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Chapter 6
Ex Vivo Enzymatic Conversion of Non-food Cellulose Biomass to Starch

Chun You and Y.H. Percival Zhang

Abstract To meet the world's rising future food/feed needs, outputs of modern agriculture must grow substantially while minimizing agriculture's environmental footprint and conserving biodiversity. In this chapter, we propose an ex vivo synthetic enzymatic pathway to enable the transformation of non-food cellulose to amylose, a high-value linear starch, meanwhile glucose released by enzymatic hydrolysis of cellulose is used to produce ethanol and/or single-cell protein by yeast fermentation in the same vessel. The strategy of simultaneous enzymatic biotransformation and microbial fermentation is the basis of new biomass biorefineries that would address the food, fuels, and environment trilemma by coproducing food/feed, biomaterials, and biofuels from the most abundant renewable bioresource—non-food lignocellulosic biomass. Toward this development, new directions pertaining to pretreatment of lignocellulosic biomass and advanced enzyme engineering are discussed to increase the efficiency of saccharification.

Keywords Bioeconomy · New biorefinery · Food and feed · Invitro synthetic biology · Synthetic amylose · Food-energy-water nexus

6.1 Introduction

The continuing growth of population and food consumption per capita means that the global demand for food could increase by 70 % by 2050 [1, 2]. In the developed countries, starch provides at least 35 % of man's daily intake, whereas, this value may

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© Springer-Verlag GmbH Germany 2017

P.C.K. Lau (ed.), *Quality Living Through Chemurgy and Green Chemistry*,
Green Chemistry and Sustainable Technology,
DOI 10.1007/978-3-662-53704-6_6

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Layout: T1 Standard | Book ID: 337251_1_En | Book ISBN: 978-3-662-53702-2
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go to 80 % in most developing countries, especially in Africa and the far East. Increase in the supply of starch food to fight against hunger is becoming a global challenge. However, approximately 30 % of the world's arable land and 70 % of the world's fresh water withdrawals are being used for the production of food and feed to support seven billion people [3]. It is difficult to greatly increase arable land to agricultural use due to its other use and increase freshwater withdrawals. Some solutions were proposed to solve potential food crisis, like increasing agricultural resource efficiency, closing yield gaps on underperforming lands, reducing food waste, and developing genetically modified (GM) crops. However, long-term impacts of GM cereals as staple food on human health are not clear and their wide acceptance is in heated debates, especially in China and Europe [4, 5]. On the other hand, modern agriculture is also environmentally destructive, because some forests have been cut down and wetlands have been drained, more use of fertilizers and pesticides have caused serious environmental problems, such as changes in ecosystems, nonpoint water runoff pollution, climate changes, and decreased biodiversity [6].

Cellulose and starch are both anhydroglucose polymers, which are major products derived to plant photosynthesis. Cellulose is the supporting material of plant cell walls and the most abundant renewable bioresource on earth. It is a linear glucan linked by β-1,4-glucosidic bonds. Annual production of cellulose is estimated to be 100 billion tonnes [7]. It can be derived from a variety of sources, such as wood, agriculture residues, perennial plants, and so on. Humans, unlike herbivores, cannot utilize cellulose-containing biomass as a food source. Starch is the most important diet component for humans because it provides more than 50 % calories of healthy diets. It is a polysaccharide consisting of a large number of anhydroglucose units joined together primarily by α-1,4-glucosidic bonds and α-1,6-glucosidic bonds. Three major crops for producing starch-rich seeds are maize, rice, and wheat. However, dedicated cereal crops produce approximately 2.8 billion tonnes of starch-rich cereals annually, much less than annual cellulose production (Fig. 6.1). Due to the same building block (i.e., anhydroglucose) of cellulose and starch, the cost-effective transformation of non-food cellulose to edible starch could drastically enhance food security, while revolutionizing agriculture from cultivating annual seed-harvesting plants to perennial cellulose-rich plants, maintaining biodiversity, minimizing agriculture's environmental footprint, decrease inputs of fertilizers and herbicide, lowering energy consumption of farming machinery, and conserving fresh water [8]. This transformation cannot be done efficiently by microorganisms because of inherent side-reactions of microorganisms and cellular membrane that can prevent large molecular weight polymeric compounds such as cellulose and starch to go through. However, ex vivo synthetic enzymatic biosystems can implement this seemingly mission impossible task by assembling cascade enzymes outside cell membranes. Such ex vivo systems have many appealing advantages, like high product yield, faster reaction rate, great engineering flexibility, and high tolerance to toxic compounds [9–15].

