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Bacterial Cellulose

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A-BC	bacterial cellulose from agitated culture
ATP	adenosine triphosphate
BC	bacterial cellulose
CBH	cellobiohydrolase
c-di-GMP	cyclic diguanosine monophosphate
Cel^-	cellulose-negative mutant
Cel6A, Cel7A	cellobiohydrolases belonging to 6A and 7A families, respectively
CM	carboxymethyl-
CS	cellulose synthase
CSL	corn steep liquor
D	aspartic acid

DMSO	dimethyl sulfoxide
DP	degree of polymerization
E	glutamic acid
FBP	fructose-1,6-biphosphate phosphatase
FK	fructokinase
Fru-bi-P	fructose-1,6-biphosphate
Fru-6-P	fructose-6-phosphate
G	guanine
GK	glucokinase
Glc	glucose
Glc-6(1)-P	glucose-6(1)-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
H-S medium	Hestrin and Schramm medium (1954)
IS	insertion sequence
Lip	lipid
LP	UDPGPT: lipid pyrophosphate: UDPGlc- phosphotransferase
LPP	lipid pyrophosphate phosphohydrolase
Man	mannose
NMR	nuclear magnetic resonance
PC	plant cellulose
PDEA	phosphodiesterase A
PDEB	phosphodiesterase B
Pel^-	pellicle non-forming
1PFK	fructose-1-phosphate kinase
PGA	phosphogluconic acid
PGI	phosphoglucoisomerase
PMG	phosphoglucomutase
PTS	system of phosphotransferases
Q	glutamine
R	arginine
Rha	rhamnose
Rib	D-ribose
S	serine
S-BC	bacterial cellulose from static culture
TC	terminal complexe
U	uridine
UDP	uridine diphosphate
UDPGlc	uridine diphosphoglucose
UGP	pyrophosphorylase uridine diphosphoglucose
UMP	uridine monophosphate
v.v.m.	volume per volume per minute
W	tryptophan

1 Introduction

Cellulose is the most abundant biopolymer on earth, recognized as the major component of plant biomass, but also a representative of microbial extracellular polymers. Bacterial cellulose (BC) belongs to specific products of primary metabolism and is mainly a protective coating, whereas plant cellulose (PC) plays a structural role.

Cellulose is synthesized by bacteria belonging to the genera *Acetobacter*, *Rhizobium*, *Agrobacterium*, and *Sarcina* (Jonas and Farah, 1998). Its most efficient producers are Gram-negative, acetic acid bacteria *Acetobacter xylinum* (reclassified as *Gluconacetobacter xylinus*, Yamada et al., 1997; Yamada, 2000), which have been applied as model microorganisms for basic and applied studies on cellulose (Cannon and Anderson, 1991). Investigations have been focused on the mechanism of biopolymer synthesis, as well as on its structure and properties, which determine practical use (Legge, 1990; Ross et al., 1991). One of the most important features of BC is its chemical purity, which distinguishes this cellulose from that from plants, usually associated with hemicelluloses and lignin, removal of which is inherently difficult.

Because of the unique properties, resulting from the ultrafine reticulated structure, BC has found a multitude of applications in paper, textile, and food industries, and as a biomaterial in cosmetics and medicine (Ring et al., 1986). Wider application of this polysaccharide is obviously dependent on the scale of production and its cost. Therefore, basic studies run together with intensive research on strain improvement and production process development.

2 Historical Outline

Although synthesis of an extracellular gelatinous mat by *A. xylinum* was reported for the first time in 1886 by A. J. Brown, BC attracted more attention in the second half of the 20th century. Intensive studies on BC synthesis, using *A. xylinum* as a model bacterium, were started by Hestrin et al. (1947, 1954), who proved that resting and lyophilized *Acetobacter* cells synthesized cellulose in the presence of glucose and oxygen. Next, Colvin (1957) detected cellulose synthesis in samples containing cell-free extract of *A. xylinum*, glucose, and ATP. Further milestones in studies on BC synthesis, presented in this review, contributed to the elucidation of mechanisms governing not only the biogenesis of the bacterial polymer, but also that of plants, thus leading to the understanding of one of the most important processes in nature. The true historical outline is presented throughout all the paragraphs below, including the references.

