From plant biomass to biofuels and bio-based chemicals with microbial cell factories

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Abstract: Global energy demands and global warming represent key challenges of the future of human society. Continous renewable energy supply is key for sustainable economy development. Waste plant biomass represent abundant source of renewable energy that can be transformed to biofuels and other value-added products, which is currently limited due to the lack of cost-effective biocatalysts. The bottleneck of this process is the degradation of structural polysaccharides of plant cell walls to soluble compounds that can be fermented to solvents or transformed to biogas via methanogenesis and can be used as biofuels or chemical raw materials. In order to replace traditional physical and chemical methods of lignocellulose pretreatment with more environmentally friendly biological approaches, native microbial enzyme systems are increasingly being explored as potential biocatalysts that could be used in these processes. Microbial enzymes are useful either as catalysts in the enzymatic hydrolysis of lignocelluloses or as components incorporated in engineered microbes for consolidated bioprocessing of lignocelluloses. The unprecedented development of tools for genetic and metabolic engineering for a wide range of microorganisms enabled significant progress in the development of microbial cell factories optimized for the producton of biofuels. One of the most promising strategies aimed towards this goal, i.e. systematic design and heterologous expression of »designer cellulosomes« in industrial solventogenic strains is adressed in detail.

Key words: waste plant biomass; biocatalysts biofuels; solvents; microbial cell factories

Od rastlinske biomase do biogoriv in bio-surovin z mikrobnimi celičnimi tovarnami

Izvleček: Naraščajoče potrebe po obnovljivih virih energije in globalno segrevanje predstavljata ključna izziva prihodnosti človeške družbe. Za trajnostni razvoj industrije je nujna neprekinjena oskrba z obnovljivo energijo. Odpadna rastlinska biomasa predstavlja velik rezervoar obnovljive energije, ki jo je mogoče pretvoriti v biogoriva in druge produkte z dodano vrednostjo, vendar trenutno za tovrstno pridobivanje energentov še ni na voljo dovolj učinkovitih biokatalizatorjev. Omejujoča stopnja mikrobiološke pretvorbe odpadne rastlinske biomase v produkte z veliko dodano vrednostjo je hidroliza strukturnih polimerov do topnih podenot, ki predstavljajo substrat za fermentacijo do biogoriv in biosurovin z večjo dodano vrednostjo. V želji, da bi drage in neekološke tradicionalne postopke fizikalne in kemične predobdelave lignoceluloznih substratov nadomestili z okoljevarstveno sprejemljivejšimi biološkimi, v zadnjem desetletju poteka intenzivno proučevanju mikrobnih encimskih sistemov za razgradnjo rastlinske biomase. Vzporedno je nesluten razvoj orodij za gensko in metabolno spreminjanje mikrobnih celic omogočil pomembne dosežke na področju načrtovanja mikrobnih celičnih tovarn za optimizirano proizvodnjo biogoriv. Ena od obetavnejših naprednih strategij usmerjenih k temu cilju je tudi sistematično načrtovanje in ekspresija celulosomov »po meri« v solventogenih industrijskih sevih, čemur smo v pregledu posvetili največ pozornosti.

Ključne besede: odpadna rastlinska biomasa; biokatalizatorji, biogoriva; topila;mikrobne celične tovarne