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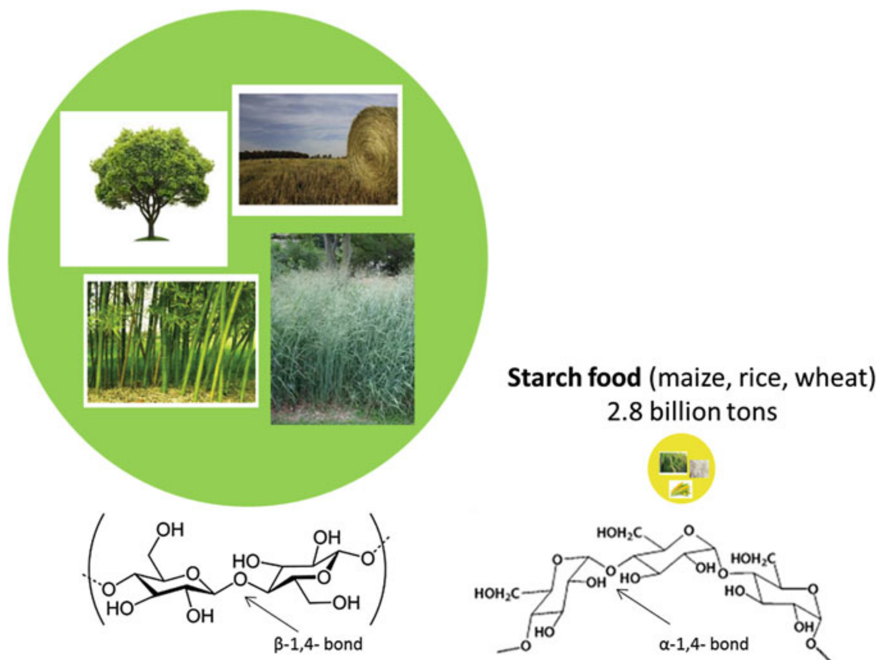

Fig. 6.1 The annual production of cellulose and starch on earth

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67 Here we demonstrate simultaneous (ex vivo) enzymatic transformation and
68 microbial fermentation that can transform cellulosic materials to starch, ethanol, and
69 single-cell protein in one pot.

70 6.2 Transform Cellulose to Starch

71 An ex vivo enzymatic pathway has been designed to transform cellulose to syn-
72 thetic amylose in an aqueous solution (Fig. 6.2a) [16]. This enzyme cocktail has
73 two modules: (1) partial hydrolysis of cellulose to cellobiose by an optimized
74 mixture of cellobiohydrolase (CBH) and endoglucanase (Endo), and (2) amylose
75 synthesis by utilizing cellobiose phosphorylase (CBP) and potato alpha-glucan
76 phosphorylase (PGP). In this system, CBP converts cellobiose to glucose
77 1-phosphate (G1P) and glucose in the presence of phosphate ions; PGP adds one
78 glucose unit from G1P at the nonreducing end of amylose, and phosphate is
79 recycled to maintain nearly constant pH and phosphate levels (Fig. 6.2a). To
80 eliminate glucose inhibition, the ethanol-producing yeast *Saccharomyces cerevisiae*
81 is added to the vessel because the yeast cannot utilize cellobiose and G1P [17, 18].

