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Recent Advances in Microbial Raw Starch Degrading Enzymes

Haiyan Sun · Pingjuan Zhao · Xiangyang Ge · Yongjun Xia · Zhikui Hao · Jianwen Liu · Ming Peng

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Abstract Raw starch degrading enzymes (RSDE) refer to enzymes that can directly degrade raw starch granules below the gelatinization temperature of starch. These promising enzymes can significantly reduce energy and simplify the process in starch industry. RSDE are ubiquitous and produced by plants, animals, and microorganisms. However, microbial sources are the most preferred one for large-scale production. During the past few decades, RSDE have been studied extensively. This paper reviews the recent development in the production, purification, properties, and application of microbial RSDE. This is the first review on microbial RSDE to date.

Keywords Raw starch degrading enzymes · Production · Purification · Properties · Application

Introduction

Starch is the major carbohydrate reserve polymer in maize, wheat, oat, rice, potato, cassava, etc. It is a potential substrate for the production of gaseous or liquid fuels, proteins, sugars, and chemicals [1]. The conventional conversion of starch to glucose requires a two-step process, namely, liquefaction and saccharification. This process is energy-intensive, thus

H. Sun · P. Zhao · M. Peng (✉)
Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture,
Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences,
Haikou 571101, China
e-mail: mmpeng_2000@yahoo.com

X. Ge · Y. Xia · Z. Hao
The Key Laboratory of Industrial Biotechnology, Education Ministry, School of Biotechnology,
Jiangnan University, Wuxi, China

J. Liu
Research Center of Enzymes, AsiaPac Bio-technology Co. Ltd., Dongguan, Guangdong 523808, China

increasing the production cost of starch-based products. Raw starch degrading enzymes (RSDE) can act directly on raw starch granules below the gelatinization temperature of starch. Therefore, RSDE significantly reduce the requirements of energy and simplify the process. Robertson et al. [2] estimated that the reduction in energy usage achieved by using RSDE in ethanol production is 10–20%. The exhaustion of fossil fuels in the foreseeable future has sparked a global effort to develop alternative energy-saving process. Hence, with the view of reducing the energy cost of starch processing and process simplicity, conversion of raw starch directly to monosaccharide by RSDE is superior to the conventional process. Furthermore, the absence of high-temperature cooking minimizes the formation of undesirable Maillard reaction byproducts that reduce yield [3]. Currently, there is an ongoing search for efficient RSDE, especially since the oil crisis of 1973. RSDE are ubiquitous and produced by plants [4], animals [5], and microorganisms [6]. In spite of the wide distribution of RSDE, microbial sources have many advantages for the industrial production such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization. Therefore, microbial sources are the most preferred one for large-scale production. Much work has been carried out regarding the production, purification, properties, and application of microbial RSDE in recent years. This review illustrates an overview of recent advances in microbial RSDE.

Production of RSDE

Screening for the potent RSDE producers is a key step for RSDE production. As listed in Table 1, a number of microorganisms including fungi, yeasts, and bacteria have been reported to produce RSDE. The RSDE producers are widely distributed in the nature. These strains have been isolated mainly from rotting starchy material, soil, and air. Among them, *Aspergillus* sp. and *Rhizopus* sp. and *Bacillus* sp. are apparently the most common choices.

Like other enzymes, RSDE production is generally influenced by the strain, composition of the medium, and cultural conditions. As summarized in Table 2, many researchers have conducted extensive investigations into enhancing the productivity and economy of RSDE production, including screening potent producers, optimization of the medium composition, and cultural conditions.

RSDE production is also influenced by different fermentation modes, such as solid-state fermentation (SSF) or submerged fermentation (SmF) or batch or fed-batch fermentation. Morita et al. made a comparison of RSDE production in liquid and solid cultures by *Rhizopus* strains. It was observed that RSDE productivity of the liquid culture was about 4.4 times higher than that of the solid-state culture, based on the unit starch amount in the liquid and solid media carbon source [42]. However, SSF attained new attention in recent years due to its advantages such as its simplicity and closeness to the natural growth conditions of many microorganisms, especially fungi. Wang et al. maximized the production of RSDE by *Rhizopus* sp. using SSF combined with SmF. The yield of RSDE from the combined system were over 18- and 4-fold higher than the results obtained by SmF and SSF systems singly, respectively [44]. Batch fermentation is used in most cases for RSDE production because of its ease of control. However, fed-batch culture is superior to batch fermentation for RSDE production by *Penicillium* sp. S-22. When partially hydrolyzed raw yam starch and peptone were fed together in a pH-stat mode, RSDE activity and productivity increased by 22.9- and 17.8-fold greater than the values obtained in the batch culture, respectively [35].