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1   INTRODUCTION

Global society is confronted with climate change, which is frequently associated with excessive consumption of fossil fuels, currently the most important source of energy on the planet. Moreover, the price of fossil fuels will continue to rise gradually as global demand grows and supply decreases (Shafiee and Topal, 2009). Some forcasts estimate consumption growth of up to 56 % between 2010 and 2040 (Azad et al., 2019). Consequently, new renewable and environmentally friendly energy resources are gaining in importance. One example of such bio-based energy resources are biofuels, which are based on microbial processes for transforming various types of biomass into energy-rich solvents or gases (Zhang et al., 2019). The first generation of biofuels was based on the use of easily degradable substrates (corn, grain, sugar cane, etc.). However, this strategy has ethical and ecological disadvantages, as it leads to the cultivation of monocultures. This could lead to an unacceptable reduction of agricultural land used for food production, with the potential to cause higher food prices and a reduction in biodiversity or even deforestation (Subramaniam et al., 2020, Hahn-Haegerdal et al., 2006). Therefore, it was soon recognized that biofuels should be produced from waste products instead of food crops. One such option is waste plant biomass, accumulating as a by-product of agriculture and food industry. The biggest bottleneck in the production of these, so-called second generation biofuels, is the conversion of the lignocellulosic biomass into soluble compounds that could easily be fermented to biofuels or other bio-based raw materials (chemicals and industrially-relevant enzymes). This step is enzymatically demanding and cannot be performed by most of the naturally solventogenic strains used in industrial processes. To facilitate the degradation of lignocellulose to fermentable compounds, pretreatment with concentrated chemicals (acids, bases, organic solvents or oxidants) and/or physical pretreatments (treatment with hot steam under pressure, pyrolysis, ultrasonic treatment) has traditionally been used. Such procedures are associated with environmental and cost burden (Chen et al., 2017). On the other hand, cost-efficiency of biological pretreatment processes is still too low for a wider industrial use.

To solve this issue, a considerable part of research is focused on the discovery and optimization of biocatalysts that would make this step economically viable for industrial production (Klein-Marcuschamer et al., 2011). Apart from their use for second generation biofuel production, optimized enzyme coctails could also improve the production of third-generation biofuels based on microalgal biomass (Venkata Mohan et al., 2016).

Over the last ten years, celullolytic systems have attracted a great deal of interest from the energetic industry. As many anaerobic cellulolytic microorganisms naturally convert (hemi)cellulose to ethanol they represent a promising alternative to conventional processes. However, the production yields in wild strains are not satisfactory for industrial applications.

In order to achieve the highest possible yields of biofuels, research is concentrating on the development of strains with improved properties. Various strategies are used for this purpose, in particular targeted evolution (random mutagenesis and screening for usable mutants) or targeted genetic manipulation of wild strains (Choi et al., 2020). A prerequisite for the second approach is the adequate characterization of restriction modification, transport and cellulolytic systems and metabolic pathways involved in the fermentation of sugars (Majidian et al., 2018). Exhaustive basic knowledge of native cellulolytic systems as well as potential production strains is essential for the development of strains with the desired properties and can be gained via genomic, transcriptomic and proteomic approaches as well as *in vitro* studies of interactions between individual enzymes. This review is focused on important scientific findings in the field of microbial enzyme systems for the degradation of lignocellulosic biomass and selected results of metabolic engineering experiments to design microbial cell factories for the production of second generation biofuels.

2   NATIVE MICROBIAL SYSTEMS FOR LIGNOCELLULOSIC BIOMASS DEGRADATION

The main component of accumulating plant biomass are the cell walls. This is associated with its structural recalcitrance, preventing most (micro)organisms to decompose this abundant energy source. The most abundant compund in the plant cell walls is cellulose, which typically accounts for 20 % to 90 % of the dry mass of plant tissues. This homopolysaccharide consists of 100 - 20 000 D-glucopyranose residues linked by 1-4 β-glycosidic bonds (O'Sullivan, 1997). Uniform structure and charge distribution in glucan chains enables the formation of multiple intramolecular hydrogen bonds, Van der Walls and hydrophobic interactions, These are responsible for tight packing of these molecules to microfibrils (30-36 glucan chains) and further to macrofibrils, rendering cellulose insoluble and inaccessible to enzymes (Keegstra, 2010). In most natural cellulose substrates, regions with a high order rate (crystalline regions) predominate and are partially interrupted by disordered (amorphous) regions. For an efficient decomposition of natural plant biomass the synergistic action of several en-

