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ARTICLE TYPE

A highly efficient designer cell for enantioselective reduction of ketones

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A designer cell, surf-crs-gdh coexpressing carbonyl reductase (crs) and glucose dehydrogenase (gdh) on cell surface has been constructed and its enzyme activities compared with corresponding cell, cyto-crs-gdh coexpressing crs and gdh in cytotosol. For various ketones, surf-crs-gdh exhibited 48 to 265-fold higher crs activity per unit protein compared to cyto-crs-gdh.

The cofactor-dependent asymmetric reduction of ketones catalysed by alcohol dehydrogenases represents a valuable method for the synthesis of optically active alcohols.[1,2] Isolated enzymes as well as whole-cell biocatalysts have been used for this purpose. However, the utility of these systems in technical applications has remained limited due to poor catalytic efficiency, especially when compared with well-established metal catalysed asymmetric reductions.[3,4] Recently, genes encoding two enzymes involved in carbonyl reduction have been cooverexpressed in suitable host cells and applications of such "designer cells" have been demonstrated for asymmetric reduction of ketones,[4] α-halo ketones,[5] α-hydroxy ketones,[6] α-ketoesters[7] and reductive amination of α-keto esters.[8] Although, these designer cells perform much better than the natural whole-cell biocatalysts in biotransformations, they still suffers from drawback of lower efficiency due to limits imposed by cellular membrane on substrate/cosubstrate uptake and product/coproduct efflux, which also result in complex kinetics of the overall process.[9]

To overcome this major drawback of designer whole-cell systems, we proposed to express these enzymes on the surface of cell, i.e. freely hanging in the media but firmly anchored to the outer membrane. An enzyme expressed in such a manner is expected to behave like a pure, immobilized enzyme, thereby obviating the need for cost-intensive isolation, purification and stabilization of the enzyme. Moreover, kinetics in such a system is expected to be much simpler because of the fact that substrate uptake and product efflux across cellular membrane is not required for the reaction to occur.

The art of expressing proteins including enzymes on surface of cells is well known and has been used in a wide range of biotechnological and industrial applications like whole-cell biocatalysis, bioadsorbents for the removal of harmful chemicals and heavy metals, screening of human antibody libraries, mutation detection, biosensor development, etc.[10]

We report here a designer cell, coexpressing carbonyl reductase (crs) and glucose dehydrogenase (gdh) firmly anchored to the surface of the *E. coli* cell (Fig. 1). The crs activity per unit

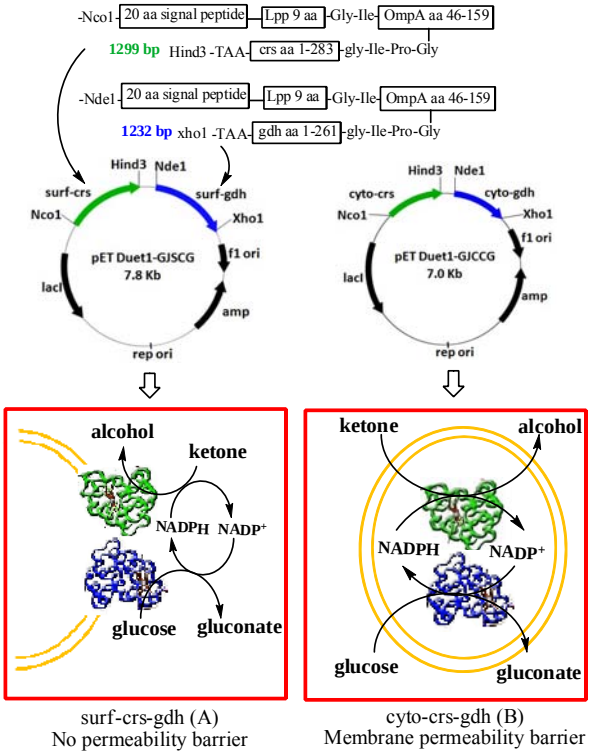


Fig. 1 Schematic representation showing design, construction and advantage of designer cell coexpressing crs and gdh on cell surface over corresponding cell coexpressing crs and gdh in cytosol

protein for the recombinant cells coexpressing crs and gdh on surface was up to 265-fold higher compared to recombinant strain coexpressing crs and gdh intracellularly. Similarly, the activity per unit protein for gdh in recombinant *E. coli* strain coexpressing crs and gdh on surface was 203-fold higher compared to recombinant strain coexpressing crs and gdh intracellularly. The designer cell reduced a variety of aliphatic and aromatic ketones to furnish corresponding alcohol in 95 to >99% ee and 100% conversion.

