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Overview of systems and techniques for surface display of recombinant proteins in yeast *S. cerevisiae*

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Abstract

In the past decade much effort has been devoted to the development of new expression systems and novel techniques for the surface display of heterologous proteins in yeast in order to improve their applications in biotechnology, food technology, pharmacology and medicine. Heterologous protein-encoding genes are generally fused with genes coding for yeast cell wall proteins or their fragments required for anchoring. The variety of reactions by which a protein can be displayed at the cell surface enables finding the appropriate one for each individual protein. However, it is still challenging how to improve the efficiency of display of protein complexes and increase the quantity of protein displayed on the yeast surface. Recently, synthetic protein chimeras that self-assemble into the scaffolds on the yeast surface displaying different proteins have been constructed. This review focuses on systems and techniques for display of recombinant proteins on the yeast cell surfaces and applications afforded by this technology.

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1. Introduction

The expression and incorporation of recombinant proteins into the cell wall of *Saccharomyces cerevisiae* represents an outstanding tool for displaying and engineering of many proteins. In the past decade much effort has been devoted to the development of new expression systems and novel techniques for the display of heterologous proteins, so the yeast surface display nowadays is a well-established technology. Heterologous protein encoding genes are generally fused with genes coding for yeast cell wall proteins or their fragments required for anchoring [1-4]. The anchor protein should enable transport of the fusion protein through the secretory pathway, proper folding and stability of the heterologous protein and firm binding of the heterologous protein at the cell surface. Depending on the characteristics of the protein to be immobilized fusion can be done either at the C- or the N-terminal end of the protein, or the protein could be inserted within the ″carrier protein″ sequence. The location of the fusion might influence immobilization efficiency, stability, post-translational modifications of the protein and its specific activity. Since the recombinant enzymes are genetically self-immobilized on the yeast cell surface, the activities of the enzymes are retained as long as the yeast continues to grow, while the activity of enzymes secreted into the medium is poorly maintained over a long reaction period. Furthermore, reutilization of the yeast cells enables reuse of the enzymes displayed on their cell surface.

However, it is still challenging how to improve the efficiency of display of protein complexes and increase the quantity of protein displayed on the

yeast surface. Recently, protein building blocks of natural protein complexes have become exploited to create synthetic protein chimeras that self-assemble into the scaffolds on the yeast surface displaying different proteins [5-7].

2. Yeast cell surface proteins and systems used to display recombinant proteins

The outer layer of the yeast cell wall is composed of manno-proteins which are attached to β-1,3-glucan by at least three and most probably four different ways. Some of the proteins are simply adsorbed to β-1,3-glucan chains, while the others are covalently linked, either through glycosyl phosphatidyl inositol (GPI)-anchors and β-1,6-glucan, or through an ester linkage between glutamate and glucose. Non-covalently attached cell wall proteins are transported through the plasma membrane and released into the periplasmic space where β-1,3-glucan adsorbs them by forming hydrogen bonds. Heterologous protein-encoding genes are generally fused with genes coding for host cell surface proteins or their fragments required for anchoring. Depending on the characteristics of the protein to be immobilized, fusion can be done either at the C- or the N-terminal end of the protein of interest, or the protein could be inserted within the carrier protein sequence (Figure 1). Most frequently used cell wall proteins for yeast surface display are α-agglutinin, a-agglutinin and Flo1p. A lectin-like cell wall protein Flo1p contains the flocculation functional domain near the N-terminus that adheres non-covalently to the cell wall mannans of other cells [8]. Matsumoto et al. developed a cell-surface display system by which the N-terminus of the passenger protein, *Rhizopus oryzae* lipase, is fused to the Flo1p flocculation domain and the recombinant protein is expressed under the control of the appropriate inducible promoter [2]. Flo1 system was used for surface-display of gluco-amylase [9], carboxyl esterase EstA [10], α-amylase [11] and few lipases [12-14]. Other yeast cell wall proteins are covalently bonded in one of the two ways. Some proteins are linked to β-1,3-glucan through GPI anchors and β-1,6-glucan, while the other group, so-called Pir protein family, is bound through ester linkages formed between particular glutamates contained in characteristic repeating units and glucose. GPI-anchored proteins used as sources of GPI anchoring signals for surface display of heterologous proteins are agglutinin [15-21], Sed1p [4,22] and Cwp2p [4,10,23]. A particular way of covalent immobilization of heterologous proteins in the yeast cell wall employs yeast α-agglutinin consisting of two subunits. The bigger, Aga1p, is anchored to the cell wall via GPI anchor, while the smaller, Aga2p, is linked to the Aga1p through disulphide bridges. Thus, fusing the passenger to Aga2p would lead to its exposure at the cell surface [24-29]. Unlike GPI anchors, Pir repetitive sequences are not sole requirements for the incorporation of proteins in the cell wall and their addition to the N-terminus of another protein would not lead to its covalent attachment to glucan. Actually, only the complete Pir proteins in native conformation were found to be efficiently linked [30]. Therefore, to achieve a successful surface display of a protein through its N-terminus, a chimeric protein consisting of the whole Pir and the desired passenger protein has to be created [1,31-35]. The simplest examples of yeast strains displaying recombinant proteins on the cell surface are those displaying single recombinant protein encoded by a gene expressed either from an episomal plasmid or integrated into the yeast genome.