3 Structure of BC

Cellulose is an unbranched polymer of β-1,4-linked glucopyranose residues. Extensive research on BC revealed that it is chemically identical to PC, but its macromolecular structure and properties differ from the latter (Figure 1). Nascent chains of BC aggregate to form subfibrils, which have a width of approximately 1.5 nm and belong to the thinnest naturally occurring fibers, comparable only to subelemental fibers of cellulose detected in the cambium of some plants and in quinee mucous (Kudlicka, 1989). BC subfibrils are crystallized into microfibrils (Jonas and Farah, 1998), these into bundles, and the latter into ribbons (Yamanaka et al.,

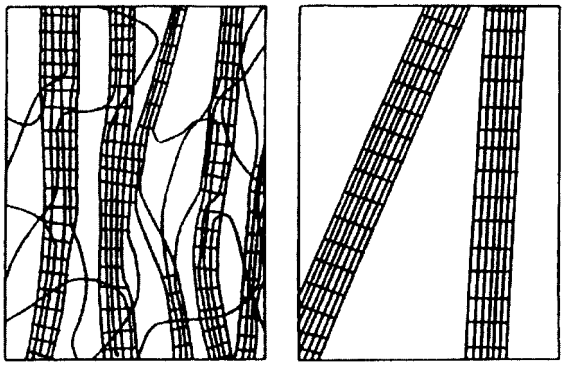

Fig. 1 Schematic model of BC microfibrils (right) drawn in comparison with the 'fringed micelles'; of PC fibrils (Iguchi et al., 2000; with kind permission).

2000). Dimensions of the ribbons are 3–4 (thickness) × 70–80 nm (width), according to Zaar (1977), 3.2 × 133 nm, according to Brown et al. (1976), or 4.1 × 117 nm, according to Yamanaka et al. (2000), whereas the width of cellulose fibers produced by pulping of birch or pine wood is two orders of magnitude larger ($1.4–4.0 \times 10^{-2}$ and $3.0–7.5 \times 10^{-2}$ mm, respectively). The ultrafine ribbons of microbial cellulose, the length of which ranges from 1 to 9 μm, form a dense reticulated structure (Figure 2), stabilized by extensive hydrogen bonding (Figure 3). BC is also distinguished from its plant counterpart by a high crystallinity index (above 60%) and different degree of polymerization (DP), usually between 2000 and 6000 (Jonas and Farah, 1998), but in some cases reaching even 16,000 or 20,000 (Watanabe et al., 1998b), whereas the average DP of plant polymer varies from 13,000 to 14,000 (Teeri, 1997).

Macroscopic morphology of BC strictly depends on culture conditions (Watanabe et al., 1998a; Yamanaka et al., 2000). In static conditions (Figure 4), bacteria accumulate cellulose mats (S-BC) on the surface of nutrient broth, at the oxygen-rich air–liquid interface. The subfibrils of cellulose are continuously extruded from linearly ordered pores at the surface of the bacterial cell, crystallized into microfibrils, and forced deeper into the growth medium. Therefore, the leather-like pellicle, supporting the population of *A. xylinum* cells, consists of overlapping and intertwisted cellulose rib-

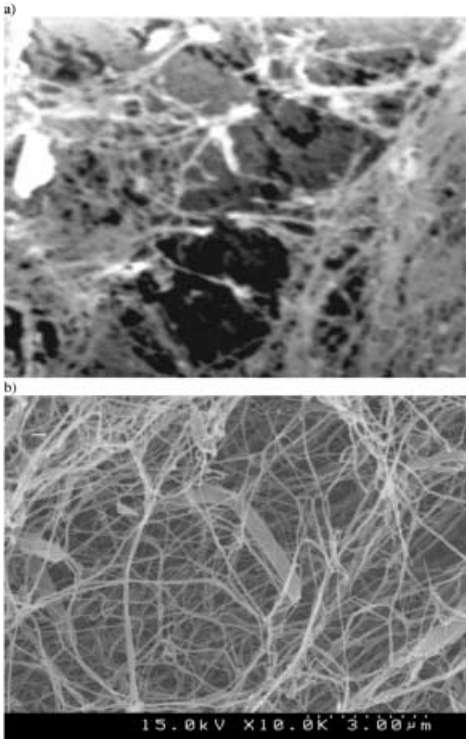

Fig. 2 Scanning electron microscopy images of BC membrane from static culture of *A. xylinum* (a) and bacterial cell with attached cellulose ribbons (b).

