



Construction of the yeast whole-cell *Rhizopus oryzae* lipase biocatalyst with high activity*

Mei-ling CHEN¹, Qin GUO², Rui-zhi WANG¹, Juan XU¹, Chen-wei ZHOU¹, Hui RUAN^{†‡1}, Guo-qing HE¹

¹Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China

²School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, China

[†]E-mail: ruanhui98@yahoo.com.cn

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Abstract: Surface display is effectively utilized to construct a whole-cell biocatalyst. Codon optimization has been proven to be effective in maximizing production of heterologous proteins in yeast. Here, the cDNA sequence of *Rhizopus oryzae* lipase (ROL) was optimized and synthesized according to the codon bias of *Saccharomyces cerevisiae*, and based on the *Saccharomyces cerevisiae* cell surface display system with α -agglutinin as an anchor, recombinant yeast displaying fully codon-optimized ROL with high activity was successfully constructed. Compared with the wild-type ROL-displaying yeast, the activity of the codon-optimized ROL yeast whole-cell biocatalyst (25 U/g dried cells) was 12.8-fold higher in a hydrolysis reaction using *p*-nitrophenyl palmitate (pNPP) as the substrate. To our knowledge, this was the first attempt to combine the techniques of yeast surface display and codon optimization for whole-cell biocatalyst construction. Consequently, the yeast whole-cell ROL biocatalyst was constructed with high activity. The optimum pH and temperature for the yeast whole-cell ROL biocatalyst were pH 7.0 and 40 °C. Furthermore, this whole-cell biocatalyst was applied to the hydrolysis of tributyrin and the resulted conversion of butyric acid reached 96.91% after 144 h.

Key words: *Rhizopus oryzae* lipase (ROL), Yeast surface display, Codon optimization, Whole-cell biocatalyst
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1 Introduction

Lipases (EC 3.1.1.3) catalyze not only hydrolysis of ester bonds under aqueous conditions but also synthesis of ester bonds under microaqueous conditions, being the biocatalysts with broad use (Zaks and Klivanov, 1988; Klivanov, 2001). Despite being widely spread in several species, microbial lipases have more potential for industrial application than those from other sources due to their high productivity, high catalytic specificity, ability to remain active

in organic solvents, and ease of genetic manipulation (Hasan *et al.*, 2006). Among the microbial lipases, that from *Rhizopus oryzae* is one of the most famous and versatile lipases. There are extensive reports on *Rhizopus oryzae* lipase (ROL) application in bioconversion processes, such as the productions of bio-diesel (Matsumoto *et al.*, 2001; Hama *et al.*, 2004; Tamalampudi *et al.*, 2008), 2-monoacylglycerol (2-MAG) (Kaewthong *et al.*, 2005; Esteban *et al.*, 2009) and 1,3-diacylglycerol (1,3-DAG) (Lo *et al.*, 2004; Kristensen *et al.*, 2005). Thus, constructing ROL biocatalyst with high activity could facilitate its industrial application.

Enzyme-displaying yeast, as the application of the yeast cell surface display system, is one of the most attractive biocatalyst systems for bioconversion processes. Enzymes displayed on yeast as whole-cell biocatalysts have many advantages such as simple

[‡] Corresponding author

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Table 1 Nucleotide sequence comparison between the non-optimized pro and codon-optimized pro sequences of ROL