2.1. Surface display of proteins using α-agglutinin system

Most heterologous proteins constructed for yeast surface display are GPI-anchored to the cell wall (Table 1). Most frequently used GPI-anchored yeast protein for this purpose is α-agglutinin. Like other yeast cell wall GPI-anchored proteins, α-agglutinin is transported to the outer side of the plasma membrane and then bound to β-1,6-glucan [36], so the foreign protein fused to the N-terminus of α-agglutinin will be anchored covalently at the cell surface. The protein can either be fused with the entire α-agglutinin, or with a part of it providing the GPI-anchoring signal.

Some examples of protein fusions to α-agglutinin are β-galactosidase [15], gluco-amylase [16], CM-cellulase [37], *R. oryzae* lipase [17], β-glucosidase [18], laccase from white-rot fungus [19], *Rhizomucor miehei* lipase (RML) [20] and *Candida albicans* lipase B (CALB) [21]. Further improvement of the CALB activity on the yeast surface was achieved by introduction of an artificially synthesized CelA linker (based on *Neocallimastix patriciarum* Cellulase A (CelA) with a FLAG peptide at the C-terminus) between the CALB and α-agglutinin anchor [38]. *S. cerevisiae* cells displaying the hybrid protein successfully catalyzed the esterification of hexanoic acid and ethanol to ethyl hexanoate in *n*-heptane, and showed to be promising in industrial production due to good operational and storage stability.

Nakamura et al. [3] and Shimojyo et al. [39] employed cell-surface engineering using the C-terminal half of α-agglutinin or Flo1p respectively to construct a yeast strain displaying the ZZ domain derived from *Staphylococcus aureus*, which binds to the Fc part of immunoglobulin G (IgG) and has been used as an affinity tag to purify recombinant proteins and for immunoassays.

Recently, xylose isomerase from *Clostridium cellulovorans* has been successfully displayed in active form on the surface of the *S. cerevisiae*. The constructed xylose isomerase displaying yeast could grow in medium containing xylose as the sole carbon source and directly produce ethanol from xylose [40]. Furthermore, there are some applications suggesting the potential for using displaying yeasts as.

Table 1. Heterologous proteins expressed and immobilized in the *S. cerevisiae* cell wall

Anchor source	Passenger	Reference
α-agglutinin	lipase B *(CALB) / Candida antarctica*	21, 38
α-agglutinin	endoglucanase / *Trichoderma reesei*	58, 65, 66,70
α-agglutinin	cellobiohydrolase / *Trichoderma reesei*	58, 65, 66
α-agglutinin	SWOI / *Trichoderma reesei*	66
α-agglutinin	Aoelp I / *Aspergilus oryzae*	66
α-agglutinin	lipase ROL / *Rhizopus oryzae*	17
α-agglutinin	lipase RML / *Rhizomucor miehei*	20
α-agglutinin	β-glucosidase / *Aspergillus aculeatus*	18, 53, 58, 65, 66, 69
α-agglutinin	cellodextrin transporter / *Neurospora crassa*	65
α-agglutinin	gluco-amylase / *Rhizopus oryzae*	11, 16, 54, 57, 71
α-agglutinin	CM-cellulase / *Aspergillus aculeatus*	37, 53
α-agglutinin	laccase / white-rot fungus	19
α-agglutinin	β-galactosidase / guar	15
α-agglutinin	ZZ-domain / *Staphylococcus aureus*	3
α-agglutinin	xylose isomerase / *Clostridium cellulovorans*	40
α-agglutinin	ModE / *Escherichia coli*	42
α-agglutinin	α-amylase / *Bacillus stearothermophilus*	54
α-agglutinin	endoglucanase II / *Trichoderma reesei*	55, 56, 59, 69
α-agglutinin	β-glucosydase 1 / *Aspergillus aculeatus*	55, 56, 59, 64,70
α-agglutinin	cellobiohydrolase II / *Trichoderma reesei*	56, 59, 69, 70
α-agglutinin	α-amylase / *Streptococcus bovis*	71
α-agglutinin	CBD of cellobiohydrolase I / *Trichoderma reesei*	57
α-agglutinin	CBD1 of cellobiohydrolase I / *Trichoderma reesei*	57
α-agglutinin	CBD of cellobiohydrolase II / *Trichoderma reesei*	57
α-agglutinin	CBD2 of cellobiohydrolase II / *Trichoderma reesei*	57
α-agglutinin	endoglucanase I / *Thermoascus aurantiacus*	59
α-agglutinin	xylanase II / *Trichoderma reesei*	63
α-agglutinin	β-xylosidase / *Aspergillus oryzae*	63
a-agglutinin	endoglucanase CelA / *Clostridium thermocellum*	59
a-agglutinin	endoglucanase CelD / *Clostridium thermocellum*	59
a-agglutinin	trans-sialidase / *Trypanosoma cruzi*	24
a-agglutinin	CD47 / mammalian	26
a-agglutinin	Heavy chain of antistreptavidin / human	27
a-agglutinin	MHC α and β chain / human	29
a-agglutinin	lipase ANL / *Aspergillus niger*	25
Cwp2	Lip2 / Yarrowia lipolytica	23
Cwp2	carboxylesterase EstA / *Burkholderia gladioli*	10
Ccw12	RNase Rny1 / *S.cerevisiae*	49
FLO1	lipase B *(CALB) / Candida antarctica*	13
FLO1	ROL / *Rhizopus oryzae*	2, 12
FLO1	LipB52/ *Pseudomonas fluorescens* B52	14
FLO1	gluco-amylase / *Ryzopus oryzae*	9
FLO1	carboxylesterase EstA / *Burkholderia gladioli*	10
FLO1	α-amylase / *Streptococcus bovis*	11
PIR1	α-1,2-galactosyltransferase /*Shizosaccharomyces pombe*	31
PIR1	α-1,2-mannosyltransferase /*S. cerevisiae*	31
PIR2	α-1,3-mannosyltransferase /*S. cerevisiae*	31
PIR4	xylanase A / *Bacillus sp. BP-7*	33
PIR2	α-2,3-sialyltransferase / human	52
PIR2	α-1,3-fucosyltransferase VII / human	52
PIR4	VP8* fragment of human rotavirus	34

biosensors for non-biological targets and bio-adsorption of toxic, as well as rare-metal ions