Table 1 RSDE-producing microorganisms so far reported.

Microorganism	Source	Type	Reference
Fungi			
Acremonium sp.	Forest tree	GA	[7]
Aspergillus awamori	–	–	[8]
Aspergillus awamori var. *kawachi*	–	–	[9–11]
Aspergillus awamori KT-11	air	α	[12]
Aspergillus carbonarius	rotten cassava	β	[13]
Aspergillus cinnamomeus	–	GA	[14]
Aspergillus ficum	–	α	[15]
Aspergillus niger	–	GA	[16, 17]
Aspergillus niger	Cassava waste	–	[18]
Aspergillus niger AM07	Soil	α, GA	[19]
Aspergillus niger NIAB 280	–	GA	[20]
Aspergillus niger sl. 1	Soil	–	[21]
Aspergillus oryzae	–	GA	[22]
Aspergillus sp. N-2	Cassava chips	GA	[23]
Aspergillus sp GP-21	Garden soil	GA	[24]
Aspergillus sp K-27			[25, 26]
Aspergillus terreus	Cassava waste	–	[18]
Chalara paradoxa	–	–	[27–29]
Cladosporium gossypiicola ATCC 38026	–	GA	[30]
Corticium rolfsii	Tomato stem	GA	[31–33]
Gibberella pulicaris	Tree	GA	[7]
Nodilusporium sp.	–	GA	[7]
Penicillium brunneum No. 24	–	α	[34]
Penicillium sp. S-22	Soil	–	[35]
Penicillium sp. X-1	–	GA	[36]
Rhizoctonia solani	–	GA	[37]
Rhizomucor pusillus	–	GA	[38]
Rhizopus niveus	–	GA	[39]
Rhizopus sp.	–	GA	[40]
Rhizopus sp.	–	GA	[41]
Rhizopus sp. A-11	Ragi	GA	[42]
Rhizopus sp. MKU 40	–	GA	[43]
Rhizopus sp. W-08	Mildewed corn	GA	[44]
Rhizopus stolonifer	Cassava waste	–	[18]
Synnematous sp.	–	α	[7]
Streptcoccus bovis	–	–	[45]
Streptomyces precox NA-273	–	α	[46]
Streptomyces sp.E-2248	Mud	–	[47]
Streptomyces sp no. 4	Soil	α	[48]
Thermomucor indicae-seudaticae	Soil	GA	[49]
Thermomyces lanuginosus F1	Municipal compost	α	[50]
Yeast			
Aureobasidium pullulans N13d	Deep sea	GA	[51]

Table 1 (continued)

Microorganism	Source	Type	Reference
Cryptococcus sp. S-2	–	α	[52]
Endomycopsis fibuligera	–	GA	[53]
Saccharomycopsis fibuligera	–	GA	[54]
Bacteria	–		
Anoxybacillus contaminans	–	α	[55]
Bacillus amyloliquefaciens	Soil	α	[56]
Bacillus cereus	–	β	[57]
Bacillus circulans F-2	Potato starch	–	[58]
Bacillus firmus	Soil	CTG	[59]
Bacillus licheniformis	–	α	[60]
Bacillus macerans BE101	–	CGT	[61]
Bacillus polymyxa	–	β	[62]
Bacillus sp. B1018	–	α	[63]
Bacillus sp. I-3	–	α	[64]
Bacillus sp. IMD 434	–	α	[65]
Bacillus sp. IMD 435.	Mushroom compost	α	[66]
Bacillus sp. TS-23	–	α	[67]
Bacillus sp. WN11	Hot spring	α	[68]
Bacillus sp. YX-1	Soil	α	[6]
Bacillus stearothermophilus	–	α	[69]
Bacillus subtilis 65	–	α	[70]
Bacillus subtilis IFO 3108	–	α	[71]
Clostridium butyricum T-7	Mesophilic methane sludge	α	[72]
Cytophaga sp.	Soil	α	[73]
Geobacillus thermodenitrificans HRO10	–	α	[74]
Klebsiella pneumoniae AS-22	–	CGT	[75]
Lactobacillus amylophilus GV6	Starchy waste	AMP	[76]
Rhodopseudomonas sp T-20	–	–	[77]
Streptococcus bovis 148	–	α	[78]
Thermoanaerobacter sp.	–	CGT	[79]