Pro	Sequence	Length (bp)
Non-optimized_pro	G TTCCTGTTTCTGGTAAATCTGGATCTTCCAACACCGCCGTCTCTGCATC	50
Optimized_pro	----- a ----- g ----- t ----- t ----- t - t - t ----- t - -	50
Non-optimized_pro	TGACAATGCTGCCCTCCCTCCTCTCATCTCCAGCCGTTGTGCTCCTCCTT	100
Optimized_pro	----- c ----- t t - g -- a -- a t - g -- t -- t t c t a - a ----- a -- a -	100
Non-optimized_pro	CTAACAAGGGAAGTAAAAGCGATCTCCAAGCTGAACCTTACAACATGCAA	150
Optimized_pro	----- t t c --- g t c t - - c t - g - ----- a -----	150
Non-optimized_pro	AAGAATACAGAATGGTATGAGTCCCATGGTGGCAACCTGACATCCATCGG	200
Optimized_pro	----- c - t ----- c -- a - t - c ----- t - - t - - t - - t - - t - -	200
Non-optimized_pro	AAAGCGTGATGACAACCTGGTTGGTGGCATGACTTTGGACTTACCCAGCG	250
Optimized_pro	t - - - a - a - - c ----- t ----- g - - a t c t -	250
Non-optimized_pro	ATGCTCCTCTATCAGCCTCTCTAGCTCTACCAACAGCGCC	291
Optimized_pro	- c ----- a - - a - - t t c t t - g - - - g - a - - c - - t - - t c t - - t	291

Table 2 Nucleotide sequence comparison between the non-optimized ROL and codon-optimized ROL sequences

ROL	Sequence	Length (bp)
Non-optimized_ROL	TCTGATGGTGGTAAGGTTGTTGCTGCTACTACTGCTCAGATCCAAGAGTT	50
Optimized_ROL	----- c ----- a - t ----- a - -	50
Non-optimized_ROL	CACCAAGTATGCTGGTATCGCTGCCACTGCCTACTGTCGTTCTGTTGTCC	100
Optimized_ROL	- - - t - - - - c - - - - - t - - - - t - - - - t - - - - a - a - - - - - t -	100
Non-optimized_ROL	CTGGTAACAAGTGGGATTGTGTCCAATGTCAAAAGTGGGTTCTGATGGC	150
Optimized_ROL	- a - - - - - c - - - - - t - - - - - a - - c - - t	150
Non-optimized_ROL	AAGATCATCACTACCTTTACCTCCTTGCTTTCCGATACAAAATGGTTACGT	200
Optimized_ROL	----- t - - t - - - - t - - c - - t - - t - - t - g - - t - - c - - t - - c - - - - -	200
Non-optimized_ROL	CTTGAGAAGTGATAAACAAGACCATTTATCTTGTTTTCCGTGGTACCA	250
Optimized_ROL	t - - - - - t c - - - c - - g - - - - - t - - - - - c t - g - - - - - a - a - - - - t -	250
Non-optimized_ROL	ACTCCTTCAGAAGTGCCATCACTGATATCGTCTTCAACTTTTCTGACTAC	300
Optimized_ROL	- - - - t - - - - - t c - - - t - - t - - - - c - - t - - t - - - - - c - - - - -	300
Non-optimized_ROL	AAGCCTGTCAAGGGCGCCAAAGTTCATGCTGGTTTCCTTTCTCTTATGA	350
Optimized_ROL	----- a - t - - - - t - - t - - g - - - - c - - - - - t - g - - t - - - c - -	350
Non-optimized_ROL	GCAAGTTGTCAATGACTATTTCCCTGTCGTCCAAGAACAATTGACCGCCC	400
Optimized_ROL	a - - - - - t - - c - - - - c - - - - a - - t - - t - - - - - t - - t -	400
Non-optimized_ROL	ACCCTACTTATAAGGTCATCGTTACCGGTCCTCACTCGGTGGTGCACAA	450
Optimized_ROL	- - - - a - - - - - c - - - - t - - t - - - - t - - - - - t t - g - - - - - t - - -	450
Non-optimized_ROL	GCTTTGCTTGCCGGTATGGATCTCTACCAACGTGAACCAAGATTGTCTCC	500
Optimized_ROL	----- t - g - - t - - - - - c t - g - - - - a - a - - - - -	500
Non-optimized_ROL	CAAGAATTTGAGCATCTTCACTGTCGGTGGTCTCGTGTGGTAACCCCA	550
Optimized_ROL	a - - - - - c - - - t c t - - t - - - - - t - - - - - a a - a - - - - - a -	550
Non-optimized_ROL	CCTTTGCTTACTATGTTGAATCCACCGGTATCCCTTTCCAACGTACCGTT	600
Optimized_ROL	- t - c - - - - - c - - - - - t - - t - - - - t - - a - - - - - a - a - t - - -	600
Non-optimized_ROL	CACAAGAGAGATATCGTTCCTCACGTTCCCTCCTCAATCCTTCGGATTCCT	650
Optimized_ROL	----- c - - t - - - - a - - - - - a - a - - - - t - - - - t - - t -	650
Non-optimized_ROL	TCATCCCAGGTTGAATCTTGATCAAGTCTGGTACTTCCAACGTTCAAA	700
Optimized_ROL	g - - - c - - a - - - - - t - - - - - t - - - - -	700
Non-optimized_ROL	TCTGTACTTCTGAAATTGAAACCAAGGATTGCAGTAACTCTATCGTTCTCCT	750
Optimized_ROL	- t - - - - - t - - - - - t - - - - - c - - t t c - - - - - t - - - - - a	750
Non-optimized_ROL	TTCACCTCTATCCTTGACCACTTGAGTTACTTTGATATCAACGAAGGAAG	800
Optimized_ROL	----- t - - - - - t t - g - - - - - t c - - - - - c - - c - - t - - - - - t t c	800
Non-optimized_ROL	CTGTTTG	807
Optimized_ROL	t - - - c - -	807