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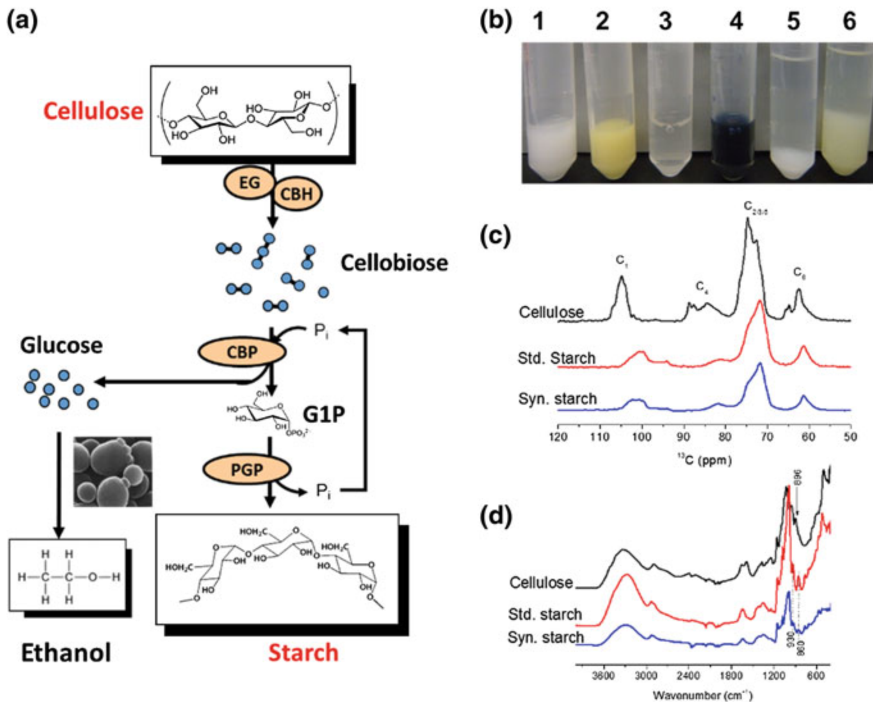

Fig. 6.2 The enzymatic transformation of converting cellulose to starch by endoglucanases (EGs), cellobiohydrolases (CBHs), cellobiose phosphorylase (CBP), and potato alpha-glucan phosphorylase (PGP), and the residual glucose was taken up by yeast to produce ethanol (**a**). Characterization of synthetic starch by iodine test (**b**), cross-polarization magic angle spinning (CP/M AS) ^{13}C-NMR (**c**) and FTIR (**d**). Tube 1: cellulose-suspended solution; tube 2: cellulose solution plus iodine/potassium iodide; tube 3: water-soluble synthetic starch solution made from cellulose mediated by the four-enzyme cocktail; tube 4: synthetic starch solution plus iodine/potassium iodide; tube 5: precipitated starch by ethanol addition; and tube 6: precipitated starch when the mixture was supplemented with glucose oxidase [16]

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82 This bioprocess called simultaneous enzymatic biotransformation and microbial
83 fermentation can transform pretreated biomass to amylose, ethanol, and yeast as a
84 single-cell protein in one bioreactor.

85 It is important to identify the right enzymes suitable for this ex vivo biotrans-
86 formation. Enzymatic cellulose hydrolysis usually requires synergistic action of
87 EG, CBH, and beta-glucosidase (BG) to yield glucose. But in our process,
88 beta-glucosidase is not allowed because this enzyme hydrolyzes cellobiose to
89 glucose, which cannot be added to starch chains. We tested the combinations of two
90 EGs [family 5 *Bacillus subtilis* EG (BsCel5) and *Trichoderma* spp. EG II
91 (TrCel5A)] and three CBHs family 7 *Trichoderma* spp. CBH (TrCel7A), family 9

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92 *Clostridium phytofermentans* CBH (CpCel9), and family 48 *C. phytofermentans*
93 CBH (CpCel48). Based on cellobiose yield and the cellulose degradation, the best
94 cellulase combination on regenerated amorphous cellulose [19] (RAC, high surface
95 area, prepared from Avicel) to cellobiose was bacterial BsCel5 and fungal TrCel7A.
96 For starch synthesis, three combinations of the *Clostridium thermocellum* CBP
97 (CtCBP) and one of the three αGP (from potato, *Solanumtuberosum* and two
98 thermophilic bacteria, *C. thermocellum* and *Thermotogamaritima*) were tested for
99 the synthesis of amylose fromcellobiose. Potato αGP (PGP) has the strongest ability
100 to generate amylose from low concentration of cellobiose (5 mM), while αGP from
101 *T. maritima* can only generate a little amylose from high concentration of cellobiose
102 (50 mM); and αGP from *C. thermocellum* cannot generate any amylose from very
103 high concentration (200 mM) of cellobiose. This study suggests the importance of
104 identification of right enzymes used in such ex vivo pathways.
105 One-pot biotransformation of RAC to amylose was performed by four enzymes,
106 Bscel5, TrCel5A, CtCBP, and PGP, in 0.5 ml of reaction volume (Fig. 6.2b).
107 The RAC slurry (Fig. 6.2b, tube 1) was completely hydrolyzed and then converted
108 into amylose (tube 3). The synthetic amylose exhibited a deep blue color in the
109 presence of iodine (tube 4), whereas the negative control (cellulose/iodine) was
110 yellow (tube 2). The soluble amylose was precipitated by the addition of ethanol
111 (tube 5). The amylose yield was 14.4 % (wt/wt) (i.e., 0.144 g of amylose per gram