Surface expression of crs and gdh required designing of non-

natural gene sequences for each protein. The sequence designed based on literature report[11] consisted of (i) N-terminal 20-amino acid signal sequence linked to first nine N-terminal residues of mature *E. coli* lipoprotein (Lpp). The 9-amino acid residue sequence will help anchoring the passenger protein to the outer membrane, (ii) residues 46-159 of *E. coli* outer membrane protein A (OmpA), which is expected to transport the passenger protein fused at its C-terminal across the membrane and (iii) full sequence of crs (or gdh). The 1st 29 aa residue signal + Lpp peptide was linked to 114 aa OmpA residue through Gly-Ile linker, which in turn was attached to N-terminal of crs (or gdh) through Gly-Ile-Pro-Gly. The corresponding *E. coli* strain expressing these proteins in cytoplasm was also constructed for direct comparison of activities.[12] Carbonyl reductase (crs) from *Candida magnoliae* was chosen as enzyme for asymmetric reduction of ketones.[13] Glucose dehydrogenase (gdh) from *Bacillus megaterium* was selected as enzyme of choice for *in situ* cofactor recycling.[14] *E. coli* DH5α and *E. coli* BL21(DE3) were chosen as host-cells for cloning and expression of enzymes, respectively.

Our ultimate aim was to co-express both crs and gdh together on the surface of *E. coli* cells. However, *a priori* it was not possible to predict whether or not the surface expressed crs and gdh would adopt native like confirmation and remain in active form. Therefore, as a first step we expressed crs alone on the surface of the cell to test the feasibility of the proposed study. The recombinant *E. coli* strain harbouring synthetic gene for surface expression of crs has been designated as surf-crs. Corresponding strain harbouring gene for cytoplasmic expression of crs has been designated as cyto-crs. The expression of protein in recombinant strains was confirmed by SDS-PAGE. The surface expression was confirmed by EM immunogold labelling studies (see supplementary information for details).

Being different systems, the levels of crs-protein expressed in surf-crs and cyto-crs may not be similar. To find the quantum of limits imposed by cellular membrane on the efficiency of crs, it was necessary to estimate relative amounts of crs expressed in two strains. The relative expression levels of crs were determined by immuno-enzymatic method as described in supplementary information. Surface expression level of crs was found to be 17.9-fold lower compared to intracellular expression level of this protein. However, recombinant *E. coli* strain expressing crs on surface showed 15.7-fold higher activity for substrate **1a** than recombinant strain expressing crs intracellularly. Thus, activity per unit crs-protein for recombinant strain expressing crs on surface was 275-fold higher compared to recombinant strain expressing crs intracellularly.

The permeability of cellular membrane is expected to vary depending on the structure of the substrate. Therefore, we tested both surf-crs and cyto-crs for reduction of a variety of ketones. The results are summarized in Table 1. As expected, surf-crs was much more efficient than cyto-crs in reduction of all the aliphatic as well as aromatic ketones studied. The increase in activity was in the range of 50 to 275-fold per unit crs-protein.

Next, we compared recombinant strain expressing gdh on the surface with corresponding strain expressing gdh in cytosol. Surface expression level of gdh was found to be 13.8-fold lower compared to intracellular expression level of these proteins (see supplementary information). However, recombinant *E. coli* strain expressing gdh on surface showed 16.3-fold higher activity than recombinant strain expressing crs intracellularly. Thus, activity per unit gdh-protein for recombinant strain expressing crs on surface was 225-fold higher compared to recombinant strain expressing gdh intracellularly.

Table 1 Relative increase in activity of surface expressed crs over cytosol expressed crs for various aliphatic and aromatic ketones

Ketone	surf-crs/cyto-crs		surf-crs-gdh/cyto-crs-gdh	
	Fold increase per unit		Fold increase per unit	
	cell mass	crs-protein[a]	cell mass	crs-protein[a]
1a	12.72	275	14.84	265.5
1b	5.37	96.1	4.84	86.64
1c	3.33	59.6	3.00	53.70
1d	12.25	219.3	11.01	197.01
1e	5.71	102.2	5.23	93.62
1f	2.86	51.2	3.04	54.42
3a	3.37	60.3	3.59	64.25
3b	4.88	87.3	4.56	81.60
3c	5.38	96.3	5.50	98.45
3d	8.96	160.4	8.16	146.06
3e	2.82	50.5	2.67	47.79
3f	3.41	61.0	3.07	54.97
3g	10.06	180.1	9.27	165.93
3h	8.06	144.3	7.42	132.82

[a]crs-protein expression per unit cell mass is 17.9-fold lower in surf-crs strain compared to cyto-crs strain