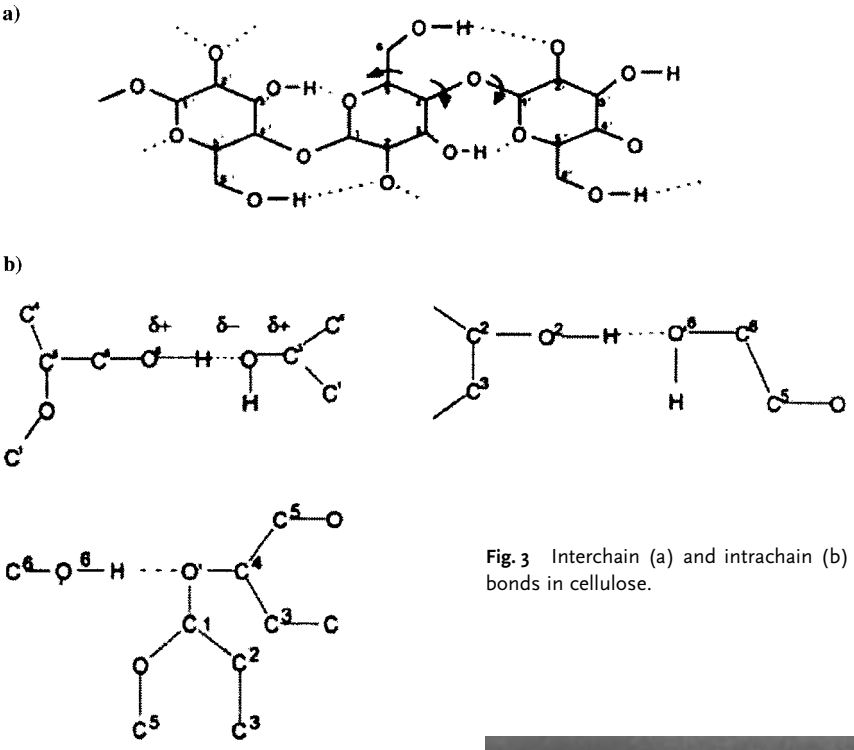

Fig. 3 Interchain (a) and intrachain (b) hydrogen bonds in cellulose.

bons, forming parallel but disorganized planes (Jonas and Farah, 1998). The adjacent S-BC strands branch and interconnect less frequently than these in BC produced in agitated culture (A-BC), in a form of irregular granules, stellate and fibrous strands, well-dispersed in culture broth (Figure 5) (Vandamme et al., 1998). The strands of reticulated A-BC interconnect to form a grid-like pattern, and have both roughly perpendicular and roughly parallel orientations (Watanabe et al., 1998a).

Differences in three-dimensional structure of A-BC and S-BC are noticeable in their scanning electron micrographs. The S-BC fibrils are more extended and piled above one another in a criss-crossing manner. Strands of A-BC are entangled and curved (Johnson and Neogi, 1989). Besides, they

Fig. 4 BC pellicle formed in static culture.

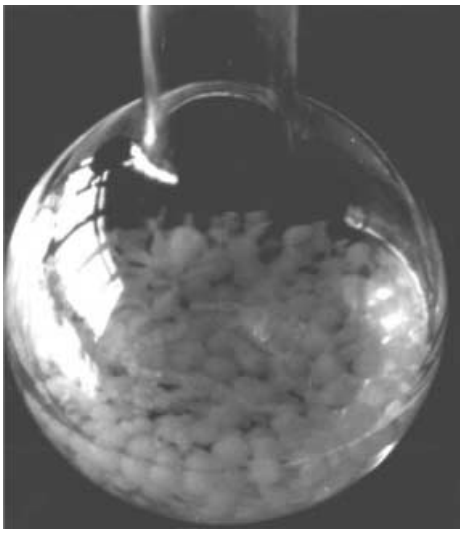

Fig. 5 BC pellets formed in agitated culture.

have a larger cross-sectional width (0.1–0.2 μm) than S-BC fibrils (usually 0.05–0.10 μm). Morphological differences between S-BC and A-BC contribute to varying degrees of crystallinity, different crystallite size and I_α cellulose content.