In order to recover metal ions from the environment microorganisms can either adsorb metals to the cell surface components or accumulate them in the cells. Surface adsorption has several advantages compared to accumulation process, like elimination of the rate-limiting step (crossing the membrane barrier), selective adsorption and possibility to remove adsorbed metal ions from the cell surface without cell disruption, so the cells could be reused. The cell surface display of metal-binding proteins/peptides enables rapid and selective adsorption of target metal ions on the cell surface and repeated use of yeasts. Kuroda et al. [41] constructed a yeast strain capable of adsorption and recovery of copper ions by displaying histidine hexapeptide fused with the C-terminal half of α-agglutinin on the surface of *S. cerevisiae*. To introduce aggregation ability of the cells only in the presence of copper ion they co-expressed copper-responsive zinc-finger transcription factor *GTS1* (required for induction of cell aggregation) under the control of the copper-inducible yeast *CUP1* promoter. The yeast constructed in this way was shown to both adsorb copper on the cell surface and self-aggregate, enabling simple and fast removal from the solution phase. The C-terminal domain of *E. coli* transcription factor ModE, which binds molybdate, has been displayed on *S. cerevisiae* using the α-agglutinin-display system [42]. Furthermore, a single amino acid mutation ($T_{163}Y$) of the metal-binding pocket of ModE was efficient in converting it to a selective binder of tungstate [43]. Different fluorescent proteins under the control of different promoters were displayed on the cell surface of *S. cerevisiae* and used as a reporter system to develop the biosensors of environmental changes. In this way levels of intra- or extracellular glucose [44], phosphate and ammonium ions [45] could be estimated by measuring the fluorescence intensities of GFP and BFP [44], ECFP and EYFP [45], respectively.

In order to develop alternative coupling technique for the production of biosensors, recombinant proteins marked with different peptide tags were constructed and immobilized on the yeast cell surface and after that adhesion of cells to metal oxide surfaces was attempted [46]. Cell detachment assays showed that the strength of cell adhesion increased with the increase of basicity of expressed peptides. According to that finding, a peptide tag $(GlyLys)_6$ was cloned onto the C-terminus of maltose-binding protein (MBP), expressed on yeast cell surface and used to bound cells to a model metal oxide Al_2O_3 [46]. Surface display of proteins with peptide tags may provide a simple one-step coupling of the cells to metal surfaces or electrodes for the formation of different biosensors.

2.2. Surface display of proteins using Aga2p system

Covalent immobilization of heterologous proteins in the yeast cell wall employing yeast a-agglutinin system, containing Aga1p (a GPI/β-1,6-glucan-anchored protein) and Aga2p, anchors the protein on the surface of yeast via disulfide bonding. The flexibility of the Aga2p protein allows C- or N-terminal fusions of proteins of interest. C-terminal fusion of a *trans*-sialidase from *Trypanosoma cruzi* with Aga2p has been performed, and this yeast was used in the sialylation of synthetic oligosaccharides [24]. *Aspergillus niger* lipase (ANL), a widely used hydrolase, was displayed on the surface of *S. cerevisiae* using a-agglutinin (Aga2 subunit) as an anchor protein [25]. Besides, this approach was used to display a ubiquitous mammalian membrane protein, CD47, which was implicated in cancer, immune-compatibility, and motility [26].

For the purposes of sensitive toxin detection, antagonistic activity and tumor targeting an increasing number of antibodies and receptors, summarized in recent reviews on this topic [47, 48], have been engineered and exposed at the yeast cell surface using Aga2 display system.

Besides monomeric proteins, yeast can be used to display homo and heterodimeric proteins since the eukaryotic yeast secretory machinery allows oligomeric mammalian proteins to assemble and export to the surface in a native-like conformation. Van den Beucken et al. [27] created a single vector encoding the heavy (HC) and light chains (LC) of an anti-streptavidin antigen-binding fragment with a HC-Aga2p fusion and soluble LC under separate GAL1 promoters. HC and LC assemble through the native inter-chain disulfide bond and are displayed as a full antigen-binding fragment.

Lin et al. [28] displayed a fully assembled catalytic antigen-binding fragment on the yeast surface, using Aga2p display system, capable of catalyzing the formation of chloramphenicol from a chloramphenicol monoester derivative.

Class II major histocompatibility complex (MHC) is the first example of non-covalent hetero-dimer expression. Boder et al. [29] displayed it by a system that co-expresses MHC α chain and MHC β chain fused to Aga2p from a dicistronic plasmid employing the bidirectional GAL1-10 promoter.

2.3. Surface display of proteins using other yeast GPI-anchored proteins as anchors

Other GPI-anchored yeast cell wall proteins such as Cwp1p, Cwp2p, Tip1p, Sed1p and Tir1p have been proven capable of displaying ?-galactosidase [4], or GFP [22] on the yeast surface. Some of them, as Cwp2p and Sed1p, were shown to be better carriers than the commonly used α-agglutinin giving six- to eightfold higher levels of displayed heterologous protein at cell surface [4].

