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Substrate channeling and enzyme complexes for biotechnological applications

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ABSTRACT

Substrate channeling is a process of transferring the product of one enzyme to an adjacent cascade enzyme or cell without complete mixing with the bulk phase. Such phenomena can occur *in vivo, in vitro, or ex vivo*. Enzyme–enzyme or enzyme–cell complexes may be static or transient. In addition to enhanced reaction rates through substrate channeling in complexes, numerous potential benefits of such complexes are protection of unstable substrates, circumvention of unfavorable equilibrium and kinetics imposed, forestallment of substrate competition among different pathways, regulation of metabolic fluxes, mitigation of toxic metabolite inhibition, and so on. Here we review numerous examples of natural and synthetic complexes featuring substrate channeling. Constructing synthetic *in vivo, in vitro* or *ex vivo* complexes for substrate channeling would have great biotechnological potentials in metabolic engineering, multi-enzyme-mediated biocatalysis, and cell-free synthetic pathway biotransformation (SyPaB).

Contents

1. Introduction

Substrate channeling is a process of direct transfer of the product of an enzyme to another proximate enzyme or cell as its substrate without equilibration with the bulk phase (Geck and Kirsch, 1999; Miles et al., 1999; Spivey and Ovádi, 1999). Such phenomena occur only when a distance between two entities are close enough (Fig. 1B) but not when the distance between cascade entities is far away (Fig. 1A). As a result, reaction rates among cascade entities may be accelerated greatly. For example, adjacent different active sites can be located on either separate domains in a multifunctional enzyme, separate subunits in a multi-enzyme complex, or separate enzymes that are spatially close enough.

Substrate channeling can occur *in vitro*, *ex vivo*, or *in vivo*. For example, enzymatic hydrolysis of solid cellulose requires extracellular

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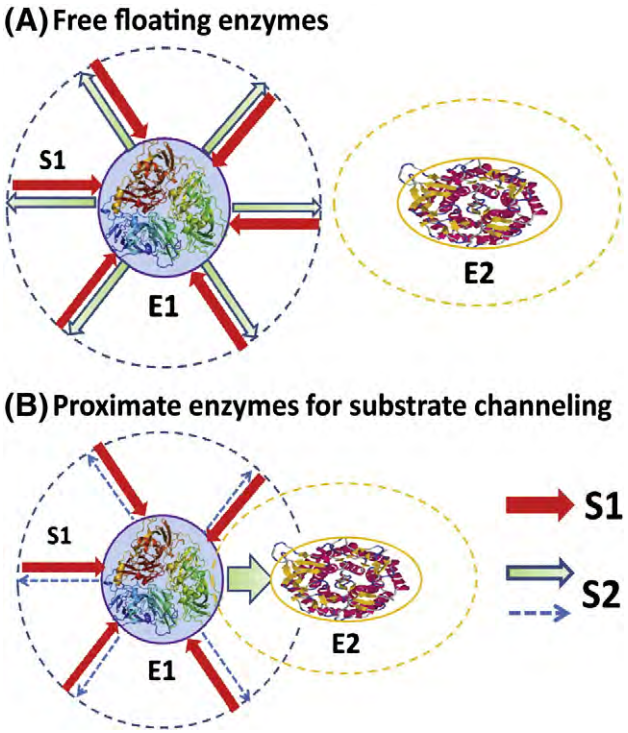

Fig. 1. Scheme of free floating cascade enzymes E1 and E2 (A) and proximate E1/E2 enzymes for potential substrate channeling (B), where diffusions of the substrate and product of E1 are assumed to be all directional.

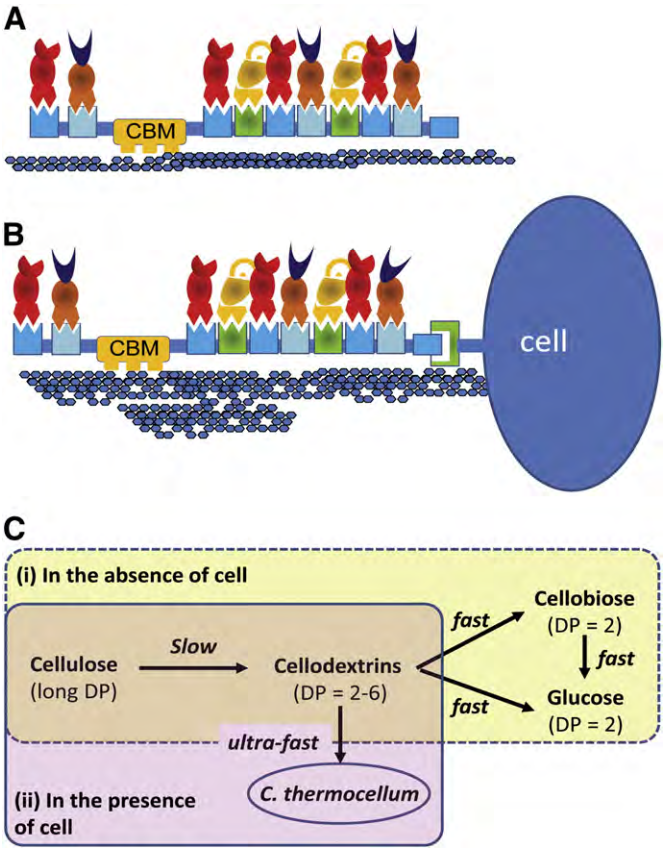

Fig. 2. Scheme of the *Clostridium thermocellum* cellulosome containing endoglucanases, exoglucanses, and a non-hydrolytic scaffoldin for facilitating enzymatic cellulose hydrolysis (A), cellulose–enzyme–microbe (CEM) complex through cell-associated cellulosome (B), and soluble cellodextrin update (C) for enzymatic cellulose hydrolysis in the absence of cell (i) and cellodextrin channeling in the presence of cell (ii).