zymes with different specificities is necessary. Only some bacteria, fungi and very few protists contain this type of enzyme consortia. Two main types of cellulolytic systems that are found in nature are (1) free enzyme systems and (2) the cellulosomes. Free enzyme systems are typically found in aerobic cellulolytic microorganisms, which produce large amounts of free or membrane proteins that bind separately to the substrate. On the other hand, some anaerobes developed a more efficient strategy based on combining enzymes in complexes, namely cellulosomes (Vodovnik and Marinšek Logar, 2010). A limiting factor in the process of enzymatic decomposition of cellulose is poor access of glucan chains within highly ordered and strongly connected regions of microfibrils. Loosening of cellulose structure is therefore crucial first step of its degradation, as it makes a larger surface area available for hydrolytic and oxidative enzymes. This process is called amorphogenesis and is still being investigated. Cellulose binding modules (CBMs) of enzymes and cellulosomic structural proteins (scaffoldins) have been shown to play an important role, as well as specialized proteins called expansinsins. (Arantes in Saddler, 2010). After the cellulose network is loosened, hydrolytic enzymes (β-1,4-glucanases) can access individual cellulose chains and cleave them to soluble cellodextrins with a degree of polymerisation of 2-6. These are easily converted to glucose before or during the transfer across the cell wall and enter central fermentation pathways. Considering the mode of action and specificity toward the substrate, the cellulases can be classified as endoglucanases and exoglucanases (cellobiohydrolases). Endo-β-glucanases produce cello-oligosaccharides by randomly acting on the cellulosic chain within amorphous regions of cellulose. Released free ends represent the substrate for exoglucanases which release sugars from the end of the molecules. Cellulolytic microorganisms always produce both types of the cellulases that work synergistically (Moraïs et al., 2016). Traditionally, cellulases have been classified as glycoside hydrolases. Only recently, oxidative activity was detected in some of these enzymes. These enzymes were found in some aerobic microorganisms and were classified as lytic monooxygenases (Frandsen et al., 2016).

Cellulose fibrils in plant cell walls are incorporated in a matrix of hemicellulose and pectin, in addition to various glycoproteins and sometimes lignin. Consequently, the vast majority of cellulolytic microorganisms also produces enzymes necessary for the degradation of these molecules, particularly hemicellulases. One of the main components of the hemicellulose matrix in plant cell walls is xylan. This is branched molecule, with diverse substituents. Endoxylanases catalyze the hydrolysis of 1,4-β-glycosidic bonds within the main xylan backbone, releasing various oligoxylosaccharides, xylobiose and xylose. Exoxylanases cleave successive residues of D-xylose from the ends of the main xylan molecule. Finally, β-xylosidases hydrolise shorter oligoxylosaccharides (mostly xylobiose) to xylose (Malgas et al., 2019). These enzymes are present in most hemicellulolytic microorganisms and are usually cell-bound or released to the supernatant. The efficiency of xylanases depends on synergistic activity with auxiliary hemicellulytic enzymes (arabinofuranosidases, glucorunidases, esterases, etc.) which cleave side chains (arabinose, glucuronic acids, fenol and acetyl substituents) from the xylan backbone and separate it from lignin. In addition to hemicellulases, some aerobic microorganisms (mostly fungi) also produce ligninolytic oxidative enzymes, lacases and peroxidases (Plácido et al., 2015). In nature, decomposition of the complex matrix in plant cell walls is usually performed by coordinated action of different microorganisms, that either produce enzymes actively involved in the decomposition of polysaccharides or indirectly influence the degradation, e.g. by consuming end products to avoid catabolic repression. Such systems are too complex to be directly used for industrial applications, so different strategies are being used to develop better adapted, cost-efficient catalysts. Some major approaches will be discussed in the next chapters.

3 DEVELOPMENT OF CONSOLIDATED BIOPROCESSES

As mentioned above, one of the main strategies striving towards more efficient conversion of lignocellulose to biofuels is the development of consolidated bioprocesses. These are processes which alow direct (one-step) conversion of biomass to biofuel or other value-added compounds within a single system. Two approaches are commonly used to create such system. One option is to complement selected industrial solvent-producing strain with the genes encoding (ligno)cellulolytic enzymes. Alternatively, metabolic pathways of native cellulolytic strains can be manipulated towards increased production of the desired products. In both cases, the strains also need to be adapted to tolerate process conditions and high concentrations of produced solvents. This can be done by metabolic engineering or directed evolution coupled to strain selection (Olson et al., 2012).