were amplified by PCR using the *Saccharomyces cerevisiae* MS-1 genomic DNA as the template, and the *kanMX* gene was amplified from the plasmid pUG6 (Guldener *et al.*, 1996), with recognition sites of relevant restriction enzymes added to the primers in 5'-ends and 3'-ends, respectively. The resulting plasmid was first amplified in *Escherichia coli* and then extracted and transformed into the *Saccharomyces cerevisiae* MS-1 by electroporation (Thompson *et al.*, 1998).

2.4 Screening by halo assay

Transformants were spread on YGCG plate-medium containing 0.2% tributyrin as a substrate. Colonies hydrolyzing tributyrin were identified by the formation of clear halos. *Saccharomyces cerevisiae* MS-1 harboring pGMRAK (wild ROL) and pGMRAK (optimized ROL) and *Saccharomyces cerevisiae* MS-1 harboring pGMAK (MS-1/pGMAK) as the control were spread on the medium, respectively. The activities of the lipases were examined by the halo formed around the colony.

2.5 Preparation of ROL-displaying yeast

For whole-cell catalyst preparation, the wild-type ROL and codon-optimized ROL-displaying yeasts were cultivated in YGCG medium at 30 °C for 120 h (stationary phase), respectively. After cultivation, the cells were collected by centrifugation (3000×g, 15 min), washed with distilled water and 50 mmol/L phosphate buffered solution (PBS; pH 7.0), and then lyophilized for 24 h by a freeze-drying system.

2.6 Measurement of lipase activity in yeast

The hydrolysis activities of the codon-optimized ROL and the wild-type ROL displayed on yeast cell surface were measured with *p*-nitrophenyl palmitate (pNPP) as the substrate (Prim *et al.*, 2000). The assay was conducted in aqueous medium with 1 ml 50 mmol/L PBS (pH 7.0) containing 0.03% (v/v) pNPP, 4 g/L Triton-X, and 1 g/L arabic gum. Reaction was initiated by the addition of 100 µl 1 mg/ml lyophilized ROL-displaying yeast cells and was incubated at 30 °C for 15 min with agitation (150 r/min). After the reaction was complete, 200 µl aliquot of the reaction solution was placed in a 96-well plate to measure the absorbance value of *p*-nitrophenyl (pNP) released at 410 nm in a microplate reader Multiskan

Asent (Labsystems). One unit of hydrolysis activity was defined as the amount of enzyme that released 1 µmol of pNP per minute under the assay conditions described ($n=3$, relative standard deviation (RSD) was estimated less than 10%).