α α-amylase, *AMP* amylopullulanase, *CGT* cyclomaltodextrin glucanotransferase, *β* β-amylase, *GA* glucoamylase

Purification of RSDE

Various purification strategies adopted for microbial RSDE were summarized in Table 3. The methods used to purify RSDE can vary considerably, but most purification protocols involve a series of steps. The purification of extracellular RSDE from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from the fermentation broth by centrifugation and/or ultrafiltration, selective concentration by precipitation using ammonium sulfate, or organic solvents such as chilled acetone or ethanol. The crude enzyme is then subjected to chromatography, usually affinity, ion exchange, and/or gel filtration. Intracellular RSDE need the usual step of cell wall

Table 2 Carbon sources, nitrogen sources, and cultural conditions of some RSDE-producing strains.

Microorganism	Carbon and nitrogen sources	Cultural conditions	Reference
Aspergillus carbonarius	Potato starch, peptone	30 °C, 100 rpm, 96–144 h	[13]
Aspergillus niger	Wheat bran, yeast extract	30 °C, pH 6.5, 150 rpm, 80–96 h	[20]
Aspergillus niger and *Saccharomyces cerevisae*	Sorghum pomace	30 °C, pH 5.0, 100 rpm, 72 h	[21]
Aspergillus niger	Cassava waste	28 °C, static culture, 10 days	[18]
Aspergillus terreus			
Rhizopus stolonifer			
Aspergillus niger	Cassava starch, yeast extract	30 °C, 100 rpm, 96 h	[80]
Aspergillus sp GP-21	Wheat bran	30 °C, 72 h	[24]
Aureobasidium pullulans N13d	Soluble starch, peptone	28 °C, pH 4.0, 250 rpm; aeration 6 l/min, 56 h	[51]
Bacillus firmus	Soluble starch, yeast extract, peptone	30 °C, 200 rpm	[81]
Bacillus sp. IMD 435	Lactose, yeast extract	40 °C, pH 7.0, 200 rpm, 41 h	[66]
Bacillus sp. IMD434	Soluble starch, yeatex, corn steep liquor	40 °C, pH 7.0, 200 rpm, 41 h	[65]
Bacillus sp. TS-23	Soluble starch, yeast extract, peptone	55 °C, pH 8.5, 150 rpm, 18 h	[67]
Bacillus sp. YX-1	Soluble starch, peptone, corn syrup	45 °C, pH 4.5, 150 rpm, 44 h	[6]
Bacillus sp.I-3	Galactose, soyabean meal	37 °C, pH 7.0, 150 rpm, 48 h	[64]
Cladosporium gossypiicola ATCC 38026	Corn starch, soya bean meal	25 °C, pH 5.5, shaking, 120 h	[30]
Cryptococcus sp. S-2	Maltose, yeast extract	pH 4.5	[52]
Cytophaga sp.	Corn starch, polypeptone, yeast extract	35 °C, pH 6.5, 100 rpm, 24 h	[82]
Gibberella pulicaris	Sago starch, peptone, sodium nitrate	27 °C, pH 4.5, 120 rpm, 5 days	[83]
Klebsiella neumonia AS- 22	Soluble starch, yeast extract, peptone	30 °C, pH 7.0, 400 rpm, airflow rate 8 l/min,18 h	[75]
Nodilusporium sp.	Sago starch, peptone, sodium nitrate	27 °C, pH 4.5, 120 rpm, 6 days	[7]
Penicillium sp. S-22	Yam starch, peptone	30 °C, pH 5.5, 600 rpm, aeration: 5.5 l/min, 96 h	[35]
Penicillium sp. X–1	Wheat bran, maltotetraose, peptone	30 °C, pH 6.0, 36 h	[36]
Rhizopus sp. A-11	Liquid culture: cassava starch, CH_3COONH_4	30 °C, pH 6.0, 600 rpm, 1 vvm, 30 h	[42]
	Solid culture: cassava starch, wheat bran	30 °C, 7 days	
Rhizopus sp. W-08	Corn flour, wheat bran, $(NH_4)_2SO_4$	30 °C, stationary for 36 h, then 140 rpm for 36 h	[44]
Streptomyces sp. No. 4	Sweet potato starch, soybean meal	30 °C, pH 6.0, shaking, 3 days	[48]
Synnematous sp.	Raw sago starch, peptone, sodium nitrate	27 °C, pH 4.5, 120 rpm, 7 days	[7]