Apart from strain development by metabolic engineeging, consolidated bioprocesses can also take place in stable mixed culture (co-culture), composed of two or more different strains or species specialized for different process stages (Yang et al., 2015). Argyros et al. (2011), for example, developed a co-culture system with the cellulolytic bacteria *Clostridium thermocellum* and the

solvent-producing *Thermoanaerobacter saccharolyticum* and achieved about 80 % of the theoretical maximum ethanol yield from microcrystalline cellulose (Avicel substrate). A co-culture system of *C. thermocellum* combined with *C. thermohydrosulfuricum* or *Thermoanaerobacter pseudoethanolicus* growing on cellulose substrate has also been reported to achieve better ethanol conversion than *C. thermocellum* as a monoculture (Levin et al., 2015). One of the main advantages or even the main benefit of the mixed culture system is the reduction of catabolic suppression of enzymes when less recalcitrant carbon sources are also present in the substrates(Liu et al., 2019).

4 MICROBIAL CELL FACTORIES FOR THE PRODUCTION OF BIOFUELS

Microbial cell factory design is an approach to bioengineering which considers microbial cells as a production facility in which the optimization process largely depends on metabolic engineering. Microbial cell factories are used for many applications in industrial microbiology and also hold promise for bioenergetics. With the intention of developing industrial strains for cost-efficient conversion of lignocellulosic waste to biofuels and other value-added products, research focuses on the adaptation of selected strains with addition, silencing, modification or overexpression of different genes involved in target metabolic pathways (Das et al., 2020). Among the strategies used for metabolic engineering to enhance biofuel production and facilitate utilization of non-edible low-value carbon sources are carbon flux rerouting, reducing power enhancement, enzyme engineering, pathway design/discovery, overcoming product toxicity and protein secretion optimization were already applied (Choi et al., 2020).

The biosynthesis of lignocellulolytic enzymes in micro-organisms is regulated by transcription factors that are activated in the presence of lignocellulosic materials (Aro et al., 2005). Genes encoding these proteins thus represent a potential target for increasing the production of targeted enzymes through genetic modification. Su et al (2009) created artificial imitations of transcription factors involved in the regulation of lignocellulolytic glycoside hydrolases and achieved a higher production these enzymes in strains with artificial regulation.

To facilitate the utilization of lignocellulosic derivatives, heterologous machineries for catabolizing xylose, arabinose, cellulose and lignin have also been screened and introduced to the production hosts. Furthermore, engineering of protein folding and post-translational modification systems to enhance overexpression of the heterologous enzymes, genome scale modelling and ALE to overcome catabolite repression have so far been effective in enhancing utilization of lignocellulosic derivatives (Choi et al., 2020).

Furthermore, a very promising strategy to improve the yield of target enzymes focuses on the regulation of their transport and secretion from cells (Idiris et al. 2010; Reed and Chen, 2013). Pakula et al (2005) investigated the secretome of the hemicellulase producing fungus *Trichoderma reesei* Simons and noticed that only a small fraction of produced enzymes is actually secreted out of the cells, which confirmed that secretion is one of the bottlenecks in protein production and can be targeted for improvement. Furthermore, recombinant enzymes are useful either as catalysts in the enzymatic hydrolysis of lignocelluloses or as components incorporated in engineered microbes for consolidated bioprocessing of lignocelluloses. If microbial cells expressing recombinant proteins are used to interact with polymeric material, such as lignocelluloses, extracellular secretion of proteins is necessary due to the inability of microbial cells to uptake polymer substrates. In the case of applications where purified recombinant proteins are used directly, secretion of these proteins extracellularly could significantly reduce the complexity of a production process by eliminating the need for cell lysis and reducing the burden of removing host proteins. In addition, secretion of highly expressed proteins minimizes formation of inclusion bodies due to environment better suited for folding and disulfide bond formation, reducing the effects of intracellular protein degradation and lessening the detrimental effects of cytotoxic proteins (Reed and Chen, 2013; Burdette et al., 2018). The signal peptide sequence is one of the most important factors influencing secretion efficieny. A common approach is to fuse an optimized signal sequence of the expression host to the target protein. Such endogenous signal peptide fusion approach has for example been used in engineering *B. subtilis* (Ehrenberg 1835) Cohn 1872 secreting *Clostridium cellulovorans* Sleat et al. 1985 mini-cellulosome (Arai et al., 2007). Signal peptides from microorganisms other than expression host may also be used (exogenous peptides). As with the endogenous pepides, selection of exogenous signal sequences often entail screening a large library of candidate signal sequences (Reed & Chen, 2013).