Breinig et al. [10] made in-frame fusion of the bacterial carboxyl esterase EstA from *Burkholderia gladioli* to the endogenous yeast proteins Cwp2p and Flo1p. EstA expression resulted in about two times higher cell wall-associated esterase activities for EstA-Cwp2p than for EstA-Flo1p. Lipase Lip2 from *Yarro-wia lipolytica* was displayed on the cell surface of *S. cerevisiae* using Cwp2 as an anchor protein [23].

Genetic immobilization of the yeast RNase Rny1p was performed by creating a hybrid protein containing the signal sequence of the *S. cerevisiae* cell wall protein Ccw12p followed by the catalytic part of the Rny1p and additional 73 amino acids of the Ccw12p including the GPI-anchoring signal [49].

2.4. Surface display of proteins using Flo1p system

The protein of interest can also be fused C-terminal to the flocculation domain of Flo1p (Figure 1), which adheres noncovalently to the cell wall mannan [50].

Sato et al. [9] showed that a long anchor enhances the activity of cell surface-displayed enzyme to polymer substrates by using a gluco-amylase-Flo1p fusion. Another construct showing high cell surface lipase activity was made by fusion of Flo1p as cell wall anchor and *R. oryzae* lipase [2, 12]. The lipase of *R. oryzae* exhibits relatively high activity compared to different available lipases and is suitable for human oral use and, therefore, for food production.

Tanino et al. [13] successfully constructed the *Candida antarctica* lipase B (CALB)-displaying yeast whole-cell biocatalysts using the Flo1p anchor system.

Jiang et al. [14] displayed lipase LipB52, isolated from *Pseudomonas fluorescens* B52, utilizing flocculation functional domain of Flo1p protein anchoring system. Flo1p system was also used to display homodimeric streptavidin using two vectors, one encoding soluble streptavidin and the other encoding streptavidin fused to the C-terminus of Flo1p. The construct expressing both soluble and Flo1p-anchored streptavidin showed significant biotinylated fluorescein iso-thiocyanate binding [51].

2.5. Surface display of proteins using PIR system

Display systems with Pir-proteins as cell wall anchors are suitable for enzymes whose active sites are located near their C-termini (Figure 1). Deletion of endogenous *PIR* genes increased display efficiency of fusion proteins about three-fold compared to wild type cells, and further increase in display efficiency is possible if two Pir proteins fused with the enzyme of interest are co-used and simultaneously expressed in the yeast cell [1]. Comparison of several Pir fusion proteins showed higher activities for the *PIR3* fusion than for the *PIR1* and *PIR4* fusions, confirming the more efficient and homogenous localization of *PIR3* fusions in the cell wall [32].

Abe et al. [31] fused three glycosyl transferases (α-1,2-galactosyltransferase, α-1,2-mannosyltransferase and α-1,3-mannosyltransferase) to the Pir1p or Pir2p respectively. Yeast cells transformed in this way were successfully used as a biocatalyst for sequential synthesis of oligosaccharides identical to those synthesized in vivo.

Andres et al. [33] used Pir4p for targeting of xylanase A from *Bacillus sp.* BP-7 on the cell wall. Constructs were made by insertion of a xylanase-encoding gene in the coding region of *PIR4* gene after the repetitive sequence or on the C-terminus. Salo et al. [52] fused two mammalian glycosyl transferases in the yeast cell wall for the synthesis of specific oligosaccharides (sLe^x) that can be used as anti-inflammatory therapy. Namely, sLe^x bind to selectins and inhibit the adhesion of leukocytes on endothelial cells decreasing the possibility of inflammation during organ rejection and tissue damages during other inflammation processes. The antigenic VP8* fragment of the rotavirus spike protein was expressed as a fusion protein with Pir4p in yeast [34] for the development of a vaccine against rotavirus infections.

Shimma et al. [35] fused catalytic regions of 51 various human sialyl-, fucosyl-, galactosyl-, N-acetyl galactosaminyl-, and *N*-acetyl glucoseaminyl transferases with Pir1p, Pir3p and/ or Pir4p proteins, 40 of which showed activity. Moreover, *PIR1* and *PIR3* fusions showed synergistic effect when expressed simultaneously.

3. Systems used to co-display recombinant proteins

Apart from the production of different heterologous proteins displayed at the yeast surface that can be used in a wide variety of applications (production of pharmaceutical chemicals, detergent enzymes, food and feed enzymes, diagnostic, development of vaccines *etc.*) in the past decade much effort has been devoted to the study of expression systems for the efficient utilization of biomass. Current technologies include physical and/or chemical pre-treatment of biomass to reduce the polymeric sugar into a form usable for enzymatic processes. Improvements of biomass conversion technology would therefore greatly reduce processing cost. One option is to use mixed cultures of microorganisms that produce different enzymes needed for degradation of polysaccharides and for fermentation. However, the use of mixed cultures of microorganisms on industrial scale is problematic due to the divergent optimal growth and processing conditions, and maintaining the optimal ratios of the organisms involved. Alternative possibility to solve this problem is to engineer a consortium of yeast strains displaying and/or

secreting enzymes that can efficiently de-polymerize biomass polysaccharides to fermentable sugars and simultaneously ferment this mixed-sugar hydrolysate to final product. To date many efforts are given to the construction of multifunctional mini-cellulosomes on *S. cerevisiae*, accomplishing the combining of cellulase production, cellulose hydrolysis, and ethanol fermentation into a single step. The challenges in this field are finding the genes compatible with expression in yeast, improving specific activities of the heterologous enzymes, and maintaining optimal ratios of enzymes in mini-cellulosomes and yeast strains in consortia.