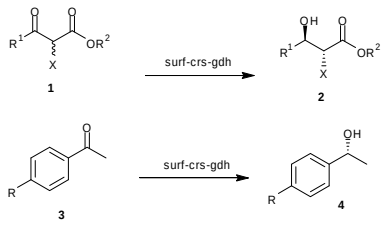
Finally, we constructed recombinant strain coexpressing both crs and gdh on the surface of cells and designated it as surf-crs-gdh. The expression level and activity of enzymes in the surf-crs-gdh strain coexpressing both crs and gdh on surface of cell was compared with cyto-crs-gdh strain coexpressing both crs and gdh in the cytosol of cells. The crs activity per unit crs-protein for surf-crs-gdh strain was 265-fold higher compared to cyto-crs-gdh strain for substrate **1a**. The crs activity per unit crs-protein for substrates **1** and **3** for surf-crs-gdh was 48 to 265-fold higher compared to cyto-crs-gdh (Table 1), which is similar to that observed for surf-crs compared to cyto-crs. The gdh activity was about 203-fold higher per unit gdh-protein in surf-crs-gdh compared to cyto-crs-gdh. Enantiomeric excess and configuration of the products (**2** and **4**) obtained from various ketones with surf-crs-gdh is shown in Table 2.

An important feature from practical point of view is that the concentration of NADPH should never become limiting for efficient conversion of ketones to alcohols. This is possible only when the enzyme responsible for recycling of cofactor has higher activity for $NADP^+$ to NADPH conversion than the enzyme responsible for conversion of ketone to alcohol. Gratefully, the gdh activity was about 1.9-fold higher than crs activity in surf-crs-gdh strain co-expressing both these enzymes, which is sufficient for efficient recycling of cofactor.

We tested the developed surf-crs-gdh biocatalyst for the production of industrially important ethyl (*S*)-4-chloro-3-hydroxybutyrate (**2a**). For industrial scale applications, it is necessary to carry out the reactions at high substrate concentration. However, enzymes in general require aqueous

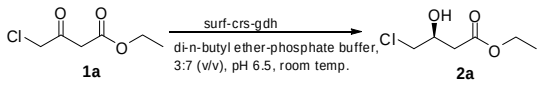
environments in which most organic substrates are poorly soluble. Aqueous-organic biphasic systems have been successfully employed to solve this problem.[12,15] We selected di-n-butyl ether as solvent of choice after screening various short-chain ether and ester solvents. In di-n-butyl ether-aq. phosphate buffer biphasic system, the strain surf-crs-gdh at 20 gL^{-1} cell concentration was able to convert about 165gL^{-1} (1M) of substrate **1a** in 10.5 h (Scheme1). Whereas, under similar conditions cyto-crs-gdh could convert a maximum of 8.25gL^{-1} (0.05M) of substrate **1a.**

Table 2 Designer cell surf-crs-gdh catalysed enatioselective reduction of ketones



Ketone	R_1	R_2	X	Yield[a] %	E.e. %	Conf.
1a	CH_2Cl	CH_2CH_3	H	96	>99	*S*
1b	CH_3	CH_2CH_3	Cl	91	98[b]	*2R,3R*
1c	CH_3	CH_2CH_3	H	89	95	*R*
1d	$(CH_3)CH$	CH_2CH_3	H	92	>99	*S*
1e	CH_2Cl	n-C_8H_{17}	H	88	>99	*S*
1f	CF_3	CH_2CH_3	H	85	>99	*S*
3a	H	CH_2CH_3	H	89	99	*R*
3b	Cl	CH_2CH_3	H	92	99	*R*
3c	Br	CH_2CH_3	H	89	97	*R*
3d	F	CH_2CH_3	H	90	97	*R*
3e	CH_3	CH_2CH_3	H	85	99	*R*
3f	OCH_3	CH_2CH_3	H	87	98	*R*
3g	CF_3	CH_2CH_3	H	94	96	*R*
3h	NO_2	CH_2CH_3	H	95	99	*R*

[a]Yield of isolated product at 100% conversion (see supplementary information for reaction conditions and conversion rates). [b]de 99% (anti)



Substrate Conc.	wet cell mass	time	conversion rate	ee	yield
165gL^{-1}	20 gL^{-1}	10.5 h	4.76	100%	96%

(conversion rate: $mmolh^{-1}g^{-1}$ wet cell mass)

Scheme 1 Designer cell catalysed production of ethyl (S)-4-chloro-3-hydroxybutyrate.

In summary, we have shown that the recombinant *E. coli* strain, surf-crs-gdh coexpressing carbonyl reductase (crs) and glucose dehydrogenase (gdh) on the surface of cell exhibit 48 to 265-fold higher crs activity (depending on the substrate) per unit crs-protein and 203-fold higher gdh activity per unit gdh-protein compared to corresponding *E. coli* coexpressing crs and gdh, within (i.e. cytosol) the cells. Accordingly, recombinant *E. coli* strain surf-crs-gdh may be regarded as highly efficient designer whole-cell biocatalyst for preparation of industrially important chiral alcohols in high enantiomeric purity.

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† Electronic Supplementary Information (ESI) available: expression and immolocalization of crs and gdh on cell surface, experimental protocols, analytical methods, analytical data, plasmid maps, NMR spectra and gene sequence listing. See DOI: 10.1039/b000000x/

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