Recent research has shown that the efficiency of cellulose degradation depends more on the degree of synergy between different enzymes than on the absolute activity of individual enzymes. This knowledge led to the development of designer cellulosomes – artificially constructed enzyme complexes tailored for optimized degradation of different lignocellulose substrates. In these recombinant cellulosomes, chimeric subunits from

different microorganisms are combined for increased efficiency of target substrate degradation (Figure 1). Studies with designer minicellulosomes, incorporating different enzyme combinations allow the identification of catalytic subunit sets with superb activity (Leis et al., 2018). At present, designed minicellulosomes are mainly used for interaction studies, but increasing number of experiments performed suggests a possible future industrial application. The genes encoding cellulosomes with the desired subunits have already been transfered to selected microorganisms without natural ability to degrade cellulose, which are able to produce a wide range of useful chemicals as fermentation products (ethanol, buthanol, acetone, etc.) (Lütke-Eversloh, 2014, Willson et al., 2016). In recent research Moraïs et al. (2016) constructed a minicellulosome combining exoglucanase Cel48S, endoglucanase Cel8A and β-glucosidase BglA from the bacterium *Clostridium termocellum* NCBI, that have previously been improved by targeted and random mutagenesis. The complexes demonstrated improved stability and activity at high temperatures than original cellulosomes, which is particularly promising for application in thermophilic industrial processes.

The expression of minicellulosomes in industrial strains of fungi, has also been reported. *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen, for example, has been used for thousands of years for the production of ethanol. It is one of the most important industrial microorganisms, not only due to its efficient production of ethanol, but also due to its rapid growth, flexible metabolism, ability to grow in anaerobic environments and tolerance for higher concentrations of ethanol than most of bacteria (Nevoigt, 2008). However, this yeast does not have the ability to degrade plant cell walls (Piškur in Langkjaer, 2004). Tsai et al

(2009) reported succesfull expression of a minicellulosome combining enzymes from two different *Clostridia* and *Ruminococcus* within *S. cerevisie*. The recombinant strain was capable of simultaneous cellulose saccharification and fermentation of the produced sugars, which makes it interesting for industrial production of bioethanol.

Apart from ethanol, buthanol is also considered a promising biofuel. Moreover, compared to ethanol, buthanol is more energy-dense, less corrosive and less volatile, which makes it even more appealing for industrial applications (da Silva Trinidade and dos Santos, 2017). Buthanol and some other higher alcohols are also more compatible with current infrastructure and mechanisation (Kótai et al., 2013). The most important current producer of biobuthanol is the thermophilic bacterium *Clostridium acetobutylium* Mccoy et al. 1926 emend. Keis et al. 2001, which is known for its bi-phasic fermentation: acidogenic and solventogenic. In acidogenic phase the cells convert substrate into acetic and butyric acid, while in the solventogenic phase they convert it into acetone, ethanol and buthanol in a ratio of 3:6:1. This phase is also known as ABE fermentation (Xue et al., 2017). Native species, however, can only ferment sugars to usable solvents and are therefore not suitable for consolidated bioprocesses leading to the production of second-generation biofuels. To overcome this obstacle, Willson et al (2016) inserted synthetic gene constructs encoding cellulosomic structural and enzyme subunits from the related cellulolytic *C. cellulolyticum* to the native *C. acetobutylicum* strain. One of the main challenges limiting the industrial production of biobuthanol is the toxicity of this solvent to most of the producing strains. Consequently, the production is stopped at lower concentrations than in case of bioethanol, leading to higher purification costs. Most of the efforts are therefore focused
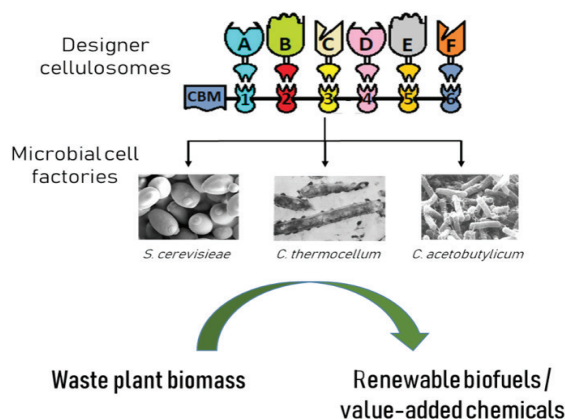