Table 3 Purification strategies employed for RSDE.

Microorganism	Purification strategy	Purification fold/yield (%)	Reference
Aspergillus carbonarius	4 M sucrose concentration, S-Sepharose (fast flow) column, Q-Sepharose (fast flow) column	2.8/12.0	[13]
	Purification of glucoamylase: 60% $(NH_4)_2SO_4$ precipitation, 2-propanol precipitation, cG_6-Sepharose 6B column, DEAE-Toyopearl 650M	8.5/60.5	
Bacillus firmus	Ultrafiltration, starch affinity chromatography, gel filtration (Sephacryl S-200)	400/64	[59]
Bacillus sp. IMD 370	Ethanol precipitation, first DEAE BioGel A chromatography, second DEAE BioGel A chromatography, Superose 12 gel filtration	104.3/14.9	[84]
Bacillus sp. IMD 434	Conventional method: acetone precipitation, ion-exchange (Resource Q column), hydrophobic interaction chromatography (phenyl-sepharose)	266/10	[65]
	One-step affinity method: α-CD Sepharose 6B affinity chromatography	375/65	[85]
Bacillus sp. IMD 435	α-CD Sepharose 6B affinity chromatography	774/65	[66]
Bacillus sp. TS-23	Raw starch adoption-elution, Sephacryl S-100 HR, HiTrap	708.5/13.2	[67]
Bacillus sp. WN11	60% $(NH_4)_2SO_4$ fractionation, first DEAE-Sephadex, second DEAE-Sephadex, Gel filtration(Sephadex G-75)	E I: 65.0/13.0 E II: 40.7/9.5	[68]
Bacillus sp. YX-1	60% $(NH_4)_2SO_4$ fractionation, DEAE-Sepharose, Sephadex G-75	34/6.6	[6]
Corticium rolfsii	Purification of G1and G2:	G1: 34.1/16	[31]
	80% $(NH_4)_2SO_4$ fractionation, first DEAE-	G2:32.5/13	
	Toyopearl 650M, DEAE-Sepharose CL -6B, second		
	DEAE-Toyopearl 650M		
	Purification of G3:		
	80% $(NH_4)_2SO_4$ fractionation, first DEAE-Toyopearl 650M, second DEAE-Toyopearl 650M, SP-Toyopearl 650m, DEAE-Sepharose CL-6B	31.7/1.2	
Cryptococcus sp. S-2	Ultrafiltration, α-cyclodextrin-Sepharose 6B	141/78	[52]
Klebsiella pneumoniae AS-22	Ultrafiltration, starch affinity chromatography, gel filtration (Sephacryl S-200)	738/68	[75]
Penicillium sp. X-1	65% $(NH_4)_2SO_4$ precipitation	4.3/91	[36]
Rhizopus sp. A-I1	80% $(NH_4)_2SO_4$ precipitation, CM-Sephadex C-50 chromatography	5.4/72.9	[42]
Streptomyces sp. E-2248	Starch adoption, DEAE-Toyopearl, Toyopearl-HW55S	2130/60.1	[47]
Streptomyces sp. no. 4	Purification of amylase-1	126/2.0	[48]
	Starch adsorption, α-CD Sepharose 6B, DEAE-Toyopearl 650M		
	Purification of amylase-2	16.3/4.4	
	Starch adsorption, α-CD Sepharose 6B, DEAE-Toyopearl 650M, SP-Toyopearl550		