2.7 Optimum temperature and pH

The optimum temperature for the codon-optimized ROL on yeast cell surface was determined by measuring the hydrolysis activity at pH 7.0 in a water bath at temperatures ranging from 30 to 50 °C. The optimum pH was determined at 30 °C with pH buffer solutions ranging from 6.0 to 8.0. The hydrolysis activity was measured at 30 °C and pH 7.0, using pNPP as substrate.

2.8 Thermostability assay

The thermostability of the codon-optimized ROL on yeast cell surface was measured as follows: the whole-cell biocatalyst was washed twice with distilled water after centrifugation and resuspended in 50 mmol/L PBS (pH 7.0). The suspension was then incubated at 40, 50 and 60 °C for 2–6 h, respectively. Aliquots of the cell suspensions were taken out and chilled immediately on ice at regular intervals. The residual hydrolysis activity was measured at 30 °C and pH 7.0, using pNPP as substrate.

2.9 Application of the whole-cell biocatalyst

The codon-optimized ROL-displaying yeast was cultivated in 150 ml YGCG broth medium containing 20 g/L galactose as the inducer and 1% (v/v) tributyrin as the substrate. Aliquots of 1 ml were taken from the reaction mixture every 8 h. The amount of butyric acid produced by hydrolysis reaction was measured by high-performance liquid chromatography (HPLC), in an Agilent 1200 series Rapid Resolution LC System (Agilent Technologies, Palo Alto, CA). The analyses were performed isocratically at 1.2 ml/min and 40 °C with a rapid resolution high throughput (RRHT) column (Zorbax SB-C₁₈, 6×10⁷ Pa, 4.6 mm×100 mm, 1.8 µm particle size) equipped with a 0.2-µm pore size inline filter. The mobile phase was: 80% of solvent A consisting of 25 mmol/L NaH₂PO₄ with 0.05% (v/v) trifluoroacetic acid (TFA) and 1.5% (v/v) methanol, adjusted to pH 2.31, plus 20% of solvent B consisting of methanol. The retention time for butyric acid was 5.85 min.

3 Results

3.1 Comparison of wild-type ROL and codon-optimized ROL genes

As the rare codons were replaced, a fully codon-optimized pro-ROL gene has been constructed. A sequence comparison between the codon-optimized sequence and wild-type one was shown in Tables 1 and 2. Compared to the non-optimized sequence, the fully codon-optimized gene had 217 nucleotide variations in all 1098 nucleotides, resulting in 178 changes out of 366 codons. Further study demonstrated that the overall G+C content of codon-optimized pro-ROL sequence was reduced to 41.53% from 46.81% of the non-optimized sequence.

3.2 Expression of the recombinant gene

A clear halo was observed around the colonies of codon-optimized ROL-displaying yeast on plate medium containing tributyrin as substrate, while the wild-type ROL-displaying yeast showed a smaller one, but none for the control yeast (Fig. 2). The result indicates that the ROL- α -agglutinin fusion protein was expressed in an active form, and the hydrolysis activity of ROL was significantly improved by codon-optimization.

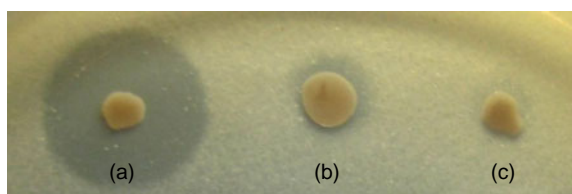


Fig. 2 Halo formation of ROL-displaying yeasts cultivated for 144 h

Transformants were spread on YGCG solid medium containing 2 g/L tributyrin as substrate.