Two common crystalline forms of cellulose, designated as I and II, are distinguishable by X-ray, nuclear magnetic resonance (NMR), Raman spectroscopy, and infrared analysis (Johnson and Neogi, 1989). It is known that in the metastable cellulose I, which is synthesized by the majority of plants and also by *A. xylinum* in static culture, parallel β-1,4-glucan chains are arranged uniaxially, whereas β-1,4-glucan chains of cellulose II are arranged in a random manner. They are mostly antiparallel and linked with a larger number of hydrogen bonds that results in higher thermodynamic stability of the cellulose II.

A-BC has a lower crystallinity index and a smaller crystallite size than S-BC (Watanabe et al., 1998a). It was also observed that a significant portion of cellulose II occurred in BC synthesized in agitated culture. In nature, cellulose II is synthesized by only a few organisms (some algae, molds, and bacteria, such as *Sarcina ventriculi*) (Jonas and Farah, 1998); the industrial production of this kind of cellulose is based on chemical conversion of PC.

Using CP/MAS ^{13}C-NMR it is possible to reveal the presence of cellulose I_α and I_β – two distinct forms of cellulose (Watanabe et al., 1998a). These forms occur in algae-, bacteria-, and plant-derived cellulose. The latter one contains less I_α cellulose than BC (Johnson and Neogi, 1989). The irreversible crystal transformation from cellulose I_α to I_β shifts the X-ray and CP/MAS ^{13}C-NMR spectra because of the difference in the unit cell. S-BC contains more cellulose I_α than A-BC. It was reported that the difference in cellulose I_α content between A-BC and S-BC exceeded that in crystallinity index (Watanabe et al., 1998a), and the mass fraction of cellulose I_α was closely related to the crystallite size.

4 Chemical Analysis and Detection

For the detection of either crystalline or amorphous cellulose, several direct dyes, specific for the linear β-1,4-glucan, are used (Mondal and Kai, 2000). All of them are fluorescent brightening agents and form dye–cellulose complexes, stabilized by van der Waals and/or hydrogen bonding. One of these dyes is the fluorescent brightener Calcofluor. The direct dyes do not only enable visualization of cellulose chains, but also have been intensively applied for studies on nascent cellulose chains association and crystallization (see Section 7.3.2).

The weight-average DP of cellulose and the DP distribution are determined by high-performance gel-permeation chromatogra-

phy (Watanabe et al., 1998a) of nitrated cellulose samples.

The differences between the reticulated structure of microbial cellulose, produced under agitated culture conditions, and the disorganized layered structure of cellulose pellicle, formed in static culture, are noticeable in scanning electron microscopy images (Johnson and Neogi, 1989).

To distinguish the parallel chain crystalline lattice of cellulose I from the antiparallel one of cellulose II, X-ray diffraction, Raman spectroscopy, infrared analysis, and NMR are applied (Johnson and Neogi, 1989). The crystallinity index and crystallite size are calculated based on X-ray diffraction measurements (Watanabe et al., 1998a).

Two distinct forms of cellulose I, i.e. cellulose I_α and I_β, are not distinguishable by X-ray diffraction, and therefore CP/MAS ^{13}C-NMR analysis, carried out on freeze-dried cellulose samples, has to be performed to determine their mass fractions (Watanabe et al., 1998a).

The physicochemical properties of cellulose such as water holding capacity, viscosity of disintegrated cellulose suspension, and the Young's modulus of dried sheets are determined using conventional methods (Watanabe et al., 1998a, Iguchi et al., 2000).

5 Occurrence

BC is synthesized by several bacterial genera, of which *Acetobacter* strains are best known. An overview of BC producers is presented in Table 1 (Jonas and Farah, 1998). The polymer structure depends on the organism, although the pathway of biosynthesis and mechanism of its regulation are probably common for the majority of BC-producing bacteria (Ross et al., 1991; Jonas and Farah, 1998).