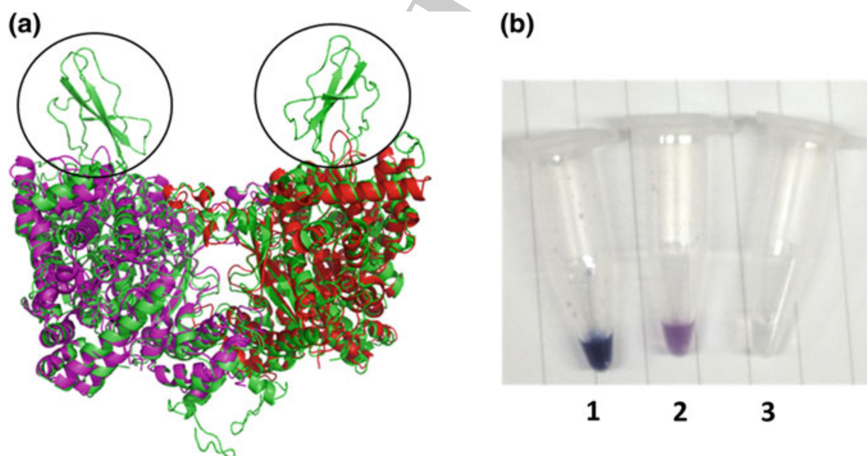

Fig. 6.3 Homology structure comparison between PGP (*green*) and *Clostridium thermocellum* alpha-glucan phosphorylase (*purple* and *red*), the circled regions represent cap structure of PGP (**a**) and photos of starch-synthesizing ability (**b**) based on cellobiose mediated by CBP and wild-type PGP (Tube 1), partially decapped PGP (Tube 2) or completely decapped PGP (Tube 3) [16]

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112 of cellulose), and the number-average degree of polymerization (DP) was ∼150.
113 The addition of glucose oxidase to remove glucose, a strong inhibitor of CBP,
114 resulted in a yield increase to 30.0 %. And the formation of amylose was also
115 validated by ^{13}C-NMR and FTIR (Fig. 6.2c, d).

116 Alpha-glucan phosphorylase from *C. thermocellum* and *T. maritima* were tested
117 for the negative production of amylose from cellulose. Comparison of the primary
118 sequences of these three αGP indicates that the residues involved in substrate
119 binding and catalysis are fairly conserved. However, homology modeling of the
120 structures of the three αGPs reveals that potato αGP has a special cap consisting of
121 residues from 478 to 561, which was absent in the other two bacterial enzymes
122 (Fig. 6.3a). This cap structure can be observed in many plant αGPs, e.g., from
123 sweet potato (*Ipomoea batatas*), spinach (*Spinacia oleracea*), rice (*Oryza sativa*),
124 and wheat (*Triticum aestivum*) but as yet reported in bacteria. We hypothesized that
125 the polypeptide cap on the catalytic site of PGP was responsible for driving low
126 concentration G1P toward the synthesis of amylose. We designed two PGP deletion
127 mutants—one had a part of the cap sequence removed and the other without the
128 entire cap. In the buffer containing cellobiose and CBP, the partially "decapped"
129 PGP (PGP-PDC) had decreased amylose synthesis ability compared to the
130 wild-type enzyme (Fig. 6.3b, tube 2), whereas, the completely decapped PGP
131 (PGP-CDC) lost the activity completely (Fig. 6.3b, tube 3). The kinetic parameters,
132 K_m and k_{cat} values ofPGP, PGP-PDC, PGP-CDC, *C. thermocellum* αGP and
133 *T. maritima* αGP are compared in Table 6.1. The complete removal of the cap of
134 PGP decreased the k_{cat}/K_m values from 3.33 to 0.43 mM^{-1} s^{-1} in the starch syn-
135 thesis direction and from 0.55 to 0.06 mM^{-1} s^{-1} in the starch degradation direction.
136 Compared to the *C. thermocellum* αGP, PGP has a higher k_{cat}/K_m value in the
137 synthesis direction and a lower k_{cat}/K_m in the degradation direction, suggesting that
138 wild-type PGP has a preferred function for starch synthesis to degradation.