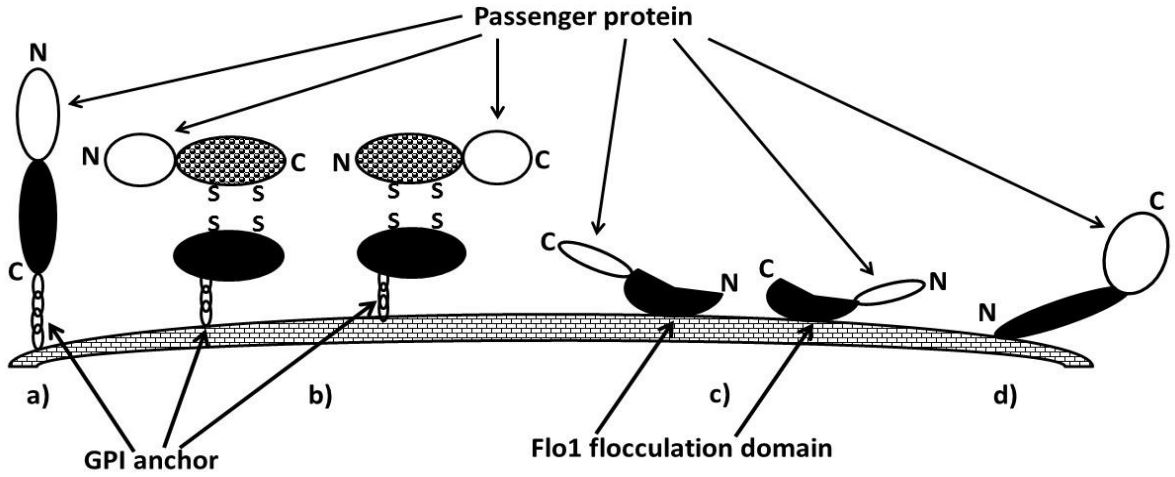

Figure 1. Cell surface display system in *S. cerevisiae:* (a) GPI display system; (b) a-agglutinin display system; (c) Flo1p display system; d) Pir display system.

In order to improve the efficiency of cell surface-displayed enzymes new display methods and systems have emerged. The first attempts to increase the efficiency in surface display of recombinant proteins resulted in yeast strains simultaneously co-displaying two or more different enzymes at the cell surface [11, 53-58]. Yeast consortia composed of three or more strains displaying and/or secreting recombinant proteins were used [6, 59-62]. Furthermore, strains co-expressing surface-displayed recombinant proteins and intracellular heterologous enzymes or protein transporters and expansines were constructed [63-66]. Finally, synthetic cellulosomes and similar scaffolding systems were constructed and displayed on the *S. cerevisiae* cell surface [5-7,60-62,67,68].

Lignocellulosic biomass has been recognized as an inexpensive and abundant source of sugar for fermentation into ethanol. For the efficient utilization of biomass, bioconversion of xylan as well as cellulose is required. Efficient degradation of such materials requires the synergistic action of different hydrolytic enzymes. Therefore, a yeast strain harboring multi-copy plasmids for the co-expression of CM-cellulase-α-agglutinin and β-glucosidase-α-agglutinin fusion proteins in the cell wall was constructed [53], and it has been shown that this strain could grow in a medium containing cellobiose as sole carbon source. On the other hand, a yeast strain co-displaying α-amylase-α-agglutinin and gluco-amylase-α-agglutinin fusion integrated into the yeast chromosome [54] was able to grow in 1% soluble starch as sole carbon source.

Furthermore, a cellulose-degrading yeast strain was constructed by genetically co-displaying *Trichoderma reesei* endoglucanase II-α-agglutinin and *A. aculeatus* β-glucosidase 1-α-agglutinin fusion proteins on the cell surface of *S. cerevisiae* [55]. The resulting yeast cells could grow in synthetic medium containing β-glucan as the sole carbon source and could directly ferment β-glucan to produce ethanol with the yield which corresponds to 93.3% of the theoretical yield. In the next step, Fujita et al. [56] co-displayed *T. reesei* endo-glucanase II and cellobiohydrolase II, and *A. aculeatus* β-glucosidase 1 simultaneously as individual fusion proteins with the C-terminal half region of α-agglutinin. Strain obtained in this way showed to be able to directly produce ethanol from the amorphous cellulose with the yield that corresponds to 88.5% of the theoretical yield.

Similar approach was used in the yeast strain that co-displayed *R. oryzae* gluco-amylase and two kinds of *T. reesei* cellulose-binding domains (CBD1, a CBD of cellobiohydrolase I (CBHI); and a CBD2, CBD of cellobiohydrolase II (CBHII)) for enzymatic desizing of starched cotton cloth [57]. Co-displaying gluco-amylase and cellulose-binding domains increased specific binding ability to cotton cloth and provided better activity than a strain displaying gluco-amylase only.

Yanase et al. [58] utilized a genome-integrated expression system for stable expression of endo-glucanase (EG) and cellobiohydrolases (CBH) from *T. reesei*, and ɒ-glucosidase (BGL) from *A. aculeatus* at the *S. cerevisiae* surface in fusion with α-agglutinin. Resulting yeast strain was used for

direct ethanol fermentation from amorphous cellulose.