Tab. 1 BC producers (Jonas and Farah, 1998, modified)

Genus	*Cellulose structure*
Acetobacter	extracellular pellicle composed of ribbons
Achromobacter	fibrils
Aerobacter	fibrils
Agrobacterium	short fibrils
Alcaligenes	fibrils
Pseudomonas	no distinct fibrils
Rhizobium	short fibrils
Sarcina	amorphous cellulose
Zoogloea	not well defined

A. xylinum (synonyms *A. aceti* ssp. xylinum, *A. xylinus*), which is the most efficient producer of cellulose, has been recently reclassified and included within the novel genus *Gluconacetobacter*, as *G. xylinus* (Yamada et al., 1998, 2000) together with some other species (*G. hansenii*, *G. europaeus*, *G. oboediens*, and *G. intermedius*).

6 Physiological Function

In natural habitats, the majority of bacteria synthesize extracellular polysaccharides, which form envelopes around the cells (Costeron, 1999). BC is an example of such a substance. Cells of cellulose-producing bacteria are entrapped in the polymer network, frequently supporting the population at the liquid–air interface (Wiliams and Cannon, 1989). Therefore, BC-forming strains can inhabit sewage (Jonas and Farah, 1998). The polymer matrix takes part in adhesion of the cells onto any accessible surface and facilitates nutrient supply, since their concentration in the polymer lattice is markedly enhanced due to its adsorptive properties, in comparison to the surrounding aqueous

environment (Jonas and Farah, 1998; Costeron, 1999). Some authors suppose that cellulose synthesized by *A. xylinum* also plays a storage role and can be utilized by the starving microorganisms. Its decomposition would be then catalyzed by exo- and endoglucanases, the co-presence of which was detected in the culture broth of some cellulose-producing *A. xylinum* strains (Okamoto et al., 1994).

Because of the viscosity and hydrophilic properties of the cellulose layer, the resistance of producing bacterial cells against unfavorable changes (a decrease in water content, variations in pH, appearance of toxic substances, pathogenic organisms, etc.) in an habitat is increased, and they can further grow and develop inside the envelope. It was also found that cellulose placed over bacterial cells protects them from ultraviolet radiation. As much as 23% of the acetic acid bacteria cells covered with BC survived a 1 h treatment with ultraviolet irradiation. Removal of the protective polysaccharide brought about a drastic decrease in their viability (3% only) (Ross et al., 1991).

7
Biosynthesis of BC

Synthesis of BC is a precisely and specifically regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins, whose supramolecular structure has not yet been well defined. The process includes the synthesis of uridine diphosphoglucose (UDPGlc), which is the cellulose precursor, followed by glucose polymerization into the β-1,4-glucan chain, and nascent chain association into characteristic ribbon-like structure, formed by hundreds or even thousands of individual cellulose chains. Pathways and mechanisms of UDPGlc synthesis are relatively well known, whereas molecular mechanisms of glucose polymerization into long and unbranched chains, their extrusion outside the cell, and self-assembly into fibrils require further elucidation.

Moreover, studies on BC synthesis may contribute to better understanding of PC biogenesis.

7.1
Synthesis of the Cellulose Precursor

Cellulose synthesized by *A. xylinum* is a final product of carbon metabolism, which depending on the physiological state of the cell involves either the pentose phosphate cycle or the Krebs cycle, coupled with gluconeogenesis (Figure 6) (Ross et al., 1991; Tonouchi et al., 1996). Glycolysis does not operate in acetic acid bacteria since they do not synthesize the crucial enzyme of this pathway – phosphofructose kinase (EC 2.7.1.56) (Ross et al., 1991). In *A. xylinum*, cellulose synthesis is tightly associated with catabolic processes of oxidation and consumes as much as 10% of energy derived from catabolic reactions (Weinhouse, 1977). BC production does not interfere with other anabolic processes, including protein synthesis (Ross et al., 1991).

A. xylinum converts various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids, into cellulose, usually with about 50% efficiency. The latter compounds enter the Krebs cycle and due to oxalacetate decarboxylation to pyruvate undergo conversion to hexoses via gluconeogenesis, similarly to glycerol, dihydroxyacetone, and intermediates of the pentose phosphate cycle (Figure 6).