endoglucanases and exoglucanases to work together (Lynd et al., 2002; Zhang et al., 2006). Sometimes, several cellulase components can form complexed cellulases, called cellulosomes, by a linkage of non-hydrolytic scaffoldins (Bayer et al., 2004; Doi and Kosugi, 2004) (Fig. 2A). Cellulosomes usually exhibit cellulose hydrolysis rates several times higher than simple individual enzyme mixtures (Fierobe et al., 2001; Moraïs et al., 2010; Vazana et al., 2010; Wu et al., 1988), suggesting that accelerating reaction rates by enzyme complexes is due to their proximity effect because endoglucanases randomly hydrolyze beta-1,4-glucosidic bonds of cellulose chains and proximate exoglucanases can conduct further hydrolysis based on new ends. When cellulosomes are associated on the surface of microorganisms (e.g. *Clostridium thermocellum*), cellulose–cellulosome–microorganism complexes are formed (Bayer et al., 1983) (Fig. 2B). Such substrate–enzyme–microbe complexes were found to expedite microbial cellulose utilization by several folds as compared to the cases that cellulosomes were not associated with a microorganism (Lu et al., 2006). Because primary hydrolysis of solid cellulose is usually much slower than secondary hydrolysis of soluble long-chain cellodextrins (Zhang and Lynd, 2004b), it is often difficult to detect accumulation of long-chain cellodextrins (e.g., cellotetraose, cellotriose) *in intro* (Fig. 2C-i) (Lamed et al., 1983). When hydrolytic intermediates of long-chain cellodextrins are so close to the microorganism, ultra-fast uptake of long-chain cellodextrins through high affinity ATP-binding cassette (ABC) transport system in *C. thermocellum* (Nataf et al., 2009) can prevent extracellular hydrolysis of cellodextrins (Fig. 2C-ii) (Lynd et al., 2002; Zhang and Lynd, 2005). Such substrate channeling *ex vivo* can decrease sugar transport bioenergetic cost per glucose unit across the membrane (Zhang and Lynd, 2005) and increase ATP gain through intracellular substrate phosphorylation (Lynd and Zhang, 2002; Zhang and Lynd, 2004a). *In vivo* substrate channeling among enzymes is facilitated by the compartmentation of a cell into different organelles and/or microcompartmentation of substructures (Miles et al., 1999). Many cellular reactions in metabolic pathways are catalyzed by multi-enzyme complexes but not by free-floating enzymes (Conrado et al., 2008). For some *in vivo* metabolic pathways, sequential enzymes may be located proximately and from structural-metabolic cellular enzyme complexes, called metabolons. At this time, such substrate channeling can be called as metabolite channeling. Metabolons are held together by non-covalent interactions. They may be stable, fragile, or transient. On a molecular level, overall reaction rates mediated by metabolons are more rapid and efficient than those where enzymes are randomly distributed in cytosol (Srere, 1985; Srivastava and Bernhard, 1986).

Natural multifunctional enzyme complexes have been discovered in both primary and secondary pathways. In cases of primary pathways, substrate channeling has been reported in the glycolysis pathway (Campanella et al., 2005; Graham et al., 2007; Shearer et al., 2005), the Calvin cycle (Suss et al., 1993), the tricarboxylic acid cycle in the mitochondrial matrix (Haggie and Verkman, 2002), the oxidative pentose phosphate pathway (Debnam et al., 1997), the gluconeogenesis pathway (Rakus et al., 2004), the heme biosynthetic pathway (Olsson et al., 2002), fatty acid oxidation (Ishikawa et al., 2004), cellulose biosynthesis (Ding and Himmel, 2006), amino acid synthesis (Welch and Gaertner, 1980), carboxysome (Yeates et al., 2008), proteasome (Schmidt et al., 2005), and so on. For effective synthesis of specific natural products (secondary metabolites) and prevention of primary metabolic interference, numerous secondary metabolites have been synthesized through enzyme complexes associated with substrate channeling effects, such as isoprenoids, alkaloids, phenylpropanoids, flavonoids, and cyanogenic glucosides (Jørgensen et al., 2005; Winkel, 2004).

Two general mechanisms for substrate channeling have been proposed (Geck and Kirsch, 1999; Spivey and Merz, 1989). One is a

direct transfer mechanism (i.e., perfect channeling) that the intermediate of the first enzyme is passed directly to the second enzyme or cell without diffusion to the bulk phase. In these cases, intermediates between complexes are difficult to detect. The other mechanism is often referred to as a proximity mechanism or leaky channeling, which is operative for any cascade reaction where the second receptive enzyme or cell is locally close to the first enzyme. For example, they can be bound at high densities on a surface (e.g., cellular membrane or solid substrate) or be loosely associated in large aggregates. The intermediate dissociated from the first enzyme has a high probability of being captured by the adjacent second receptor (Fig. 1B). In these cases, the intermediate may be detected in the bulk phase. Therefore, whether substrate channeling occurs or not should be kinetically determined with high caution (Spivey and Ovádi, 1999). In both mechanisms, diffusion of the intermediate into the bulk fluid is impeded by the juxtaposition of active sites and/or by steric hindrance (Geck and Kirsch, 1999; Spivey and Merz, 1989). Several kinetic methods have been used to determine occurrence of substrate channeling, including transient-time analysis, isotope dilution or enrichment, competing reaction method, enzyme buffer kinetics, and transient-state kinetics (check an excellent review (Spivey and Ovádi, 1999)).

In addition to accelerating reaction rates through substrate channeling, potential benefits of complexes include:

(i) Protection of unstable intermediates and/or stabilization of labile cofactors (e.g. NADP) (Geck and Kirsch, 1999; Rudolph and Stubbe, 1995; Thoden et al., 1997);
(ii) Forestallment of substrate competition among different pathways (Debnam et al., 1997; Elcock, 2002; Jørgensen et al., 2005; Miles et al., 1999);
(iii) Mitigation of toxic metabolite inhibition (Dueber et al., 2009);
(iv) Circumvention of unfavorable equilibrium and kinetics imposed by bulk-phase metabolite concentrations (Berg et al., 2002; Srivastava et al., 1989);
(v) Conservation of a scarce solvation capacity of cell (Atkinson, 1969);
(vi) Regulation of metabolic fluxes by modulating enzyme association (Debnam et al., 1997; Verkman, 2002) and increasing sensitivity to regulatory signal (Kholodenko et al., 1995);
(vii) Enhancement of biocatalysis by avoiding unfavorable energetics of desolvating substrates (Dewar and Storch, 1985); and
(viii) Conservation of bond energy stored in oligosaccharides through substrate phosphorylation for extra ATP generation (Zhang and Lynd, 2004a, 2005).

In this review, I present several examples of natural *in vitro, ex vivo, and in vivo* complexes for substrate channeling, and update latest advances in constructing synthetic complexes in biotechnological applications from metabolic engineering, multi-enzyme-mediated biocatalysis, to cell-free synthetic pathway biotransformation (SyPaB).

2. *In vivo* and *in vitro* systems

The formation of enzyme complexes greatly depends on their inherent dissociation constants among enzyme components and their environmental conditions. Therefore, it is vital to understand inherent differences between *in vivo* and *in vitro* and their impacts. Biochemists commonly study properties of enzymes with very dilute solutions (mostly up to 1–2 mg/mL for spectroscopy and usually as low as 10^{-3}–10^{-1} mg/mL for enzymatic kinetics). Such concentrations are much lower than those inside living cells. For example, cytoplasm is crowded with solutes, soluble macromolecules, such as enzymes, nucleic acids (i.e., RNA and DNA), structural proteins, and membrane. Macromolecules usually occupy 30–40% of cellular interior, where proteins account for ~70% of the total macromolecule weight, being ~200 to 300 mg/mL (Zimmerman and Trach, 1991). In mitochondria, enzymes and other proteins even constitute more than 60% of a matrix volume (Scalettar et al., 1991). At such high concentrations, macromolecules occupy a large fraction of solution volume (i.e., 30–60%), resulting in macromolecular crowding and excluded volume effect. High-density macromolecules in cells predominantly determine major characteristics of the cellular environment, such as viscosity, diffusion, and heterogeneity. Cells regardless of eukaryotes and prokaryotes are filled with structural proteins (e.g., cytoskeleton, microtrabecular lattice), on which water molecules are superposed in layers. In these layers, enzyme molecules are organized in strong complexes or rather loosely associated clusters. For example, it is found that immobilized F-actin-tropomyosin can adsorb aldolase (ALD), glyceraldhyde-3-phosphate dehydrogenase (G3PDH), lactate dehydrogenase (LDH), pyruvate kinase (PK), and phosphoglycerate kinase (PGK) but not phosphoglucose isomerase (PGI), triosephosphate isomerase (TIM), ennolase (EN), and phosphoglyeromutase (Bronstein and Knull, 1981). Very high catalytic rates can easily be attained with the limited number of intermediate molecules, i.e., metabolite *vs* their respective enzyme concentration (Table 1). As a result, metabolites can specifically be directed towards one or another pathway (channeling) and unstable intermediates may be protected.

