molds (Arnold, Nguyen, & Mann, 1998; Fukumoto, Tsuji, & Tsuru, 1971; Goldstein-Lifschitz & Bauer, 1976; Hiraoka, Tsuji, Fukumoto, Yamamoto, & Tsuru, 1973; Pleszczynska, Rogalski, Szczodrak, & Fiedurek, 1996; Shimizu, Unno, Ohba, & Okada, 1998; Sugiura, Ito, Ogiso, Kato, & Asano, 1973), yeasts (Koenig & Day, 1989; Ryu et al., 2000) and bacteria (Barrett, Barrett, & Curtiss, 1987; Khalikova et al., 2003; Wynter et al., 1997). These enzymes hydrolyze dextran through an endo- or exowise mechanism (Okada, Takayanagi, & Sawai, 1988; Russell & Ferretti, 1990).

Dextranases can hydrolyze various troublesome microbial dextran deposits, so they are used in the sugar industry to mitigate milling problems associated with dextran contamination of sugarcane and beets (de Bruijn, 2000; Eggleston & Monge, 2005; Roca et al., 1996). Dextran is the main composition of dental caries (Khalikova, Susi, & Korpela, 2005; Marotta et al., 2002; Ohnishi et al., 1995). Dental plaque produced by *Streptococcus mutans* and *Streptococcus sorbinus* can be removed by dextranase. Dextranase has an important function in the beverage industry for the treatment of juices and syrups, as well as in oral care products, such as mouthwash and toothpaste, for the control and removal of dental caries (Khalikova et al., 2005; Zohra et al., 2013). Moreover, this enzyme has long been used in manufacturing blood substitutes (Eggleston & Monge, 2005; Khalikova et al., 2003; Kim & Day, 1994; Lee & Fox, 1985; Mehvar, 2000).

Mold strains, such as *Chaetomium gracile* and *Chaetomium erraticum*, are the primary sources of dextranases in the industry (Eggleston & Monge, 2005). However, certain safety issues exist in using mold to produce dextranase for application in the food industry. The production period is also long. Under factory conditions, commercial dextranase activity is approximately halved (~46%) for 90 days and even shows reduced activity when stored

* Corresponding author. Tel.: +86 02584396678; fax: +86 02584396678.
** Corresponding author. Tel.: +86 051885895421; fax: +86 051885895429.
E-mail addresses: sea@njau.edu.cn (Z. Liu), shujunwang86@163.com (S. Wang).

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under refrigeration (Eggleston & Monge, 2005). The development of dextranase from marine bacteria will solve these problems. Enzymes from the oceans have more unique catalytic properties and potential applications compared with terrigenous enzymes because oceans are characterized by high salt, low temperature, and alkaline pH. The strains isolated from the ocean that produced dextranase in our laboratory had low action temperature and short production time (12–30 h) and were stable under alkaline conditions. These properties suggest that marine *A. oxydans* KQ11 can provide enormous economical and social benefits.

To date, several methods are known to enhance enzyme stability. C-terminal amino acid residues (Liao, Lee, & Chiou, 2002; Yi, Pei, & Wu, 2011) or certain enzyme subunits (Crute, Seefeld, Gamble, Kemp, & Witters, 1998), salt-bridge stabilization (Chan, Yu, & Wong, 2011), random mutagenesis (Emond et al., 2008) or site-directed mutagenesis (Li et al., 2007), substituted polysaccharides (Fernandez, Villalonga, Fragoso, Cao, & Villalonga, 2004), and polyols (Devaraneni, Mishra, & Bhat, 2012) are associated with enzyme stability. Structural transformation of enzyme (Acharya, Rajakumara, Sankaranarayanan, & Rao, 2004) or gene (Hild, Brumbley, O'Shea, Nevalainen, & Bergquist, 2007) is the most common method to improve enzyme stability. Expensive structural transformation and technical difficulties are some restrictions in the study of enzyme stability. Thus, safe, economical, and effective enzymatic protective reagents (O'Fagain, 2011; Shukla, Schneider, & Trout, 2011) are significant for large-scale applications and can generate large economical benefits.

The development of enzyme preparation results in liquid mass production of some microbial enzymes. This approach prevents damage in the human respiratory system associated with the production of powdered solid enzyme and reduces environmental pollution. Liquid enzyme production process is simple and has low-energy consumption, high yield, altitudinal mechanization degree, and so on. However, liquid enzyme is easily hydrolyzed by proteases (Tombs, 1985), leading to loss of activity. In industrial production, addition of stability agents (Bhosale, Rao, Deshpande, & Srinivasan, 1995; Costa et al., 2002; Kaushik & Bhat, 1998) and antiseptics is commonly used to maintain the stability of liquid enzymes (Martinek et al., 1977; Mu, Chen, Fang, Mao, & Gao, 2012).