Figure 1: Microbial cell factories for production of second generation biofuels: heterologous expression of designer cellulosomes in industrial solventogenic strains (authors of SEM photographies: *C. thermocellum*: E. Bayer and R. Lamed, *S. cerevisiae*: Das Murtey and Ramasamy, 2016, *C. acetobutylicum*: BacMap Genome Atlas).

on developing more robust strains with higher tolerance to buthanol and ABE fermentation by-products. For this purpose, metabolic engineering approaches and random muthagenesis have already been used (Xue et al., 2017). Another strategy to increse the cost-efficiency of the processes in second-generation biofuel production is also to diminish product purification costs by reducing the formation of by-products. However, this approach has so far proved challenging. Tummala et al (2003) attempted to regulate solvent production in *Clostridium acetobutylicum* by switching off the gene for acetoacetate decarboxylase (*adc*) by small antisense RNA molecules, but were unsuccesful. However, the resutls of the study revealed that CoAT is the rate-limiting enzyme in acetone formation process. Furthermore, Jiang et al. (2009) completely disrupted *adc* gene with the insertion of a plasmid, which led to increased buthanol production (up to 70 % of fermentation products). Nevertheless, industrial strains for biobuthanol production are currently still far from being economically viable. It is clear that many improvements still need to be performed. A promising approach that may solve some current challenges associated with genetic modification of solventogenic *Clostridia* involves use of CRISPR-Cas system, which has already been used to improve some related *species* (Xue et al., 2017; Das et al. 2020).

5 IMPROVEMENT THE BIOMASS DECOMPOSITION PROPERTIES

One of the approaches aimed for optimizing the production of biofuels from plant biomass is also genetic modification of the plants. Huang et al (2019), for example, created transgenic rice plants by overexpresseing two glycoside hydrolases involved in modification of cellulose-containing microfibrils. The plant cell wall of modified plants had lowered crystallinity index and cellulose depolarisation factor, which resulted in higher digestibility and increased bioethanol yields.

Many similar experiments to modify the biomass by regulating the expression of intrinsic plant (hemi)cellulases were carried out, but are always challenged by the side effects, particularly deterioration in the mechanical stability and crop yield. In addition, due to strict regulation associated with the use of genetic manipulation of plants currently limits the use of these plants for the general production of second-generation biofuels from waste plant biomass (Joshi in Nookaraju, 2012).

6 CONCLUSION

In December 2018, the revised renewable energy directive 2018/2001/EU entered into force, as part of the Clean energy for all Europeans package, aimed at keeping the EU a global leader in renewables and, more broadly, helping the EU to meet its emissions reduction commitments under the Paris Agreement. The new directive establishes a new binding renewable energy target for the EU for 2030 of at least 32 %, with a clause for a possible upwards revision by 2023. The directive also encourages permament development for EU to change the direction of bioenergy towards greater savings in greenhouse gas emissions and the reduction of undesirable effects on the environment, particularly in the case of indirect changes in the use of agricultural land for the production of biofuels from crops despite the use of this land as food or feed. The development of more efficient biocatalysts and systems that will allow for the economically viable production of sustainable energy from plant biomass is certainly one of the promising approaches toward these goals. New discoveries resulting from intensive research in the field of microbial systems for the degradation of plant biomass by applying system's biology approaches combined with new tools for the genetic modification of microbial cells (including CRISPR-Cas system) will certainly play a crucial role towards optimization of individual enzymes as well as the entire microbial cells.

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