Shigechi et al. [11] co-expressed α-amylase from *Streptococcus bovis* using the Flo1p-based display system in combination with gluco-amylase-α-agglutinin-based display system. This strain produces ethanol from raw corn starch with the yield that corresponds to 86.5% of theoretical yield.

Baek et al. [59] expressed on the yeast surface two fungal endoglucanases, *Thermoascus aurantiacus* EGI and *T. reesei* EGII, and two bacterial endoglucanases, *C. thermocellum* CelA and CelD in fusion with Aga2, and compared their activities. According to the results obtained in the next step, the authors used EGI-displaying strain and combined three types of yeast cells, displaying *T. aurantiacus* EGI, *T. reesei* CBHII (exo-glucanase) and *A. aculeatus* BGL1, respectively, instead of co-expressing these enzymes in a single cell. A mixture of cells with the optimized EGI:CBHII:BGLI ratio of 6:2:1 produced 1.3-fold more ethanol than the mixture composed of an equal amount of each cell type. This method allows convenient optimization of ethanol production by adjusting the combination ratio of each cell type.

Matano et al. [69] integrated the *T. reesei* genes EG2 and CBH2, and *A. aculeatus* BGL1 with a sequence encoding the C-terminal half of α-agglutinin into the genome of *S. cerevisiae*. In order to improve cellulase activity on the cell surface of the recombinant strain, additional integrations of cellulase genes (2 or 3 copies of CBH2 or both CBH2 and EG2) were performed, and the obtained results indicate that increasing the copy numbers of both EG2 and CBH2 led to higher saccharification efficiency.

S. cerevisiae co-displaying *T. reesei* xylanase II and *A. oryzae* β-xylosidase was constructed using the α-agglutinin-based display system [63]. Furthermore, intracellular co-expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Pichia stipitis* and xylulokinase (XK) from *S. cerevisiae* in the xylanase II and β-xylosidase co-displaying yeast resulted in the yeast strain capable of simultaneous saccharification and fermentation of birchwood xylan to ethanol [63].

Katahira et al. [64] constructed the recombinant *Saccharomyces* strain with xylose-assimilating and cellooligosaccharide-degrading abilities by simultaneous expression of surface-displayed *A. aculeatus* BgL1 fused with C-terminal part of α-agglutinin and intracellular co-expression of XR and XDH from *Pichia stipitis*, and XK from *S. cerevisiae*. The obtained recombinant strain was capable of ethanol fermentation from a sulfuric acid hydrolysate of wood chips.

However, improvement of efficiency gained by using these techniques has not been sufficient for industrial application. In order to optimize the level of cellulase expression for cellulose degradation, a so-called cocktail δ-integration method was developed. The first report on the expression of cellulase genes by δ-integration and optimization of various foreign genes by δ-integration in yeast was given by Yamada et al. [70]. In that work three cellulase genes (*A. aculeatus* BGL-α-agglutinin, *T. reesei* EG-α-agglut-inin and CBH-α-agglutinin fusions) were introduced into yeast genome simultaneously with one marker gene. As a result, a pool of recombinants with various genes having a different number of copies was constructed. Then a transformant with optimized cellulase expression was selected and a second cocktail δ-integration was carried out using a different marker gene to obtain a transformant with higher degradation ability.

Next strategy used to improve the levels of amylase gene expression combined δ-integration and polyploidization through cell fusion [71]. *Streptococcus bovis* α-amylase and *R. oryzae* gluco-amylase-α-agglutinin fusion was integrated into haploid yeast strains. Diploid strains were constructed from these haploid strains by mating, and then a tetraploid strain was constructed by cell fusion. The diploid and tetraploid strains proliferated faster and fermented glucose more effectively than the haploid strain. Ethanol productivity from raw starch was improved with increased ploidy [71].

In order to improve the efficiency and yield of direct ethanol production from cellulose, Yamada et al. [65] constructed a *S. cerevisiae* strain that co-expresses genes for cell surface-displayed cellulases (*A. aculeatus* BGL, *T. reesei* EG and CBH) intracellular β-glucosidase (*A. aculeatus* BGL1 gene without secretion signal) and a *Neurospora crassa* cellodextrin transporter-α-agluttinin fusion using cocktail δ-integration method. The cellulase/ cellodextrin transporter-coexpressing strain produced 1.7-fold more ethanol than a strain expressing cellulase only.

The genes encoding the expansin-like proteins SWOI from *T. reesei* and Aoelp I from *A. oryzae* were fused to 3′ half of the α-agglutinin gene in universal δ-integrative plasmid, and a *S. cerevisiae* strain co-expressing the expansin-like protein SWOI or AoelpI and *T. reesei* cellulases EG-α-agglutinin, CBH-α-agglutinin, and *A. aculeatus* BGL-α-agglutinin on the cell surface was constructed by δ-integration [66]. Although the role of expansin-like protein in degradation of cellulose has not been explained in detail yet, it is clear that the degradation and fermentation abilities of yeast strain co-expressing cellulase and expansin-like protein on the cell surface were markedly improved.

Efficient enzymatic degradation of insoluble polysaccharides requires a tight interaction between the enzymes and their substrates, and the cooperation of multiple enzymes to enhance the hydrolysis. One of the possible approaches is construction of synthetic cellulosomes, large extracellular polysaccharolytic multicomponent complexes for degradation and fermentation of xylose and cellulosic biomass (Figure 2). The

natural cellulosome is a multienzyme complex, produced by some clostridia, composed of non-enzymatic scaffolding proteins and various cellulosomal enzymes. The complex is constructed through the interaction between a cohesin module in the scaffolding protein and a cohesin binding site named dockerin in cellulosomal enzymes. The cohesin-dockerin interaction is Ca^{2+}-dependent and binding is species-specific. Recently, reconstruction of mini-cellulosome on the yeast cell surface has been attempted [5-7,60].