(a) MS-1/pGMRAK (codon-optimized ROL); (b) MS-1/pGMRAK (wild ROL); (c) MS-1/pGMAK (control)

3.3 Lipase activity and characteristics

According to the time curve of biomass of the recombinant MS-1/pGMRAK, the cells reached stationary phase after 120 h cultivation (data not shown). The hydrolysis activity of the codon-optimized ROL displayed on the yeast cell surface was 25 U/g dried cells in the stationary phase, while that of the non-optimized one was 1.96 U/g dried cells. In addition, the activity of the cell-free supernatant was

negligible throughout the entire cultivation. These results confirmed that the ROL- α -agglutinin fusion protein was not secreted into the culture medium but immobilized on the yeast cells firmly as a whole-cell biocatalyst.

According to the measurement conducted, the optimum pH and temperature for the fully codon-optimized whole-cell biocatalyst were pH 7.0 and 40 °C. As shown in Fig. 3, after 2-h incubation at 40 and 50 °C, the residual activity increased to 107% and 113%, respectively. After 6-h incubation at 40 and 50 °C, the residual activity still remained 72% and 50%, respectively. However, it reduced to 13% after 4-h incubation at 60 °C. After 6-h incubation at 60 °C, cell debris was observed in the sample, implicating that yeast cells were fractured after the heating process. As shown in Fig. 4, the fully codon-optimized

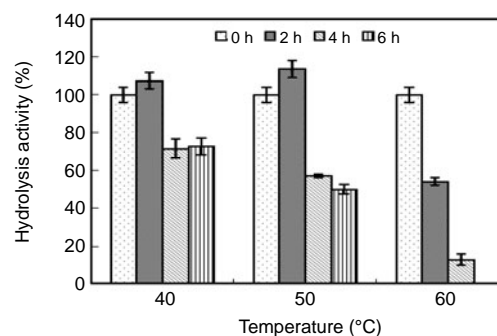


Fig. 3 Thermostability of MS-1/pGMRAK

The cells suspension was incubated at 40, 50 and 60 °C for 2–6 h, the residual hydrolysis activity of the lipase was measured by using pNPP as substrate at 30 °C and pH 7.0. Residual activity was denoted by taking the initial value at 30 °C as 100% ($n=3$)

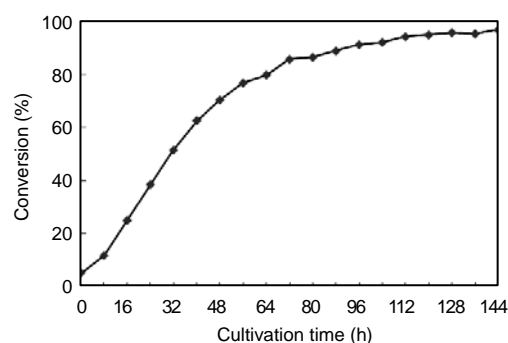


Fig. 4 Time courses of butyric acid content in the tributyrin hydrolysis reaction

The amount of butyric acid produced by hydrolysis reaction was measured by HPLC. The conversion of butyric acid reached 96.91% after 144 h

whole-cell biocatalyst was applied to tributyrin hydrolysis reaction and the conversion of butyric acid reached 96.91% after 144 h.