Macromolecular crowding accompanied with excluded volume effect creates a distinctive *in vivo* environment (Ellis, 2001a). But their impacts are often ignored by most biochemists and rarely discussed in most textbooks of biochemistry and molecular biology (Ellis, 2001a, b). Macromolecular crowding effects include (i) great decreases in diffusion rates; (ii) enhancement of protein association, especially for transient enzyme complexes, like metabolons; (iii) promotion of protein folding and aggregation; and (iv) enhancement of chaperonin action (Ellis, 2001a). The excluded volume effect leads to lower solubility of macromolecules, more favored or less favored free energy for protein interactions, and more compact macromolecular conformation (Svedruzic and Spivey, 2006).

Macromolecular crowding and excluded volume effect can greatly decrease diffusion coefficients (D) of numerous molecules, especially

Table 1
Concentration of enzymes and metabolites of the glycolytic pathway in *E. coli* (Srivastava and Bernhard, 1986).

Name	Concn. (uM)	k_{cat} (s^{-1})
Enzymes		
Phosphoglucomutase (PGM)	31.9	250
Aldolase (ALD)	809	15
α-glycerol-P-dehydrogenase (GPDH)	61.4	100
Triose phosphate isomerase (TIM)	224	5000
Glyceraldehyde-3-phosphate hydrogenase (G3PDH)	1400	50
Phosphoglycerate kinase (PGK)	134	700
Phosphoglycerate mutase (PM)	256	200
Enolase (ENL)	540	40
Pyruvate kinase (PK)	173	300
Lactate dehydrogenase (LDH)	296	600
Metabolite precursors and products		
Glucose-6-phosphate (G6P)	3900	
Fructose-6-phosphate (F6P)	1500	
ATP	8000	
Inorganic phosphate (P_i)	8000	
Metabolite intermediates		
Fructose-1,6-phosphate (F16P)	80	
Dihydroxyacetone phosphate (DHAP)	160	
Glyceraldehyde-3-phosphate (G3P)	80	
1,3-Diphosphoglycerate	50	
3-phosphoglycerate	200	
2-phophoglycerate	20	
Phosphoenol pyruvate	65	
Pyruvate	380	
NAD^+	540	
NADH	50	

for large-size molecules (e.g., proteins) (Ellis, 2001a). A comparison of diffusion coefficient values for a variety of molecules in water and in cytosol suggests that cytoplasmic structure greatly influences protein diffusion rates (Mastro et al., 1984; Mastro and Keith, 1984). For example, a diffusion coefficient of a green fluorescent protein in the cytoplasm of *E. coli* is decreased by a factor of 11 (Elowitz et al., 1999). Consequently, the moving time for a certain distance can increased by 121-fold.

Equilibrium constants for protein associations can be increased by one to three orders of magnitude. Such large variations depend on sizes and shapes of macromolecular reactants and products, and of background macromolecules. *In vitro* addition of two macromolecular crowding agents – polyethylene glycol (PEG) 6000 and PEG 35000 – leads to a sharp decrease in dissociation constants of a bacterial phosphoenol pyruvate/carbohydrate phosphotransferase system (Francke et al., 2003; Rohwer et al., 1998). Multimerization of fibrinogen is found to be strongly related with a total protein concentration. It forms a homodimer when bovine serum albumin (BSA) exceeds 40 g/L; it has high multimerization with an estimated activity coefficient of 10 when BSA concentration is 80 g/L. The activity of fibrinogen in blood plasma is an order of magnitude larger than that exhibited in dilute solutions. It is one of the reasons that transient enzyme complexes (e.g., metabolons) in cytoplasm may be difficult to observe *in vitro* by regular techniques, such as ultracentrifugation, gel filtration, non-denaturing gel electrophoresis, spectroscopy, or denaturation kinetics. By using affinity electrophoresis or chromatography, it is found that immobilized aldolase can absorb G3PDH and TIM, suggesting that they may form an enzyme complex depending on experimental conditions (Beeckmans et al., 1993). An apparent dissociation constant between ALD and G3PDH is 1 μM and their stoichiometric ratio is 1:1 (Beeckmans et al., 1993).

Macromolecular crowding can increase enzyme thermostability greatly. For example, it is found that the stability of multimer enzymes strongly depends on their protein concentrations (Myung et al., 2010, 2011; Wang and Zhang, 2009, 2010; Ye et al., 2010) and the addition of bovine serum albumin can prolong their half lifetimes (Wang and Zhang, 2010; Ye et al., 2010). The use of optically transparent silica glass for encapsulating α-lactalbumin improves its thermostability by 25–30 °C because crowding enhances the stability of the folded state relative to the unfolded state (Eggers and Valentine, 2001). Also, macromolecular crowding effect may be used to explain why not all of intracellular enzymes isolated from (hyper-)thermophilic microorganisms are thermostable *in vitro* possibly due to a lack of macromolecular crowding. For example, phosphoglucose isomerase (PGI) from a thermophilic bacterium *C. thermocellum* exhibits a very short life-time at 60 °C when its concentration is low; while immobilized PGI though affinity adsorption on the surface of cellulose (i.e., macromolecular crowding effect on a solid support) exhibits more than 80-fold enhancement in half lifetimes (Myung et al., 2011).

Intracellular substrate (metabolite) channeling may be difficult to demonstrate *in vitro*, as the degree of substrate channeling may decrease with a decrease in protein concentration. Comparison of *in vitro* enzyme kinetics and *in vivo* metabolite flux often helps identify possible substrate channeling in metabolisms (Table 1) (Srivastava and Bernhard, 1986). Taking citrate synthase in the Krebs cycle as an example, an average concentration of oxaloacetate in the mitochondrial matrix is too low to sustain the citric acid cycle at its *in vitro* measured rate. Therefore, it is suggested that a locally high concentration of oxaloacetate occurs in the microenvironment of citrate synthase for metabolite channeling (Ovadi and Saks, 2004). A theoretical paper has listed a number of enzyme complexes involved in possible metabolic channeling worthy of further investigation (Huthmacher et al., 2008).