This study reported on the stability improvement of dextranase produced by *A. oxydans* KQ11 isolated from sea mud. No reports are available on microorganisms that improve the stability of dextranase with an enzymatic protective reagent. This study also investigated the corrosion protection of dehydrogenation sodium acetate and p-hydroxy benzoic acid compound sodium acetate on enzyme preservation to provide scientific basis for the production and application of dextranase. For these reasons, improving enzyme stability and providing safe, economical, and effective enzyme reagents for industrial application are significant.

2. Materials and methods

2.1. Materials

Dextranase was prepared by bio-fermentation of *A. oxydans* KQ11, which was isolated from mud samples collected from the Yellow Sea (coastal region of Lianyungang, PR China). Sorbitol, sodium acetate, glycerin, D-ascorbic acid sodium, sodium benzoate, potassium thiocyanate, dehydrogenation sodium acetate, potassium sorbate, p-hydroxy benzoic acid compound sodium acetate, chitosan, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate were all AR grade and procured from reliable sources.

2.2. Dextranase preparation

A. oxydans KQ11 was isolated from the mud samples collected from the Yellow Sea (coastal region of Lianyungang, PR China). These microorganisms could secrete dextranase into a cultivated medium containing 0.75% soy meal, 0.75% cassava starch, 0.5% yeast extract powder, 1% wheat bran, 0.4% dextran 20000, 0.4% NaCl, and 0.04% $MgSO_4$ (pH 7.5). After 30 h of aerobic fermentation at 30 °C and 150 rpm, the thallus of the fermentation broth was removed with a diatomite filter. The liquid supernatant was then filtered by an ultrafiltration membrane and freeze dried. Freeze-dried enzyme powder was used in the stability experiment.

2.3. Dextranase activity assay

Enzyme activity was determined according to the method of Miller (Miller, 1959). Dextranase activity was measured by the production ratio of the reducing sugar in the reaction with 3,5-dinitrosalicylic acid reagent (Eggleston & Monge, 2005; Hild et al., 2007; Wang, Wang, He, & Zhang, 2012). A mixture of 0.05 ml of dextranase and 0.15 ml of 50 mM sodium acetate buffer containing 3% dextran 20000 (pH 5.5) was incubated at 50 °C for 15 min. DNS (Lever, 1972) was added into the experimental and control groups to terminate the reaction. Then, 0.05 ml of enzyme was added into the control group. The mixture was boiled for 5 min, and 3 ml of distilled water was added. The absorbance of this mixture was measured at 540 nm. One unit of dextranase enzyme activity was defined as the amount of enzyme that liberated the equivalent of 1 μmol of maltose in 1 min from dextran 20000 (Smogyi, 1952).

2.4. Dextranase storage stability

Through repeated tests, we found that the residual activity of dextranase was 50% under the condition of 55 °C and 30 min. Dextranase and different enzymatic protective reagents (Bhosale et al., 1995; Costa et al., 2002; Kaushik & Bhat, 1998) were added into the centrifuge tube with specific proportion by category. Control 1 was stored at 4 °C without enzymatic protective reagent and not treated at 55 °C for 30 min. For Control 2, no enzymatic protective reagent was added and treated at 55 °C for 30 min. The activity of control 1 group was 100%, and the retention rate of dextranase activity was calculated.

2.5. Subsequent validation experiment

Subsequent validation experiment was carried out at 25 and 37 °C to validate the best proportions obtained from the result of dextranase storage stability. Enzymatic protective reagents were added into the dextranase, which was filtered with 10 and 90 kDa ultrafiltration membranes. The enzyme without enzymatic protective reagents served as the control group. Dextranase activity was determined, and residual activity was calculated.

3. Results and discussion

3.1. Effect of different stabilizers on dextranase stability

Control 1 was stored at 4 °C without enzymatic protective reagent and not treated at 55 °C for 30 min. For control 2, no enzymatic protective reagent was added and treated at 55 °C for 30 min.

Saccharides (Kaushik & Bhat, 2003; O'Connor, Debenedetti, & Carbeck, 2007) affect enzyme stability. The stability of the enzyme structure depends on the interaction between hydroxyl groups and enzyme molecules (Zhang, 2002). In this study, different saccharides (5%) (Chen, 2006), including glucose, sucrose, maltose, lactose, dextrin and gelatin, as well as sorbitol, were added in the liquid

Table 1
Effect of different concentrations of sorbitol on dextranase stability.