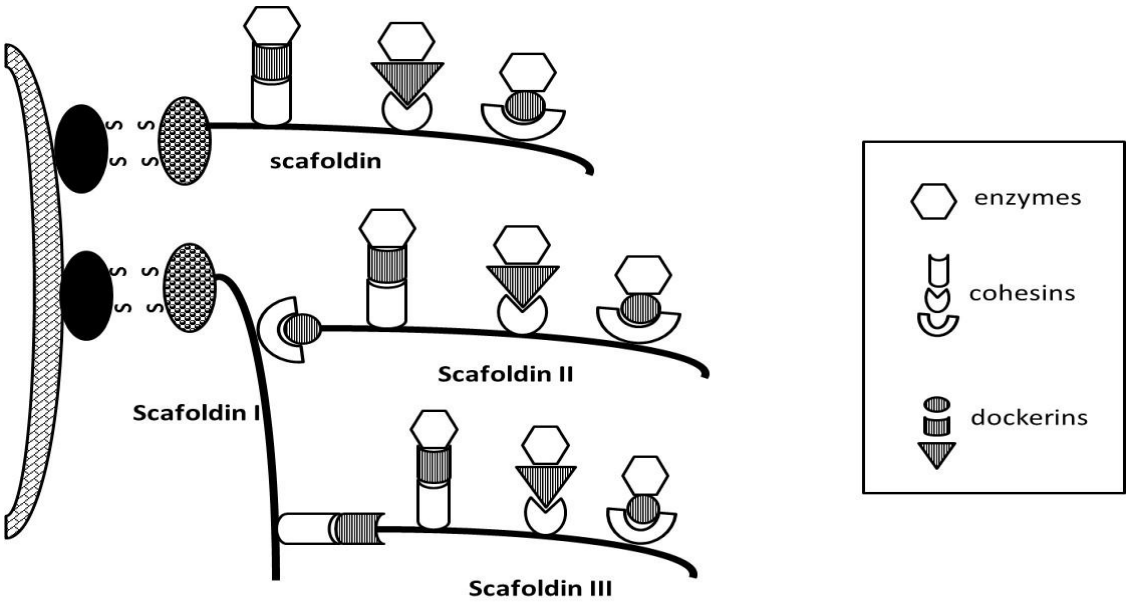

Figure 2. Surface assembly of a functional minicellulosome on the yeast surface. Scafoldin and Scaffoldin I were displayed through **a**-agglutinin display system.

Ito et al. [5] used two pairs of proteins for assembling specifically with each other - Z domain, derived from the *Staphylococcus aureus* protein A, and the Fc part of IgG, and cohesin (Coh) and dockerin (Doc) from the *C. cellulovorans* cellulosome. The scaffolding protein (fusion protein of Z and Coh) was displayed on the cell surface by fusing with the 3' half of α-agglutinin, while the *Trichoderma reesei* EGII and *A. aculeatus* BGL1 were fused with Fc or Doc. The recombinant (EGII-Fc) and BGL1-Doc fusions were secreted from the yeast cell and finally displayed on the cell surface through a scaffolding protein containing two Z and two Coh domains.

Tsai et al. [6] engineered a scaffoldin consisting of three cohesin domains derived from *C. thermocellum*, *C. cellulolyticum* and *Ruminococcus flavefaciens* and displayed it on the *S. cerevisiae* cell surface using the Aga2p as anchor. They also used *E. coli* for expression of an exo-glucanase (CelE) from *C. cellulolyticum* fused to a dockerin domain from the same species, an endo-glucanase (CelG) from *C. cellulolyticum* fused to a dockerin domain from *R. flavefaciens*, and an endo-glucanase (CelA) fused to a dockerin domain from *C. thermocellum*. Cells displaying scaffoldins on the surface were incubated directly with *E. coli* cell lysates containing enzyme-dockerin fusion proteins to form the cellulosome complex. This system demonstrated an up-to-3-fold increase in ethanol production from phosphoric acid-swollen cellulose compared with free enzymes.

Wen et al. [7] constructed a scaffoldin containing *C. thermocellum* cellulose-binding domain and three *C. thermocellum* cohesins, attached to the cell surface of *S. cerevisiae* by α-agglutinin anchor. *C. thermoscellum* dockerins were added to a *T. reesei* Cel5A (EGII) and Cel6A (CBHII), and *A. aculeatus* BGL. The scaffoldin and the three dockerin-containing cellulases were expressed from two episomal plasmids in *S. cerevisiae*. Comparison of the activity of the strains bearing uni-functional minicellulosomes and tri-functional mini-cellulosome showed that unifunctional minicellulosomes exhibited little enzyme synergy while trifunctional mini-cellulosome showed 8.8-fold enhanced activity, which is the result of both enzyme-enzyme synergy and enzyme proximity synergy. However, co-expression of all four components in a single strain resulted in relatively low levels of exo-glucanase and β-glucosidase.