139 These enzymes were used to produce amylose from pretreated natural biomass,
140 such as diluted acid-pretreated and cellulose solvent- and organic solvent-based
141 lignocellulose fractionation (COSLIF)-pretreated corn stover [20]. In those reac-
142 tions, nonutilized glucose units generated from the cellulase and CBP were
143 assimilated by the baker's yeast; the baker's yeast can produce ethanol or other
144 biochemical or single-cell proteins. Because typical baker's yeast cannot utilize
145 cellobiose and G1P, adding yeast in this reaction system can increase the product
146 yield by removing glucose which is the inhibitor of cellobiose phosphorylase [17,
147 18]. Under the tested conditions, the amylose yields were 23 and 2 % for
148 COLISF-pretreated corn stover and dilute acid pretreated corn stover, respectively.
149 This data indicated that starch can be produced from pretreated natural
150 lignocellulose.

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Table 6.1 Comparison of potato alpha-glucan phosphorylase and mutants, thermophilic *C. thermocellum* and *T. maritima* alpha-glucanphosphorylase

Enzymes	Synthesis			Degradation			Ratio (syn/deg)
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	
PGP	5.83 ± 0.34	1.76 ± 0.18	3.33	0.90 ± 0.11	1.64 ± 0.13	0.55	6.5
PGP-PDC	4.88 ± 0.27	1.92 ± 0.21	2.54	0.83 ± 0.09	2.20 ± 0.21	0.38	5.9
PGP-CDC	0.95 ± 0.11	2.22 ± 0.41	0.43	0.19 ± 0.04	3.11 ± 0.23	0.06	5.0
CthαGP	6.6 ± 0.3	1.9 ± 0.2	3.50	8.1 ± 0.2	0.39 ± 0.01	21.0	0.8
TmαGP	12.14 ± 0.68	1.03 ± 0.13	11.77	14.44 ± 1.11	1.47 ± 0.11	9.85	0.8

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6.3 Proposed Methods to Increase the Efficiency of Converting Cellulose to Starch

The cost-effective transformation of non-food cellulose to starch could revolutionize agriculture and the fledgling bioeconomy, while maintaining biodiversity, minimizing agriculture's environmental footprint, and conserving fresh water [8]. This transformation would not only promote the cultivation of plants chosen for rapid growth rather than those optimized for starch-rich seed production, but it would also efficiently utilize marginal land for the production of the biomass required to meet the increasing needs of biofuels and renewable materials [21–23]. Therefore, increasing the efficiency of converting cellulose to starch could lead to the sustainable agricultural revolution.

6.3.1 Increasing Cellulose Pretreatment Efficiency

The goal of biomass pretreatment is to improve the enzymatic digestibility of pretreated lignocellulosic biomass. Many factors, such as substrate accessibility to hydrolytic enzymes, lignin content, cellulose degree of polymerization, particle size, and so on, are related to its recalcitrance [24, 25]. Among these factors, substrate accessibility is the most important substrate property impacting the efficiency of enzymatic cellulose hydrolysis [20]. Solvent-based biomass pretreatments can greatly increase cellulose accessibility compared to conventional biomass pretreatments, e.g., dilute acid, steam explosion and hot water [26]. Solvent-based lignocellulose pretreatments by using concentrated phosphoric acid and ionic liquids have the apparent advantages of: high glucan digestibility at low enzyme loading; fast hydrolysis rate; and potential revenues from separated coproducts (e.g., hemicellulose, lignin). In such case, an ideal solvent should be able to dissolve cellulose at modest temperature (i.e., low energy input and less sugar degradation) and those of wet cellulose so that no biomass drying step is required. In addition, the solvent should be highly recyclable nonvolatile or highly volatile for easy recycling; thermostable and chemostable for an unlimited number recycling; and nontoxic to the sequential steps of enzymatic hydrolysis and microbial fermentation. High cellulose dissolution capacity (>10 wt% cellulose/vol) and fast diffusion rate in solid lignocellulose composite are additional desirable properties of such a solvent system [26].