Enzyme coimmobilization *in vitro* may be regarded to mimic cascade enzyme complexes occurring *in vivo*. Several attempts of coimmobilization have been conducted to study *in vivo* metabolisms (DeLuca, 1984; Dulik and Fenselau, 1988; Lehman et al., 1981; Srere et al., 1973). Srere and his coworkers (Srere et al., 1973) have reported that up to 100% rate enhancements are observed for coimmobilization of malate hydrogenase, citrate synthase, and lactate dehydrogenase, as compared to free enzymes. More pronounced increases in rates of up to four times are observed after addition of pyruvate that removes the accumulated end product of NADH. These results suggest the important roles of micro-environmental changes on enzyme organization and substrate channeling (Srere et al., 1973).

In addition to enhanced reaction rates, substrate channeling can decrease degradation of labile metabolites and cofactors through desolvating metabolites and binding metabolites by high concentration enzymes. Enzyme concentrations in major metabolic pathways are usually much higher than those of intermediate metabolites (Table 1) (Srere, 1967; Srivastava and Bernhard, 1986). Consequently, a majority of intermediate metabolites are not free in cytoplasm but adsorbed on enzymes. Taking labile NAD(P) as an example, NAD(P) are infamous for their degradation in acidic conditions, alkaline conditions, or neutral pH and high temperatures in Table 2 (Wong and Whitesides, 1981; Wu et al., 1986), but their stability can be enhanced greatly by reducing water activity (Grunwald et al., 1986). It is estimated that approximately 80% NAD and NADH is bound with redox enzymes (Blinova et al., 2005), mainly with a structural motif — Rossmann fold (Lesk, 1995). Such binding of metabolites by their respective enzymes may protect the degradation of labile metabolites and NAD(P) (Geck and Kirsch, 1999; Rudolph and Stubbe, 1995; Thoden et al., 1997). Extremophiles are thought to have evolved some special mechanisms protecting spontaneous degradation of some labile metabolites, for example, dihydroxyacetone phosphate (DHAP) and fructose-1,6-bisphosphate (F16P) (Fig. 3). Since DHAP is not stable (Hettwer et al., 2002), selective pressure for TIM has evolved it to be a kinetically-perfect enzyme (Berg et al., 2002) and metabolite flux potential mediated by TIM (k_{cat}*[E]) is much larger than those of the other enzymes in the glycolysis pathway (Table 1). Similar, the degradation of F16P at high temperatures may enable hyperthermophilic hosts to evolve metabolite channeling for keeping F16P at an undetected level (Say and Fuchs, 2010). Comparison of intracellular DHAP, F16P, F6P and G6P levels (Table 1) and their stability (Table 2) also implies the important role of substrate channeling in minimizing

Table 2
Stabilities of metabolites and coenzymes.

Metabolite:coenzyme	% remaining after 1 h
NAD	<5[a]
FAD	100 [a]
FMN	75 [a]
Pyridoxal phosphate	40 [a]
CoASH	100 [a]
Acetyl CoA	100 [a]
ATP	40 [a]
ADP	50 [a]
AMP	95[a]
Glucose	100 [a]
Glucose-6-phosphate (G6P)	100 [a]
Glucose-1,6-diphosphate (G16P)	90 [a]
Gluconate	100[a]
6-Phosphogluconate	100 [a]
Glycerate	100[a]
3-Phosphoglycerate	100 [a]
Acetyl phosphate	<10 [a]
Acetate	100 [a]
Fructose-1,6-diphosphate (F16P)	95.4 [b]
Fructose-6-phosphate (F6P)	98.6 [b]
Dihydroxyacetone phosphate (DHAP)	79 [c]
DHAP	97.7 [d]

[a] 95 °C, 10 mM of the solutions in distilled water containing 1 mM KI (Daniel, 2000).
[b] 80 °C, pH 7.5 (Myung et al., 2010).
[c] 37 °C, pH 6.8 (Hettwer et al., 2002).
[d] 25 °C, pH 6.8 (Hettwer et al., 2002).

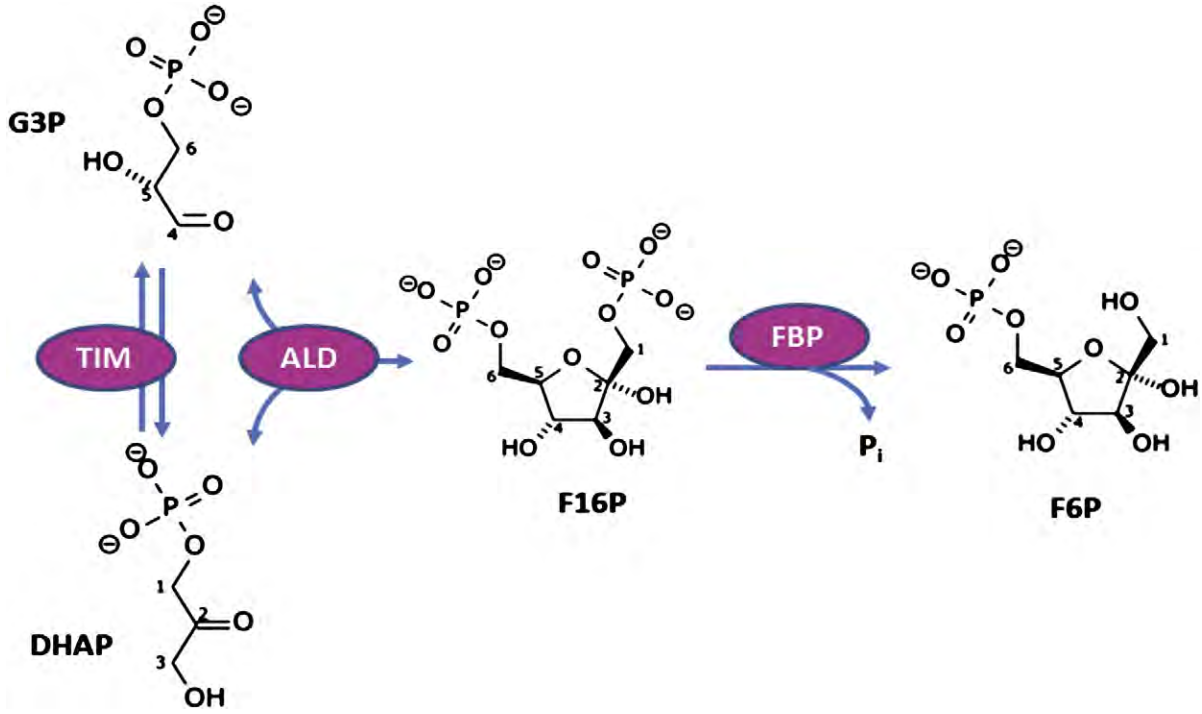

Fig. 3. Scheme of the four cascade enzymatic pathway made of triosephosphate isomerase (TIM), aldolase (ALD), and fructose-1,6-bisphosphatase (FBP) containing labile metabolites (e.g., G3P, DHAP, F16P) and stable F6P.

the degradation of labile metabolites and cofactors, even in mesophilic organisms.

3. Natural complexes for substrate channeling

Enzyme complexes vary greatly in physical stability. They can be classified into (i) static stable complexes, (ii) dynamic (transient) complexes, and (iii) catalytically induced complexes. In contrast to two latter dynamic complexes, the formation of stable complexes is independent of enzyme concentration. Typical values of degrees of substrate channeling are presented for *in vitro*, *ex vivo*, and *in vivo* complexes (Table 3).