Table 3 (continued)

Microorganism	Purification strategy	Purification fold/yield (%)	Reference
Streptomyces thermocyneoviolaceus IFO 14271	Conventional method:	161/21	[86]
	$(NH_4)_2SO_4$ fractionation, Raw corn starch treatment, Utrogel AcA34(1), FPLC MONO Q HR 5/5, Phenyl-sepharose, Utrogel AcA34(2)		
	Raw starch affinity method:	194/26	
	$(NH_4)_2SO_4$ fractionation, Raw corn starch affinity column, FPLC MONO Q HR 5/5		
Thermomucor indicae-seudaticae	Lyophilization and acetone precipitation, SP-sepharose, sephadex G-50	9.84/1.03	[49]

destruction and then followed the similar procedures. Fractionation by ammonium sulfate or acetone precipitation, which was reported as the easiest techniques, proved unsuccessful for RSDE from *Aspergillus carbonarius*, as the enzyme lost activity rapidly even when maintained at low temperature (0–4 °C). Sucrose solution was found to effectively concentrate the amylase with minimum loss of activity [13]. Most RSDE can adsorb on starch granules, so the starch adoption–elution was an effective method [47]. Mai et al. [86] compared the purification of RSDE by conventional method and raw starch affinity method. The purification folds were 161 and 192, respectively. α-CD Sepharose 6B affinity was also a good alternative for purification of RSDE [65, 66]. As shown in Table 3, the purification fold/yield of RSDE by *Bacillus* sp. IMD 434 from α-CD Sepharose 6B affinity method and conventional method were 375/65% and 266/10%, respectively [65].

Properties of RSDE

The biochemical properties of RSED from different microorganisms have been studied extensively. A summary is presented in Table 4.

pH Optima and Stability

As shown in Table 4, most RSDE had optimum pH in the acidic to neutral range. However, alkaliphilic RSDE from *Bacillus* sp IMD 370 and *Bacillus* sp.TS-23 had optimum pH of 8.0 and 9.0, respectively [67, 84]. Optimum pH of RSDE from *Bacillus* sp. IMD 370 varied from substrate to substrate. The pH optimum for raw starch hydrolysis was pH 8.0 while pH 10.0 for soluble starch hydrolysis [88]. pH stability is important for the conservation and application of RSDE. Broad pH stability of RSDE from *A. carbonarius* [13], *Bacillus* sp. IMD434 [65], *Bacillus* sp. YX-1 [6], *Penicillium* sp. X-1 [36] was observed. pH stability of RSDE from *Bacillus firmus* was dependent on temperature. It was stable over the pH range 7–11 at 10 °C and stable at pH 7.0 at 60 °C [59].

Temperature Optima and Stability

As listed in Table 4, most RSDE are known to exhibit temperature optima between 40 and 65 °C. Exceptionally, the temperature optima for RSDE from thermophilic *Bacillus* sp.TS-23, *Bacillus* sp. I-3 and *Bacillus* sp. WN11 were 70, 70, and 75–80 °C, respectively [64, 67, 68].

Table 4 Properties of some microbial RSDE.