6.3.2 Increasing PGP Thermostability

Because PGP lose its activity quickly above 45 °C, the reaction is better be performed at 37 °C despite the other enzymes can work at about 50 °C, resulting in

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186 low reaction rate of the whole process. High thermostable αGP which favors starch
187 synthesis is required to increase the efficiency of this ex vivo enzymatic pathway.
188 Plant αGPs should have the best ability for starch synthesis compared to microbial
189 homologs. However, few plant αGPs can work at >50 °C. In lieu of using naturally
190 occurring enzymes, an option is to engineer current PGP to a thermostable one by
191 protein engineering via directed evolution or rational design. Accordingly, a mutant
192 library of PGP was generated by error-prone PCR, and the mutated plasmid library
193 was transformed in *Escherichia coli* BL21 (DE3). Screening for the more stable
194 PGP mutants can be conducted at 50 °C on agar plates containing 0.05 % soluble
195 starch had been described by Yanase et al. [27]. Blue spots stained by iodine
196 solution on the filter indicated the presence of active PGP after heat treatment.
197 Three mutations (F39L, N135S, and T706I) combined together enhanced the
198 thermostability of PGP significantly without compromising activity, retaining
199 almost all the activity after heat treatment at 60 °C for 2 h while wild-type enzyme
200 was completely inactivated.

201 *6.3.3 Use of Commercial Cellulases*

202 In our previous work, we used a bacterial cellulase, BsCel5 endoglucanase that was
203 expressed and purified from recombinant *E. coli* strain, and a purified *Trichoderma*
204 cellobiohydrolase (TrCel7A). Bacterial cellulase is not as good as the secreted
205 *Trichoderma* cellulase, because of its high production cost based on *E. coli* cell
206 culture. Novozymes and Genecor (Dupont) areselling less costly (e.g., $20/kg dry
207 weight of cellulase) cellulase mixture products, like CTec2, HTec2, etc., that are
208 highly efficient in converting amorphous cellulose to glucose. However, these
209 commercial cellulases contain β-glucosidase activities that are designed to hydro-
210 lyze cellobiose to glucose. Hence, it is important to selectively remove
211 β-glucosidase or produce β-glucosidase-free cellulase cocktail for meeting our
212 special need here.

213 *6.3.4 Engineering of Yeast*

214 The yeast *S. cerevisiae* used in SEHF could be further improved by strain devel-
215 opment. In general, yeast has been engineered to produce ethanol from cellulose
216 directly by the expression of foreign cellulases including EG, CBH, and EG [28–
217 33]. These three enzymes could be assembled as an enzyme complex by the
218 interaction between cohesin and dockerin through synthetic mini-scaffolding that is
219 displayed on the yeast cell surface. Instead, we just need to replace BG with CBP
220 and PGP enzymes. So four enzymes (EG, CBH, CBP, and PGP) and synthetic
221 scaffolding can be expressed in yeast, and displayed on the yeast cell surface.

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A cellulose-enzyme-microorganism (CEM) complex is expected to enable more efficient cellulose hydrolysis, starch synthesis, and ethanol production [34]. However, before this is realized, many factors should be taken into account to improve the efficiency of this ex vivo system, e.g., cellulase selection, the number of cohesions and their order in scaffolding, linker length, and enzyme orientation.

6.4 The Application of Synthetic Linear Amylose

The only way to synthesize pure amylose is by means of phosphorylase-catalyzed enzymatic polymerization [35, 36] because natural amylose isolated from seeds always contains some small α-1,6 branches. The length of synthetic linear amylose can be controlled by the concentration of primer oligosaccharides, G1P concentration, and reaction time. Synthetic amylose could have a variety of application, from low-value to high-value products (Fig. 6.4). (1) Top-quality synthetic amylose with a well-controlled degree of polymerization can be used as a chromatographic column matrix and drug capsule material in the pharmaceutical industry. (2) Amylose can be used to make biodegradable plastics, where starch-based plastics account for 50 % of the bioplastic market [37]. High-quality linear amylose is suitable for producing clear, transparent, and flexible low-oxygen diffusion plastic sheets and films [38]. (3) Amylose is an important thickener, water binder, emulsion stabilizer, and gelling agent in the food industry. (4) Food-grade amylose can be blended with regular cereals and processed to high-amylose tailored foods for meeting special dietary needs [36, 39]. These lower glycemic load foods can improve human health and lower the risk of serious noninfectious diseases (e.g., diabetes and obesity) [40]. Amylose can be processed to form a resistant starch, which resists digestion and passes through to the large intestine, where it acts as a dietary fiber [41]. Slowly digestible and resistant starch has some healthy benefits, including the prevention and alleviation of metabolic diseases and the prevention of colon cancer [42]. (5) Medium-quality amylose can be used as a high-density hydrogen carrier for the enzymatic production of hydrogen, that could solve the challenges associated with hydrogen production, hydrogen storage, infrastructure, and safety concerns [13, 43, 44]. (6) Low-quality amylose mixed with yeast cells can be used as animal feed for nonruminant animals, such as pigs and chickens, where yeast cells are a protein source. (7) Amylose could be converted into other food nutrients. To increase amylose digestion efficiency as food/feed, the addition of the starch-branching enzyme converts linear amylose to branched amylopectin [45] (Fig. 6.4) that is more water soluble and easily digested than amylose.