The evidence of static enzyme complexes is very clear and solid because dissociation constants among enzyme components are very small and crystalline structures of components or even whole complexes can be obtained. Several well-studied *in vivo* enzyme complex examples are a bacterial *Salmonella tryhimurium* tryptophan synthase (Dunn et al., 2008; Hyde et al., 1988; Miles, 2001; Miles et al., 1999), a carbamoyl phosphate synthetase (Thoden et al., 1997), and a phosphoribosylpyrophosphate amidotransferase (Krahn et al., 1997) (Fig. 4). The three-dimensional structure of the tryptophan $\alpha_2\beta_2$ synthase complex from *S. tryhimurium* reveals the presence of an intramolecular channel for indole (Hyde et al., 1988). Active sites of the α and β subunits in each αβ pair are ~25 Å apart. The hydrophobic tunnel that connects the α and β active sites provides a passageway for indole transfer from the site of its production in the α subunit to the site of tryptophan synthesis in the β subunit (Fig. 4A). This intramolecular tunneling can thereby prevent the escape of indole to

Table 3
Selected examples of enzyme complexes for substrate channeling.

Location	Name	Type[a]	Method	Assay	DSC[b]	Ref.
In vivo	3-Hexulose-6-phosphate synthase (HPS)/6-phospho-3-hexuloisomerase (PHI)	S	Fusion	*In vitro*	2	(Orita et al., 2007)
In vivo	Enzymes in the Krebs cycle	S	Co-immobilization[c]	*In vitro*	1.38–1.49[b]	(Moehlenbrock et al., 2010)
In vivo	Hydrogenase/ferredoxin	S	Fusion	*In vitro* *In vivo*	1 1–4.3	(Agapakis et al., 2010)
In vivo	Glycerol-3-P dehydrogenase/ glycerol-3-P phosphatase	S	Fusion	*In vivo*	2	(Meynial-Salles et al., 2007)
In vivo	Tryptophan synthase	S	Affinity binding	*In vitro*	Infinite	(Hyde et al., 1988)
In vivo	Numerous enzymes in the glycolysis pathway	T	Macromolecular crowding	*In vivo/in vitro*	1–25	(Srivastava and Bernhard, 1986)
Ex vivo	Cellulose–enzyme–microbe	F	Adsorption /scaffoldin	*In vivo*	2.7–4.7	(Lu et al., 2006)
In vitro	Cellulosomes	S	Scaffoldin	*In vitro*	2–6	(Fierobe et al., 2001; Fierobe et al., 2002)
In vitro	Endoglucanase/exoglucanase	S	Fusion	*In vitro*	1.9–3.5	(Riedel and Bronnenmeier, 1998)
In vitro	Glucose oxidase/horseradish peroxidase	S	Co-immobilization by DNA scaffold	*In vitro*	20–30	(Wilner et al., 2009)
In vitro	Lactate dehydrogenase/alcohol dehydrogenase	S	Site-to-site immobilization	*In vitro*	2.8	(Mansson et al., 1983)

[a] S, static; F, fragile; T, transient.
[b] DSE, degree of substrate channeling.
[c] Relative to co-immobilization.

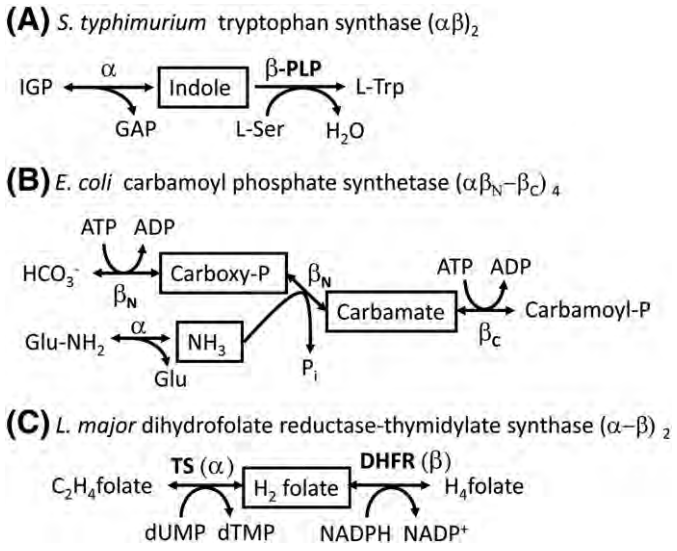

Fig. 4. Three examples of static enzyme complexes that exhibit substrate channeling in their reactions: *S. typhimurium* tryptophan synthase (A), *E. coli* carbamoyl phosphate synthetase (B), and *L. major* dihydrofolate reductase-thymidylate synthase (C). Channeled intermediates in reactions are boxed. Domains in multifunctional enzymes are separated by hyphens. Additional abbreviations used are: IGP, indole-3-glycerol phosphate; PLP, pyridoxal phosphate; G3P, D-glyceraldehyde 3-phosphate; H2 folate, dihydrofolate; H4 folate, tetrahydrofolate; and C2H4 folate, methylene tetrahydrofolate.

the solvent during the catalysis. Carbamoyl phosphate synthetase catalyzes the production of carbamoyl phosphate from bicarbonate, glutamine, and two molecules of MgATP (Fig. 4B). The 2.8 Å resolution X-ray crystal structure presents a 96 Å interior tunnel that can transport carbamoyl phosphate from one active site to the next active site (Thoden et al., 1997).

A bifunctional enzyme – dihydrofolate reductase-thymidylate synthase – catalyzes both reductive methylation of 2′-deoxyuridylate and subsequent reduces dihydrofolate for yielding 2′-deoxythymidylate and tetrahydrofolate at two discrete sites situated on different protein domains (Fig. 4C). The X-ray structure of the *Leishmania* dihydrofolate reductase-thymidylate synthase indicates that a transfer of dihydrofolate between these sites does not occur by transient binding or a tunnel at both sites but rather by movement of negatively charged dihydrofolate on the surface of the enzyme through an electrostatic highway (Atreya and Anderson, 2004; Elcock et al., 1996; Knighton et al., 1994; Liang and Anderson, 1998).

The most investigated *in vitro* static enzyme complex is cellulosomes. Cellulosomes consist of several dockerin-containing catalytic subunits, such as glycoside hydrolases (e.g., endoglucanases, exoglucanases, hemicellulases), and dokerin-containing scaffoldins through the high-affinity interaction between cohesins and dockerins (Bayer et al., 2004; Doi and Kosugi, 2004). Proximate different action-mode hydrolytic enzymes can digest plant cell walls more rapidly than free enzymes without scaffoldins (Fierobe et al., 2001). Such synergy is easily understood: an endoglucanase cuts in the middle of cellulose chains and generates two new ends, and then proximate exoglucanases conduct processive hydrolysis based on the two new ends. Without help of scaffoldins, exoglucanases may move in a much longer distance by identifying right ends before its hydrolysis. In partial support of it, solid substrate inhibition to cellulase mixtures has been reported (Huang and Penner, 1991) due to substrate surface dilution effect (Fenske et al., 1999; Zhang and Lynd, 2004b).