Strains	pI	MW (kDa)	pH optima / stability	Temperature optima/stability	Stabilizer or activator	Inhibitors	Reference
Aspergillus awamori var. kawachi HF-15	–	GAO 250	–/2.0–8.0(20h)	–/65 °C (15min)	–	–	[90]
Aspergillus niger	–	–	6.0/3.0–7.0	–	–	–	[80]
Aspergillus sp GP-21	–	–	5.5/–	65 °C/–	–	–	[24]
Rhizopus sp. A-11	–	72.4	–	–	–	–	[42]
Aspergillus oryzae	4.2	65	4.5/–	65 °C/<46 °C	–	–	[22]
Aspergillus carbonarius	–	32	6.0–7.0/3.0–9.0	40 °C/30–80 °C	Co^{2+}	EDTA, Na^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Ba^{2+}, Si^{2+}, Ag^{+}	[13]
Bacillus firmus		82	5.5–8.5/7.0–11.0 at 10 °C, 7.0 at 60 °C	65 °C/–	–	–	[59]
Bacillus amyloliqefaciens	–	Wild: 52	6.0/5.0–8.0 for 1 h	55 °C/69% residual activity after 4 h at 40 °C	Mg^{2+}, Ba^{2+}	Hg^{2}, Ag^{+}, Fe^{2+}, Zn^{2+}	[56]
	–	Mutant: 52	6.0/5.0–8.0 for 2 h	55 °C/70% residual activity after 4 h at 50 °C	Mg^{2+}	Cu^{2+}, Hg^{2+}, Ag^{+}, Fe^{2+}, Zn^{2+}	
Bacillus sp.TS-23	4.2	42	9.0/–	70 °C/–	Ca^{2+}	Hg^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, EDTA	[67]
Bacillus sp. WN11		AmyI: 76	5.5/5.5–9.0	75–80 °C/50% residual activity at 80 °C (4 h)	Ca^{2+}, Na^{+}, Co^{2+}, Ba^{2+}	Hg^{2+}, Cu^{2+}, Fe^{2+}	[68]
		AmyI I: 53	5.5/5.5–9.0	75–80 °C 50% residual activity at 80 °C (4 h)	Ca^{2+}, Na^{+}, Co^{2+}, Ba^{2+}	Hg^{2+}, Cu^{2+}, Fe^{2+}	

Bacillus sp.IMD434	5.9	69.2	6.0/4.0–9.0	65 °C/40° C (1 h), half-live at 65 °C was 18 min	Cysteine, dithiothreitol.	–	[65]
Bacillus sp.IMD435	5.6	63.0	6.0	65 °C/–			[66]
Bacillus sp. YX-1	–	56.0	5.0/4.5–11.0	40–50 °C/–	–	–	[6]
Bacillus sp. I-3	–	–	7.0/6.0–7.0	70 °C/94% residual activity after 3.5 h at 70 °C	Fe^{2+}, Cu^{2+}, Mn^{2+}	Hg^{2+}	[64]
Corticium rolfsii AHU 9627	3.85	78	4.5	60 °C/–	–	–	[31]
Cryptococcus sp. S-2	4.2	66	6.0	50 °C/60 °C	–	Hg^{2+}, Ag^{+}, Cu^{2+}, Zn^{2}	[52]
Cytophaga sp.	–	59	6.5–9.5	60 °C/–	–	Mn^{2+}, Cu^{2+}, Zn^{2+}	[87]
Gibberella pulicaris	–	–	5.5	40 °C/–	–	–	[83]
Klebsiella pneumoniae AS-22	7.3	75	7.0–7.5/6.0–9.0	45 °C/35–50 °C, 100% stable at 30°C for a month in 30% glycerol	Ca^{2+}, soluble starch	–	[75]
Penicillium sp. X-1	–	–	6.5/3.0–8.0	65 °C/<70 °C	Mn^{2+}, Ca^{2+}, Li^{2+}, Fe^{3+},Cu^{2+}	Mg^{2+}, Ni^{2+}, Zn^{2+}	[36]
Streptomyces sp. No. 4	–	E_1:56	5.5/–	50 °C/–	–	Hg^{2+}, pCMB, EDTA	[48]
	–	E_2:77	5.5/–	45 °C/–	2-mercaptoe-thanol, EDTA	–	
Streptomyces sp. E-2248		–	6.0/5.0 (1h)	50 °C/50 °C (30 min)	Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}	Fe^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}	[47]
Thermomucor indicae-seudaticae	8.2	42	7.0/–	60 °C/half-lives at 60 °C, 12 h	Co^{2+}, Fe^{2+}, Ag^{+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, dextran, trehalose	Hg^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+}, EDTA	[49]

Table 5 Cloning and expression of RSDE genes from different sources.