Added content (%)	Control 1	Control 2	10	13	15	17	19
Residual activity (%)	100	50	42	58	49.9	49.4	51.3

enzyme. Among these saccharides, glucose, sorbitol, and sucrose exhibited the best effect. Sorbitol showed the greatest influence on dextranase stability; thus, sorbitol was selected for further optimization (Table 1). Dextranase activities were measured following the aforementioned method, and residual activities were calculated. The result showed that the best group was 13% sorbitol, with a residual activity of 58%, which increased the dextranase stability by 8%. In contrast to the stabilizers below, the effect of sorbitol was not noticeable; it was not selected as a component of the stabilizers.

The effects of different polyols (Chen, 2006) on dextranase stability were examined with different additional contents (Table 2). The control group was prepared as aforementioned. Dextranase activities were measured following the previously mentioned method, and residual activities were calculated. When the concentration of glycerin was 16%, the residual activity was 93.8%, indicating that it was the best group in the experiment. Glycerin, which is categorized as a polyol (Gangadhara, Ramesh Kumar, & Prakash, 2008; Liu, Ji, Zhang, Dong, & Sun, 2010; Romero, Albis, Manuel Lozano, & Sancho, 2009), can be combined with water molecules; it reduces the dielectric constant of the medium and strengthens enzyme hydrophobicity to improve enzyme stability (Kamal, Ahmad, & Rao, 2011; Kumar, Chari, Sharma, & Kalonia, 2011; Vagenende, Yap, & Trout, 2009). Glycerin also showed significant effect on the improvement of dextranase stability as it enhanced the stability by 43.8%. The ethanediol and polyethyleneglycol groups improved dextranase stability by 40% and 18.4%, respectively. Although the ethanediol group obviously improved dextranase stability, ethanediol did not follow the principle of safety because it is toxic to humans. The polyethyleneglycol group slightly improved dextranase stability. Therefore, ethanediol and polyethyleneglycol are not suitable stabilizer components.

Certain concentrations of carboxylic compounds, such as acetic acid and sodium citrate, can stabilize the enzyme active site, as previously reported (Kaushik & Bhat, 1998). The effect of sodium acetate and sodium citrate on dextranase stability was observed with different added contents (Table 3) (Chen, 2006). Dextranase activities were measured following the previously mentioned method, and residual activities were calculated. The result showed that sodium acetate and sodium citrate had significant effects on the improvement of dextranase stability. They enhanced the stability by 22.1% and 21.3%, respectively, with the best concentration. Although the function of the common additives sodium acetate and sodium citrate was less obvious than that of glycerin, they improved dextranase stability more effectively than sorbitol. Glycerin, sodium acetate, and sodium citrate were selected for further tests of the compound stabilizer.

3.2. Effect of compound stabilizer on dextranase stability

Several kinds of stabilizers noticeably affected the stability of the enzyme based on the preceding tests. Three kinds of stabilizers that showed significant effects were selected as stabilizers for further orthogonal experiments (Table 4). The control group was prepared as previously mentioned. Dextranase activities were measured following the aforementioned method, and residual activities were calculated.

The best proportions for the three kinds of stabilizers were 16% glycerol, 18% sodium acetate, and 20% sodium citrate.

3.3. Effect of different antiseptics on dextranase stability

The production process of liquid enzyme is easier and produces lesser dust pollution than enzyme powder. However, liquid enzyme is not convenient for storage; it easily rots and loses its activity. To extend the shelf life of enzymes, an antiseptic must be added. Selecting antiseptics is important for dextranase application. These antiseptics should not affect dextranase stability and should be safe and economical for industrial application. Several antiseptics (Chen, 2006) were selected for this study, including D-sodium isoascorbate, p-hydroxy benzoic acid compound sodium acetate, potassium sorbate, chitosan, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate and sodium dehydroacetate. Dextranase activities were measured following the previously mentioned method, and residual activities were calculated.

The result showed that p-hydroxy benzoic acid compound sodium acetate, D-sodium isoascorbate, and potassium sorbate more obviously influenced dextranase stability (Table 5). p-Hydroxy benzoic acid compound sodium acetate, which is a carboxylic compound, has a mechanism similar to that of acetic acid and sodium citrate.

3.4. Effect of compound antiseptic on dextranase stability

To determine the best proportion of the three kinds of antiseptics, an orthogonal experiment (Table 6) was designed. Dextranase activities were measured using the aforementioned method, and residual activities were calculated. The compound antiseptic experiment showed that the best proportions for the three kinds of antiseptics were 0.05% p-hydroxy benzoic acid compound sodium acetate, 0.03% D-sodium isoascorbate, and 0.05% potassium sorbate. These proportions could enhance dextranase stability by 24.7%. Generally, antiseptics are applied in the food industry. This study provided technical data for the anticorrosion of dextranase.