Proximity mechanism or leaky channeling occurring to a dynamic, transient, or induced association between cascade entities is more frequently observed, compared to perfect channeling. Transient complexes offer the possibility of fast exchange of some components upon re-assembly, and thus provide a rapid fine-tuning mechanism for re-directing metabolic pathways (Debnam et al., 1997; Verkman, 2002). Due to very weak associations among complex components, a few environmental factors may influence their formation. In such cases, unambiguous experimental evidences are much more difficult to acquire and possible channeling is often criticized by some scientists (Chock and Gutfreund, 1988; Ro and Douglas, 2004; Wu et al., 1991). It is relatively straightforward to measure rates and equilibrium constants of each enzyme *in vitro*, while these quantities are generally not easy to ascertain *in vivo*. This makes quantitative simulation and prediction of behavior difficult because some functional protein interactions are transient or exist only in the presence of molecular crowding effects in living cells. Such interactions are broken during protein purification. Therefore, it is cautious to interpret *in vivo* metabolic flux based on *in vitro* data (Bujara et al., 2011; Chin, 2006; Zhang and Mielenz, 2011).

The formation of some complexes may be only transient when an association between polypeptide components is dynamic. Numerous pairs of enzyme complexes for substrate channeling have been reported, such as ALD and fructose 1,6-bisphosphatase (FBP) in muscle (Rakus et al., 2004), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase isolated from plant and yeast (Debnam et al., 1997), and α-glycerophosphate dehydrogenase and lactate dehydrogenase (Srivastava et al., 1989; Yong et al., 1993). For example, phosphoribosylamine (PRA) is the product of glutamine phosphoribosylpyrophosphate amidotransferase (PRPP-AT) and the substrate for glycinamide ribonucleotide synthetase (GAR-syn). PRA has a half-life of 5 s under the microorganism's physiological conditions. Regular technologies, such as gel chromatography, fluorescence spectroscopy, chemical cross-linking, and affinity gel chromatography, cannot validate the existence of a static enzyme complex, suggesting that PRPP-AT and GAR-syn must be a transient one (Rudolph and Stubbe, 1995).

The formation of enzyme complexes may also be controlled by substrate binding and other small molecules. For example, a conformational change in cytochrome P450 enzyme results from substrate binding and mediates the attachment of cytochrome P450 reductase (Grunau et al., 2006). Taking fumarase (FUM) and malate dehydrogenase (MDH) in the citric acid cycle as another example, their association is not influenced by either intermediates of the citric acid cycle or NAD^+, NADH, ADP, AMP and P_i. When concentrations of both ATP and GTP are below 1 mM, the association of FUM-MDH can be formed (Beeckmans et al., 1990). This induced enzyme complex formation or dissociation may be an alternative mechanism that can regulate entering the citric acid pathway or another competing pathway, like gluconeogenesis, depending on intracellular ATP/ADP ratios and levels (Beeckmans et al., 1993).

Numerous multifunctional enzyme systems have covalently attached prosthetic groups or swinging arms and their associated protein domains essential to mechanisms of active-site coupling and substrate channeling (Perham, 2000). The domains have conformationally flexible linkers so that they can move and tether them to other components of their respective multi-enzyme complexes. A typical example is pyruvate dehydrogenase complex, where a substrate is attached to a flexible arm that can move among several active sites (Perham, 2000).

Multi-functional enzymes are another strategy for achieving substrate channeling in nature. For example, archaeal groups as well as the deeply branching bacterial lineages contain a bifunctional fructose 1,6-bisphosphate (F16P) aldolase/phosphatase (Say and Fuchs, 2010). This bifunctionality ensures that heat labile metabolites — DHAP, GAP and F16P (Myung et al., 2010) are quickly removed and trapped in stabile fructose-6-phosphate (Table 2), rendering gluconeogenesis unidirectional (Myung et al., 2010), and avoiding an allosteric control of classical F16P phosphatase (Say and Fuchs, 2010). Another example is an imidazole glycerol-phosphate (IGP) synthase in the fifth step of histidine biosynthesis (Brilli and Fani, 2004). In Archaea and Bacteria, an

active form of IGP synthase is made of a stable 1:1 dimeric complex constituted by a glutamine amidotransferase (GAT) and a cyclase. In Eucarya, two genes encoding GAT and cyclase are found to be fused to an open reading frame encoding a bifunctional polypeptide encoded by HIS7 (Brilli and Fani, 2004). Aspartokinase (AK)-homoserine dehydrogenase I (HDH I) from *E. coli* is an unusual bifunctional enzyme in that it does not catalyze consecutive reactions (James and Viola, 2002). The channeling of an intermediate β-aspartyl phosphate between aspartokinase of this bifunctional enzyme and aspartate semialdehyde dehydrogenase (ASADH) has been kinetically tested by an elegant technique by increasing levels of inactivated ASADH, which competes against enzyme–enzyme interactions and direct intermediate channeling. The addition of inactivated ASADH undoubtedly leads to a decrease in the overall reaction flux through these consecutive enzymes, confirming the occurrence of substrate channeling (James and Viola, 2002).

4. Synthetic complexes in biotechnological applications

Because of numerous benefits associated with enzyme complexes, the construction of synthetic complexes has broad biotechnological applications. In general, enzyme complexes can be conducted *in vivo* through (i) simple fusion enzymes and (ii) enzyme complexes linked with scaffoldins, as well as *in vitro* through (iii) simple coimmobilization, and (iv) positionally-assembled coimmobilization (Fig. 5).

4.1. Multi-functional fusion proteins

Inspired by natural multifunctional enzymes, Bulow et al. constructed a bifunctional enzyme containing cascade reactions mediated by beta-galactosidase and galactokinase (Bulow et al., 1985). Later, the same group (Ljungcrantz et al., 1989) produced another fusion protein containing beta-galactosidase and galactose dehydrogenase. This synthetic bifunctional enzyme exhibited enhanced reaction rates by 1.5–2.4-fold over free enzyme mixtures. Such enhancement increased when lactose concentration decreased. Interestingly, substrate channeling effect was observed at pH 8.5 but disappeared at a high lactose concentration of 20 mM and pH 7.5. These changes may be explained by different optimal pHs for beta-galactosidase (pH 7.0) and galactose dehydrogenase (pH 10.5) and their apparent specific activities. But a different conclusion was made based on a study of a fusion protein — L-galactosidase and galactose dehydrogenase (Pettersson and Pettersson, 2001). They argued that no tenable kinetic evidence was available to support galactose channeling between two enzymes. Clearly, whether substrate channeling occurs *in vitro* depends on numerous environmental factors, such as substrate concentration, enzyme concentration possibly influencing enzyme multimerization, the presence of other macromolecules and salts, pH, buffer, measurement sensitivity and accuracy, and data analysis.