Gene source	RSDE	Vector	Recombinant host	Reference
Aspergillus awamori KT-11	α	pT7Blue	*E. coli*	[107]
Bacillus amyloliquefaciens	α	pKK233–2	*E. coli*	[56]
Bacillus cereus	β	pET21d	*E. coli*	[108]
Bacillus circulans F-2	α	pYKA3	*E. coli*	[109]
Bacillus sp. B1018	α	λ-ELBL3	*E. coli*	[110]
Bacillus sp. TS-23	α	pQE-30	*E. coli*	[111]
Corticium rolfsii	GA	pACG 115	S. cerevisiae	[31]
Cryptococcus sp. S-2	α	pYCDE1	S. cerevisiae	[52]
Cytophaga sp.	α	pAcUW21	*E. coli*	[73]
Saccharomycopsis Fibuligera IFO0111	GA	pGEM-T	S. cerevisiae	[112]
Streptococcus bovis 148	α	pBR322	*E. coli*	[78]

The thermostability of RSDE is affected by substrate, calcium, carbohydrate moiety, C-terminal domain, etc. RSDE from *B. firmus* and *Klebsiella pneumoniae* AS-22 rapidly lost activity when incubated in the absence of substrate [75]. The thermostability of the enzyme was improved in the presence of $CaCl_2$ for RSDE from *K. pneumoniae* AS-22 [75], *Penicillium* sp. X-1 [36], and *Bacillus* sp. I-3 [64]. The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus causing the adoption of a compact structure. Kumar and Satyanarayana reported that thermostability of RSDE from *Thermomucor indicae-seudaticae* could be due to the carbohydrate moiety associated with the enzyme [49]. Thermostability of RSDE from *Cytophaga* sp. was improved by 20-fold through polymerase chain reaction-based site-directed mutagenesis [89].

Molecular Weight

Molecular weights of microbial RSDE vary from about 32 to 250 kDa. The lowest value, 32 kDa for RSDE from *A. carbonarius* [13] and the highest of 250 kDa for RSDE from *Aspergillus awamori* var. *kawachi* HF-15 have been reported. The high molecular weight might be related to high carbohydrate content (24.3%) [90].

Synergistic Effect of Different Types of RSDE

The synergism of amylases has been well described using soluble starch as substrate. This holds true for raw starch as substrate. Raw starch, in its native form, exists in relatively inert granular structures, which are composed of macromolecules arranged in a polycrystalline state. It is difficult to hydrolyze raw starch by a single type of RSDE. The synergism of multi-RSDE might bring complete hydrolysis of raw starch. For instance, α-amylase is an endo-acting enzyme, which can cleave α-1, 4 glycosidic linkages inside the starch structure randomly and destroy the whole starch structure quickly. However, α-amylase only brought about incomplete hydrolysis with dextrin as the main product. Glucoamylase is an exo-acting enzyme, which can cleave both α-1, 4 and α-1, 6 glycosidic linkages from the non-reducing ends of starch chains and forms exclusively glucose, but glucoamylase hydrolyses

raw starch very slowly. Thus, the synergistic use of glucoamylase and α-amylase can hydrolyze raw starch completely and quickly. α-Amylase splits randomly the substrate molecules on the surface of the granules to release new non-reducing ends for gulcoamylase. Glucoamylase releases glucose successively from the granule and forms little holes from the surface to the center of the granule, which allows α-amylase into the inside of the granules and touches more glucoside bonds. This leads to physical disintegration of structure and consequent exposure of new sites susceptible to either or both enzymes. Most common synergies are completed by endo- and exo-acting RSDE [12, 26, 36, 91]. Synergic action also has been reported for two endo-RSDEs: salivary α-amylase and pullulanase [92].