The direct cellulose precursor is UDPGlc, which is a product of a conventional pathway, common of many organisms, including plants, and involving glucose phosphoryla-

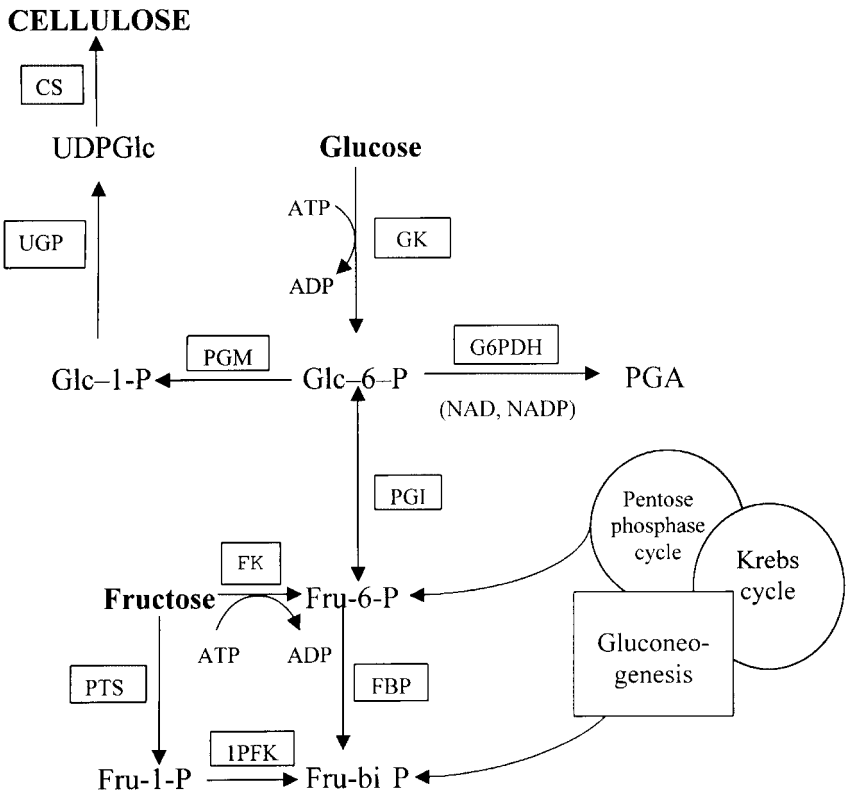

Fig. 6 Pathways of carbon metabolism in *A. xylinum*. CS, cellulose synthase (EC 2.4.2.12); FBP, fructose-1,6-biphosphate phosphatase (EC 3.1.3.11); FK, glucokinase (EC 2.7.1.2); G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 1PFK, fructose-1-phosphate kinase (EC 2.7.1.56); PGI, phosphoglucoisomerase; PMG, phosphoglucomutase (EC 5.3.1.9); PTS, system of phosphotransferases; UGP, pyrophosphorylase UDPGlc (EC 2.7.7.9); Fru-bi-P, fructose-1,6-bi-phosphate; Fru-6-P, fructose-6-phosphate; Glc-6(1)-P, glucose-6(1)-phosphate; PGA, phosphogluconic acid; UDPGlc, uridine diphosphoglucose.

tion to glucose-6-phosphate (Glc-6-P), catalyzed by glucokinase, followed by isomerization of this intermediate to Glc-α-1-P, catalyzed by phosphoglucomutase, and conversion of the latter metabolite to UDPGlc by UDPGlc pyrophosphorylase. This last enzyme seems to be the crucial one involved in cellulose synthesis, since some phenotypic cellulose-negative mutants (Cel^-) are specifically deficient in this enzyme (Valla et al., 1989), though they display cellulose synthase (CS) activity, this was confirmed *in vitro* by means of observation of cellulose synthesis, catalyzed by cell-free extracts of Cel^- strains (Saxena et al., 1989). Furthermore, the pyrophosphorylase activity varies between different *A. xylinum* strains and the highest activity was detected in the most effective cellulose producers, such as *A. xylinum* ssp. sucrofermentans BPR2001. The latter strain prefers fructose as a carbon source, displays high activity of phosphoglucoisomerase, and possesses a system of phosphotransferases, dependent on phosphoenolpyruvate. The system catalyzes conversion of fructose to fructose-1-phosphate and further to fructose-1,6-biphosphate (Figure 6).