The construction of multifunctional fusion proteins has been proposed to have a potential application in metabolic engineering because it may be beneficial in directing a substrate to a preferred pathway (Bulow and Mosbach, 1991). Later, when *E. coli* harboring galactosidase–galactokinase fusion enzymes with different linkers were grown on lactose as a sole carbon source, *E. coli* cells expressing the evolved fusion enzyme with a shorter linker showed faster growth rates (Bulow and Mosbach, 1991). By utilizing a chemostat, *E. coli* mutant with a better glycerol production ability was obtained (Meynial-Salles et al., 2007). Two genes encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase were reported to be spontaneously fused into an open reading frame encoding a bifunctional enzyme with a 4-AA linker by removing a small C-terminal fragment of glycerol-3-phosphate dehydrogenase (Meynial-Salles et al., 2007). The high efficiency of this fusion protein results in partial glycerol-3-phosphate channeling between two active sites and yields higher glycerol yields. Silver and her coworkers (Agapakis et al., 2010) tested the effects of different linker lengths (e.g., 2, 14, 24, 46, and 104 amino acids) of a fusion of hydrogenase and ferredoxin on possible substrate channeling. They found that the optimal linker length was 14 amino acids *in vivo* with an enhanced factor of more than four and no substrate channeling was observed by *in vitro* tests. The linker length and N- or C-terminal linkage between a catalytic unit and other domain or catalytic units may influence activities of fusion proteins greatly (Agapakis et al., 2010; Liu et al., 2010; Ye et al., in press). But a general rule for this design seems not available now.

In nature, numerous glycoside hydrolases contain multi-domain or multi-catalytic units. For example, the *C. thermocellum celJ* gene consists of a family 9 endoglucanase and a family 26 hemicellulase plus two X-domains and one Ig-like domain (Ahsan et al., 1996). Riedel and Bronnenmeier (1998) first attempted to link the *Clostridium stercorarium* exoglucanase CelY and endoglucanase CelZ to a large fusion protein CelYZ (170 kD). As a true multienzyme, CelYZ exhibiting both exoglucanase and endoglucanase activities had three- to four-time higher than the sum of individual activities (Riedel and

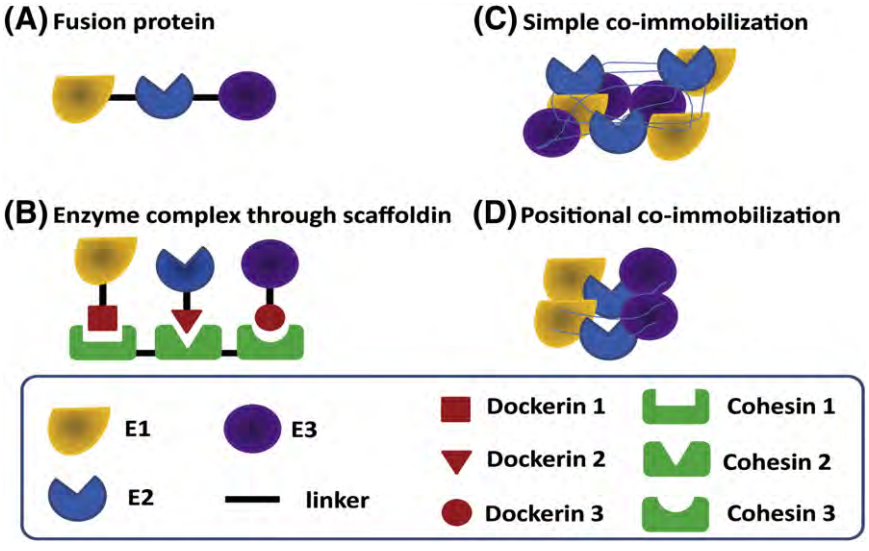

Fig. 5. Biotechnological approaches for constructing enzyme complexes for substrate channeling: a fusion protein with multiple functions (A), an enzyme complex through the linkage of scaffoldin (B), coimmobilization of randomly-mixed enzyme complexes (C), and coimmobilization of positionally assembled enzyme complexes (D).

Bronnenmeier, 1998). Two highly-active trifunctional hemicellulases were constructed by linking the catalytic portion of a xylanase with an arabinofuranosidase and a xylosidase, using either flexible peptide linkers or linkers containing a cellulose-binding module (CBM) (Fan et al., 2009). These multifunctional enzymes retained parental enzyme properties and exhibited synergistic effects in hydrolysis of natural xylans and corn stover (Fan et al., 2009). The addition of a family 9 CBM on cellodextrin phosphorylase enables it to work on insoluble cellulosic substrates (Ye et al., in press). The fusion of the *T. reesei* endoglucanase IV (EG4) with an additional catalytic module (EGIV-CM) resulted in about 4-fold enhancement in mass-specific activity (Liu et al., 2006).

4.2. Metabolic engineering

Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones by recombinant DNA technology (Bailey, 1991; Stephanopoulos, 1999). To achieve high production rates and high product titers, fluxes of enzymatic reactions in cascade pathways are required to not only limit the accumulation of intermediates, especially toxic ones, but also keep levels of labile metabolites very low (Table 1). Two classic strategies for balancing pathway fluxes are: (i) modulating expression levels of individual enzymes (e.g., through manipulating promoter strengths, ribosome binding site strengths, plasmid copy number, or tunable intergenic regions controlling mRNA processing) and (ii) improving turnover activities of rate-limiting enzymes by directed evolution or isolated from other sources. By mimicking natural enzyme complexes for substrate channeling, Keasling and his coworkers (Dueber et al., 2009) introduced a third strategies — co-localization of cascade enzymes linked by synthetic protein scaffolds (Fig. 5B). The optimization of three mevalonate biosynthetic enzymes for the formation of a synthetic complex achieved 77-fold improvement in product titer with low enzyme expression so that it reduced bioenergetic load (Dueber et al., 2009).

Since tight regulations (e.g., positive or negative feedback) are a hallmark of natural metabolisms, Silver and her co-workers attempted to insulate the production of hydrogen from its basic metabolisms through the deletion of competing reactions, rational engineering of protein interaction surfaces, direct protein fusion of interacting partners, and co-localization of pathway components on heterologous protein scaffolds (Agapakis et al., 2010), but hydrogen yields were far below the Thauer limit (i.e., four hydrogen per glucose unit) (Thauer et al., 2008). In spite of intensive metabolic engineering efforts on enhancing hydrogen production yields in microbes (Agapakis et al., 2010; Chou et al., 2008; Maeda et al., 2008a, b; Veit et al., 2008), practical hydrogen yields mediated by dark microbial fermentations do not break the Thauer limit (e.g., 4 H_2 per glucose). *In vitro* metabolic engineering (cell-free synthetic pathway biotransformation, SyPaB) evolves from cell-free ethanol fermentation (Zhang, 2010a; Zhang et al., 2010). By using this technology, nearly 12 mol of hydrogen are produced from starch or cellulosic materials and water for the first time (Ye et al., 2009; Zhang et al., 2007). SyPaB may be regarded as two-step fermentation that insulates anabolism and catabolism by process operations. At the first step, several microbes are responsible for producing high-titer recombinant enzymes only; while at the second step, the mixed purified enzymes are responsible for high-yield biotransformation only (Zhang and Mielenz, 2011).