Adorability and SBD

The ability of RSDE to adsorb onto and digest raw starch has been attributed to the presence of a raw-starch-binding domain. RSDE have been known as multi-domain enzymes consisting of a catalytic domain connected to a starch-binding domain (SBD) by an *O*-glycosylated linker region. SBD is usually a distinct sequence-structural module that improves the efficiency of an amylase so that the amylase can bind and digest raw starch. Approximately 10% of amylases contain a distinct SBD. SBD plays an active role in hydrolyzing raw starch and supports the enzyme adsorption to the cell wall where local increase of enzyme concentration may result in enhanced glucose flow to the cell [93]. SBD can be isolated by proteolysis of RSDE. The *Aspergillus niger* SBD was cloned, expressed, and purified as a fully domain in *A. niger* and *Saccharomyces cerevisiae* [94–96]. It has been demonstrated that the SBD independently retains its function even if fused to a protein other than amylase. After the deletion of SBD from RSDE, the enzyme lost its digestibility on raw starch [97]. Therefore, an important objective to enhance RSDE activity toward raw starch is to reduce protease production during the fermentation. To accomplish this, the operational approaches that have been successful include nutrient control, use of protease inhibitors, cell immobilization, growth morphology, pH control, bioreactor configuration, and screening the protease-negative producer. SBD are usually localized at the C-terminal end but in a few enzymes such as *Rhizopus oryzae* glucoamylase [98], *Lipomyces kononenkoae* α-amylase [99], and *Arxula adeninivorans* glucoamylase [100]. *Thermoactinomyces vulgaris* R-47 α-amylase [101], *Thermotoga maritima* pullulanase [102]. SBD is located in their N-terminal region. In the sequence-based classification, the individual types of SBDs have been placed into seven CBM families: CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, and CBM45. The family CBM20, known also as a classical C-terminal SBD of microbial amylases, is the most thoroughly studied. The three-dimensional structures have already been determined by X-ray crystallography or nuclear magnetic resonance for SBDs from five CBM families (CBM 20, 25, 26, 34, and 41), and the structure of the CBM21 has been modeled [103].

One potential application of SBD is enabling amylases without the ability to bind and degrade raw starch to perform these functions, e.g., α-amylase from *Bacillus subtilis* X-23 and SBD from *Bacillus* sp. A2–5a CGTase [104], glucoamylase from *S. cerevisiae* and SBD from *A. niger* glucoamylase [105], and barley α-amylase AMY1 and SBD from *A. niger* glucoamylase [106]. These results are valuable for constructing high producer of RSDE in the future. The activities of RSDE are relatively low compared with the activities of the combinations of enzymes used in the hydrolysis of gelatinized starch. Therefore, the fusion of appropriate gene of SBD and amylases, which are being used in industry, may achieve high activity RSDE producer.

Action Mechanism of RSDE

There is great difference in enzymatic hydrolysis of raw starch and gelatinized starch. Gelatinized starch in water is in a loose state, for its micelle structure has been destroyed. The enzyme can approach the inside of the starch molecules and act on them. The reaction takes place in aqueous solution. However, the enzymatic hydrolysis of raw starch is a heterogeneous reaction. The RSDE–substrate interaction must take place at the interface of the starch granules (solid state) and water (liquid state). Water molecules are associated and clustered with the hydrogen bonds in the surface of starch granules, which makes it difficult for enzyme to approach the substrate.

Cloning of RSDE Genes

Cloning of RSDE genes has been carried out for studying their sequences, characteristics, hyperproduction, expression, and enzyme engineering. As listed in Table 5, RSDE genes from different microorganisms, including fungi, yeast, and bacillus, have been cloned and expressed in appropriate host organisms using suitable vectors. *Escherichia coli* and *S. cerevisiae* were the most common hosts.

Application of RSDE

RSDE can replace current amylases to save energy and simplify the process. To date, RSDE have been tried in production of ethanol [27, 44, 113–121], organic acids [122], amino acids [123], cyclodextrin [124], and SCP [125]. Among them, the application in ethanol production was the most developed technology. Japanese Suntory Company industrialized the technology in 1980s. When raw corn starch was used as carbon source in 100-kl tank, the ethanol concentration reached 14.5% (*v*/*v*) after 90 h culture. Another potential application of RSDE is to produce porous starch, a kind of sorbent, which is widely used in the fields of food, drug, animal food, cosmetic, etc. Porous starch could be obtained from raw starch granules after partial hydrolysis by RSDE below gelatinization temperature. Compared to chemically synthesized sorbents, porous starch has no toxicity and has good biodegradability. Therefore, it is safe and environmentally friendly. With consumers' preferences for "natural," "green," and "health" products in the market, porous starch has gained much attention in recent years.

Conclusion

There have been a number of reports on RSDE in recent years. The main factors that hamper the application of RSDE is low yield and high cost. Some practical approaches could be adopted to make RSDE economically attractive: These include the use of overproducing mutant and recombinant strains, cheaper raw materials, optimized medium and culture conditions, and efficient recovery processes. Especially the application of DNA recombinant techniques to obtain high-yielding recombinant strains would be the real breakthrough in the development of RSDE in near future.

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