The production of the polysaccharides amylose and amylopectin rather than glucose is essential as key food components because over-consumption of simple sugars (e.g., glucose and fructose) is strongly associated with noninfectious diseases, such as diabetes and obesity. It is why humans have to eat starch as a major calories source instead of drinking simple sugar solutions. Starch may be an

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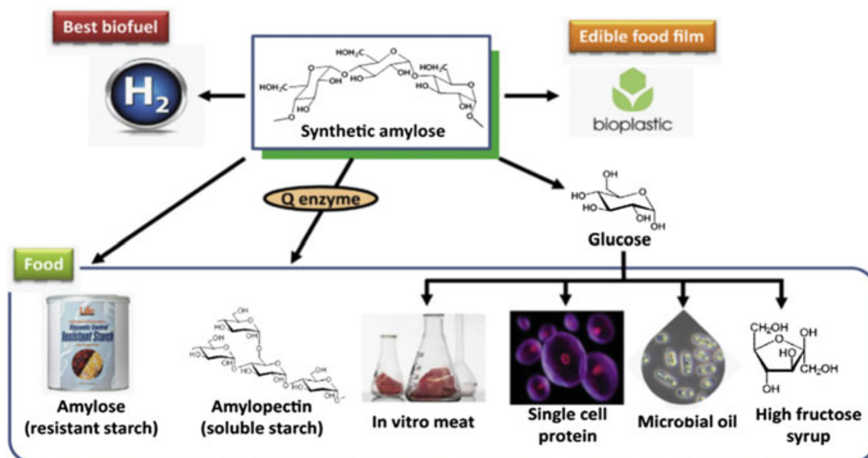

Fig. 6.4 The central role of synthetic amylose made from cellulose as new food sources (e.g., resistant starch, soluble amylopectin, in vitro meat, single-cell protein, microbialoil and high-fructose syrup), biodegradable plastic, and a hydrogen carrier. Reprinted from Ref. [6], Copyright 2015, with permission from Elsevier

262 important carbon source to produce other food nutrients, such as in vitro meat,
263 single-cell proteins, and microbial oil. For example, slowly utilized starch is a better
264 energy source for cell-free protein synthesis than glucose [46, 47].

265 6.5 Conclusion

266 To meet the growing needs of biofuels and renewable materials, as well as food and
267 feed, we proposed an ex vivo enzymatic pathway along with microbial fermentation
268 to produce edible starch from cellulose and other value-added products. This
269 method provides a potential alternative to solve the potential food crisis because
270 cellulose resource is approximately 40 times the starch food produced by cultivated
271 crops, whose production requires large amounts of arable land, water, and energy.
272 Whereas, cellulosic crops like dedicated perennial bioenergy crops (e.g., switch-
273 grass, bamboo, poplar) can grow on low-quality arable land with higher produc-
274 tivity, higher water utilization efficiency and less energy-related inputs compared to
275 cultivated annual crops. So the cost-effective transformation of non-food cellulose
276 to starch could lead to sustainable agriculture and potentially shape the bioecon-
277 omy, while maintaining biodiversity, minimizing agriculture's environmental
278 footprint, and conserving fresh water. New biorefineries based on this technology
279 could help address the food, biofuels, and environment trilemma; decrease the
280 negative impacts of growing food and feed consumption on the environment;
281 provide healthier food; and promote the bioeconomy. Because ex vivo synthetic

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biosystems cannot duplicate themselves, the large-scale implementation of cellulose-to-starch in new biorefineries would most likely not raise the questions about ethics, biosecurity, and biosafety that often confront in vivo synthetic biology projects.

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