4.3. Synthetic cellulosomes and recombinant cellulolytic microorganisms

Cellulosomes linked by non-hydrolytic scaffoldins always exhibit enhanced hydrolytic activities as compared to free cellulases due to enzyme proximity and CBM-mediated targeting effects (Elkins et al., 2010). Bayer et al.(1994) first proposed the idea of constructing designer cellulosomes with tailored subunit composition and defined spatial arrangement of enzymes for potential biotechnological applications. Fierobe and his co-workers demonstrated numerous designer cellulosomes containing cellulosomal bacterial cellulases, non-cellulosomal bacterial cellulases, fungal cellulases, and hemicellulases (Fierobe et al., 2001, 2002, 2005; Mingardon et al., 2007a, b). Later, synthetic cellulosomes containing *T. fusca* cellulases (Cel48A exoglucanase and Cel5A endoglucanase), and two *T. fusca* xylanases (endoxylanases Xyn10B and Xyn11A) exhibited approximately two-fold enhancement activities than natural non-complexed *T. fusca* enzyme mixture on wheat straw (Moraïs et al., 2010; Vazana et al., 2010). The linker length between catalytic modules and dockerins was found to have little effect on synthetic cellulosome (Caspi et al., 2009). Positioning of the dockerin on the C-terminal side of the enzyme resulted in an enhanced synergistic activity, implying the potential importance in an alignment of catalytic module and dockerin (Caspi et al., 2009). Although synthetic cellulosomes always present faster hydrolysis rates than non-complexed cellulase mixtures, it is not clear why synthetic cellulosomes constructed to date, have been much less active than their natural counterparts (Elkins et al., 2010), which might be due to less active cellulase components produced by *E. coli* or other heterologous hosts.

The formation of cellulose–cellulosome–microbe complex facilitates cellulodextrin transport from the surface of solid cellulose to adjacent cellulolytic microorganisms without further extracellular hydrolysis and exhibits several-fold higher cellulose hydrolysis rates as compared to free cellulosome along with the microorganism (Lu et al., 2006). Natural cell-surface displayed cellulosomes inspire the binding of extracellular cellulase components on the surface of hosts, including *B. subtilis* (Cho et al., 2004), *Clostridium acetobutylicum* (Mingardon et al., 2005; Perret et al., 2004), *E. coli* (Heyman et al., 2007), *S. cerevisiae* (Tsai et al., 2009; Wen et al., 2010), and *Lactococcus lactis* (Wieczorek and Martin, 2010). The mini-cellulosomes displayed on the surface of yeast exhibited significantly enhanced glucose liberation ability and produced ethanol directly from acid-treated amorphous cellulose (Tsai et al., 2009). The final ethanol concentration of 3.5 g/L was 2.6-fold higher than that using the same amount of added purified cellulases (Tsai et al., 2009). Zhao and his co-workers (Wen et al., 2010) expressed scaffoldin on the surface of yeast and co-expressed three cellulase components (*T. reesei* endoglucanase II, *T. reesei* cellobiohydrolase II, and *A. aculeatus* BGKI). Tri-functional cellulosomes showed enhanced enzyme–enzyme synergy as compared to the unifunctional and bifunctional minicellulosomes (Wen et al., 2010). We also found that cellulosome-displayed on the surface of *B. subtilis* can hydrolyze cellulose several times faster than that mediated by the same amount of mini-cellulosome plus free *B. subtilis* (in preparation for publication).

Although intensive efforts have been made to convert non-cellulolytic microorganisms to cellulose-utilizing microorganisms [see the latest reviews (Elkins et al., 2010; la Grange et al., 2010; Zhang and Zhang, 2010)], real recombinant cellulolytic microorganisms that can produce sufficient levels of a complete secretory cellulase mixture, hydrolyze cellulose to soluble sugars, and utilize sugars for supporting self-growth and cellulase production without any other organic nutrients is a great challenge mainly because of low expression levels of several recombinant secretory cellulases (Brenner et al., 2008; Lynd et al., 2005; Zhang et al., 2006). By utilizing high-protein secretion capacity *B. subtilis* along with the discovery of a high-activity endoglucanase, recombinant cellulolytic *B. subtilis* strains can grow on cellulose as a sole carbon source without any other organic nutrient for the first time (Zhang et al., 2011).

4.4. Coimmobilization of multiple enzymes

Coimmobilization of cascade enzymes can bring numerous benefits in multi-enzyme-mediated biocatalysis, such as fewer unit operations, less reactor volume, higher volumetric and space-time yields, shorter cycle times, and less waste generation (Betancor et al., 2006; Van Langen et al., 2002). Furthermore, coupling several steps

together can drive un-favorable equilibrium of reaction towards a desired product (Mateo et al., 2006).

Co-immobilization can be conducted on the surface of solid supports (Betancor et al., 2006; El-Zahab et al., 2004, 2008; van de Velde et al., 2000; van Dongen et al., 2009) or without supports (Mateo et al., 2006; Moehlenbrock et al., 2010; Shah et al., 2006; St. Clair et al., 2000). Enzyme components during coimmobilization can be randomly distributed (Betancor et al., 2006; El-Zahab et al., 2004; van de Velde et al., 2000), positionally assembled (Kim et al., 2007; van Dongen et al., 2009; Vriezema et al., 2007; Wilner et al., 2009), and even the active site of an enzyme face to the one of another enzyme (Mansson et al., 1983).

The distance between cascade enzymes may greatly influence the degree of substrate channeling. For example, a direct linkage of the Kreb cycle enzymes isolated from mitochondria of *S. cerevisea* can form static *in vitro* metabolons, which demonstrates 38–49% reaction rate enhancements as compared enzyme mixtures without such linking (Moehlenbrock et al., 2010). Through accurately controlled distances between glucose oxidase and horseradish peroxidase by using DNA scaffolds, the distances of 13 and 33 nm between two enzymes result in 25-fold and 22-fold reaction rate enhancements, respectively, as compared to free enzymes without such linkages (Wilner et al., 2009).

5. Closing remarks

Substrate channeling among enzyme complexes exhibits numerous potential benefits, such as protection and/or stabilization of labile metabolites, regulation of competing pathways for the same substrate, elimination of toxic metabolite inhibition, and so on. Therefore, the construction of synthetic complexes for substrate channeling would have great potentials in metabolic engineering (Agapakis et al., 2010; Dueber et al., 2009), multi-enzyme biocatalysis in one pot (Vriezema et al., 2007; Wilner et al., 2009), and cell-free SyPaB for low-cost biomanufacturing (Wang et al., 2011; Zhang, 2010a; Zhang et al., 2010). Different from macromolecular crowding environments created by cellular membrane that may stabilize some labile metabolites, constructing *in vitro* static enzyme complexes with metabolite channeling for accelerating reaction rates and decreasing potential metabolite degradation would be extremely important for SyPaB, especially for sugar fuel cell vehicles that would be run at elevated temperatures (Zhang, 2009, 2010b). Multifunctional enzyme complexes may be regarded like integrated circuit devices or biocatalytic modules suitably for the assembly of more complicated systems. In a word, constructing synthetic enzyme complexes or enzyme–microbe complexes with substrate channeling is becoming an emerging tool in biotechnological applications.

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