3.5. Subsequent validation experiment results

Subsequent validation experiment was carried out to validate the best proportions. The result showed that dextranase with added enzymatic protective reagents maintained 70.80% and 28.96% residual activities at the 13th week at 25 and 37 °C, respectively (Table 7). These findings provide experimental basis for dextranase

Table 2
Effect of different polyols on dextranase stability.

Residual activity (%)	Added content (%)						
	Control 1	Control 2	6	8	10	12	16
Glycerin	100	50	63.2	66.8	81.0	80.4	93.8
Ethanediol	100	50	68.4	81.8	90	61.2	32.4
Polyethylene glycol	100	50	54.4	68.4	64.1	61.7	26.8

Table 3
Effect of sodium acetate sodium citrate on dextranase stability.

Residual activity (%)	Added content (%)									
	Control 1	Control 2	8	10	12	15	18	20	24	26
Sodium acetate	100	50	35.6	48.7	54.6	61.8	72.1	65.6	65.3	–
Sodium citrate	100	50	–	35.4	49.6	60.9	65.9	68	71.3	64.3

Table 4
Effect of compound stabilizer on dextranase stability.

Added content/% Group	Glycerin	Sodium acetate	Sodium citrate	Activity (U/μmol)	Residual activity (%)
Control 1	0	0	0	6.582	100
Control 2	0	0	0	3.547	53.9
2	14	17	20	4.018	61.0
3	14	18	24	4.027	61.2
4	14	19	26	3.837	58.3
5	15	17	24	4.252	64.6
6	15	18	26	4.540	69.0
7	15	19	20	4.630	70.3
8	16	17	26	4.390	66.7
9	16	18	20	5.404	82.1
10	16	19	24	4.298	65.3

Table 5
Effect of the three best antiseptics on dextranase stability.

Antiseptic	Antiseptic (%)	Residual activity (%)	Residual activity of the control group (%)
D-Sodium isoascorbate	0.03	50.5	50
p-Hydroxy benzoic acid compound sodium acetate	0.04	74.6	50
Potassium sorbate	0.06	50.5	50
Chitosan	0.35	19.3	50
Ethyl 4-hydroxybenzoate	0.08	16.8	50
Propyl 4-hydroxybenzoate	0.07	47.2	50
Sodium dehydroacetate	0.01	26.4	50

Table 6
Effect of compound antiseptic on dextranase stability.

Group	p-Hydroxy benzoic acid compound sodium acetate (%)	D-Sodium isoascorbate (%)	Potassium sorbate (%)	Activity (U/μmol)	Residual activity (%)
Control	0.00	0.00	0.00	1.459	50
1	0.03	0.02	0.05	1.743	59.9
2	0.03	0.03	0.06	1.629	55.9
3	0.03	0.04	0.07	1.653	56.7
4	0.04	0.02	0.06	1.793	61.6
5	0.04	0.03	0.07	1.782	61.2
6	0.04	0.04	0.05	1.608	55.2
7	0.05	0.02	0.07	1.605	55.1
8	0.05	0.03	0.05	2.169	74.7
9	0.05	0.04	0.06	1.612	55.3

Table 7
Effect of enzymatic protective reagents on dextranase stability.

Weeks	Control	Residual activity (25 °C) (%)	Residual activity (37 °C) (%)
0	100	100	100
1	0	104.02	84.46
2	0	103.05	80.46
3	0	103.02	79.85
4	0	102.09	67.27
9	0	59.85	32.37
11	0	74.60	34.83
13	0	70.80	28.96
17	0	54.78	17.19

application in the production of toothpaste and mouthwash, which help clear plaque and prevent caries.

We confirm that the enzymatic protective reagents from *A. oxydans* KQ11 could remarkably improve dextranase stability. By contrast, under factory conditions, commercial dextranase activity is approximately halved (~46%) for 90 days and even shows reduced activity when stored under refrigeration (Eggleston & Monge, 2005). The enzymatic protective reagents selected were safer, more economical, and more effective compared with random and site-directed mutageneses through molecular techniques. This method has extremely important industrial and biotechnological implications because dextranase from *A. oxydans* KQ11 could be used in harsh industrial conditions without changes in its specific catalytic activity. Stability improvement of dextranase with stabilizers has not been reported to date. Moreover, no studies are available on safe, economical, and effective antiseptics that prevent microbial growth and emergence of secondary metabolites.

4. Conclusion

This study aimed to enhance dextranase stability from isolated *A. oxydans* KQ11. The reported strain is an ideal raw material

for industrial production of dextranase. *A. oxydans* KQ11, a non-pathogenic bacterium, grows at mild conditions and produces extracellular, highly substrate-specific dextranase. The addition of enzymatic protective reagents resulted in noticeably enhanced stability. These reagents can be conveniently used in various biotechnological applications.

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