Tsai et al. [60] reported the use of a synthetic yeast consortium composed of one strain displaying the tri-functional mini-scaffoldin attached to the cell surface of *S. cerevisiae* by Aga2p-display system and three strains secreting dockerin-tagged cellulases (endo-glucanase (AT), exo-glucanase (EC/CB), or β-gluco-sidase (BF)). The dockerin-cohesin pairs were from three different species, enabling specific interactions between each dockerin-tagged enzyme

and the displayed mini-scaffoldin, resulting in highly controllable ordering of each enzyme in the mini-cellulosome structure. The consortium consisting of a SC:AT:CB:BF ratio of 7:2:4:2 produced twice the level of ethanol as a consortium with an equal proportion of the different populations.

Since direct ethanol production from cellulose was achieved only using resting-cell cultures, without simultaneous growth and ethanol production, a novel yeast consortium system has been developed, in which four kinds of engineered yeasts are co-cultivated. In this system, construction of the mini-cellulosome was achieved by co-cultivation of yeast displaying the mini-scaffoldin Scaf-ctf, using the constitutive α-agglutinin anchor system, and yeasts secreting three kinds of dockerin-fused enzymes (AT, CB or BF). This synthetic yeast consortium was capable of direct growth and ethanol production [61].

Kim et al. [62] designed a scaffoldin (mini CipA) composed of the same type of cohesins, which enables the assembly of the dockerin-containing enzymes in a random manner. Mini CipA, a modified *C. thermocellum* scaffoldin containing a CBD and three cohesin domains, was expressed as an Aga2p-fusion protein on the surface of yeast. Endo-glucanase CelA from *C. thermocellum*, exo-glucanase CBHII from *Trichoderma reesei*, and β-glucosidase Bgl1 from *A. aculeatus* were secreted using α-factor prepro-peptide. CelA and CBHII were expressed with the native dockerin (DocA) and exogenous dockerin from *C. thermocellum* CelS (DocS), respectively. BGL 1 was expressed without a dockerin domain since it already has a characteristic cell adhesion without any additional anchor systems. The cellulosome activity for ethanol production was optimized by controlling the ratio among the four yeast strains, capable of either displaying the mini-CipA or secreting one of the three enzymes. A mixture of cells with the optimized mini CipA:CelA:CBHII:BGL 1 ratio of 2:3:3:0.53 showed about 20% increase compared with a consortium composed of an equal amount of each cell type.

Natural cellulolytic microorganisms usually have multiple types of scaffoldins, some of which are used for cellulosome construction, and the others for cell surface attachment, so the increase of cohesin units on anchoring scaffoldin raise considerably the cellulosome display level. Fan et al. [67] increased the display level of cellulosomes by construction and expression of two individual mini-scaffoldins-scaffoldin I and scaffoldin II. Scaffoldin I contained a C-terminal type II dockerin (DocII) a cellulose-binding domain (CBD), and three type I cohesins (CohI-1, CohI- 2, and CohI-3). The catalytic units (EG, CBH, and BGL) were cloned from mesophilic *C. cellulolyticum* and each one had a type I dockerin (DocI-1, DocI-2, and DocI-3), which could be docked individually with type I cohesins on the scaffoldin I. DocI-1/CohI-1, DocI-2/CohI-2, and DocI-3/CohI-3 were from *C. cellulovorans*, *C. cellulolyticum* and *C. thermocellum* respectively. The scaffoldin II that contained one to four repeating type II cohesins (CohII) was yeast surface-displayed using Aga2p-display system and served to anchor the whole mini-cellulosome to the yeast cell surface. Scaffoldin I and cellulases were secreted into culture medium by α-factor and assembled extracellularly. The obtained recombinant yeast was capable of producing ethanol from microcrystalline cellulose.

Liang et al. [68] coexpressed lytic polysaccharide mono-oxygenases, CBD, CBH, EG and BGL in the *S. cerevisiae*. Enzymes were secreted and docked onto surface-displayed mini-scaffoldins through cohesin-dockerin interaction to generate penta-functional minicellulosomes. The degradation of phosphoric acid-swollen cellulose and ethanol titers increased to 2.7 g^{-1}. In addition, the recombinant yeast strain was able to grow using phosphoric acid-swollen cellulose as the sole carbon source.

4. Conclusions

Number of papers dealing with yeast strains constructed for surface display of recombinant proteins and their applications in biotechnology, food technology, pharmacology and medicine is ever increasing. Cell-surface display systems must be regarded as safe to be suitable for bio-industrial processes applied to food. Since *S. cerevisiae* has ″GRAS″ status, and is already in use in traditional food preparation and food industry, it is also potentially useful in the development of cell-surface expression systems of enzymes for application in food industry like lipases, proteases, amylases, chitinases and other enzymes that might find application in production of dairy, meat products, alcoholic beverages, fruit juices, etc. Conformation of proteins self-immobilized at the microbial cell surface using standard reactions for incorporation of autochthonous cell surface proteins is usually preserved, and the stability of surface- displayed protein is usually higher than the one of the soluble form [72-74]. The variety of reactions by which a protein can be displayed at the cell surface enables finding the right one for each individual protein. However, efficiency of strains displaying a single recombinant enzyme was shown to be insufficient for industrial application. Therefore, the first attempts to increase efficiency in surface display of recombinant proteins resulted in yeast strains simultaneously co-displaying two or more different enzymes at the cell surface or in creating consortia composed of three or more strains displaying and/or secreting recombineant proteins. Furthermore, strains co-expressing surface- displayed proteins and intracellular heterologous enzymes or protein transporters and expansines were constructed. Finally, synthetic cellulosomes and other scaffolding systems were constructed and displayed on the *S. cerevisiae* cell surface.

In this way strains capable of carrying out complex chemical transformations were constructed.

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6. Conflict of interest

The authors declare no conflict of interests.

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