4 Discussion

Codon usage could play a key role in regulating gene expression, because rare codons may interrupt mRNA stability and its translation rate; moreover, high G+C content may decrease the translational efficiency or even result in failed expression (Sinclair and Choy, 2002). In this study, the satisfying expression of the modified ROL gene in *Saccharomyces cerevisiae* confirmed that the codon optimization according to the favored codon usage of *Saccharomyces cerevisiae* had a positive impact on lipase heterologous expression. As shown in the results, the hydrolysis activity of the displayed codon-optimized ROL was 25 U/g dried cells, 12.8-fold higher than that of the wild-type one, which indicates that ROL displayed in high efficiency via codon optimization. To our knowledge, this was the first example to combine the techniques of yeast surface display and codon optimization, and consequently the fully codon-optimized ROL-displaying yeast as a whole-cell biocatalyst was constructed, with much higher enzymatic activity in the *Saccharomyces cerevisiae* cell surface display systems using α -agglutinin as an anchor. We could infer that the high activity of the yeast whole-cell ROL biocatalyst is attributed to the combination of the two techniques, thus leading to highly efficient ROL display on cell surface, because the codon usage is a key factor for the mRNA translation rate and so for protein synthesis efficiency in cells (Proshkin *et al.*, 2010).

In conclusion, the strategy of combining the techniques of cell surface display and codon optimization is successfully developed, thus the yeast whole-cell ROL biocatalyst with high activity was constructed, which could be of great potential in bioconversion processes.

References

- Andersson, S.G.E., Kurland, C.G., 1990. Codon preferences in free-living microorganisms. *Microbiol. Rev.*, **54**(2): 198-210.
- Bucarey, S.A., Noriega, J., Reyes, P., Tapia, C., Saenz, L., Zuniga, A., Tobar, J.A., 2009. The optimized capsid gene of porcine circovirus type 2 expressed in yeast forms virus-like particles and elicits antibody responses in mice fed with recombinant yeast extracts. *Vaccine*, **27**(42): 5781-5790. [doi:10.1016/j.vaccine.2009.07.061]
- Das, S., Roymondal, U., Sahoo, S., 2009. Analyzing gene expression from relative codon usage bias in yeast genome: a statistical significance and biological relevance. *Gene*, **443**(1-2):121-131. [doi:10.1016/j.gene.2009.04.022]
- Esteban, L., Munio, M.D., Robles, A., Hita, E., Jimenez, M.J., Gonzalez, P.A., Camacho, B., Molina, E., 2009. Synthesis of 2-monoacylglycerols (2-MAG) by enzymatic alcoholysis of fish oils using different reactor types. *Biochem. Eng. J.*, **44**(2-3):271-279. [doi:10.1016/j.bej.2009.01.004]
- Guldener, U., Heck, S., Fiedler, T., Beinbauer, J., Hegemann, J.H., 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.*, **24**(13): 2519-2524. [doi:10.1093/nar/24.13.2519]
- Guo, Q., Zhang, W., Ma, L.L., Chen, Q.H., Chen, J.C., Zhang, H.B., Ruan, H., He, G.Q., 2010. A food-grade industrial arming yeast expressing β -1,3-1,4-glucanase with enhanced thermal stability. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **11**(1):41-51. [doi:10.1631/jzus. B0900185]
- Hama, S., Yamaji, H., Kaieda, M., Oda, M., Kondo, A., Fukuda, H., 2004. Effect of fatty acid membrane composition on whole-cell biocatalysts for biodiesel-fuel production. *Biochem. Eng. J.*, **21**(2):155-160. [doi:10.1016/j.bej.2004.05.009]
- Hasan, F., Shah, A.A., Hameed, A., 2006. Industrial applications of microbial lipases. *Enzyme Microb. Technol.*, **39**(2):235-251. [doi:10.1016/j.enzmictec.2005.10.016]
- Ikemura, T., 1985. Codon usage and transfer-RNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.*, **2**(1):13-34.
- Kaewthong, W., Sirisansaneeyakul, S., Prasertsan, P., H-Kittikun, A., 2005. Continuous production of monoacylglycerols by glycerolysis of palm olein with immobilized lipase. *Process Biochem.*, **40**(5):1525-1530. [doi:10.1016/j.procbio.2003.12.002]
- Kato, M., Fuchimoto, J., Tanino, T., Kondo, A., Fukuda, H., Ueda, M., 2007. Preparation of a whole-cell biocatalyst of mutated *Candida antarctica* lipase B (mCALB) by a yeast molecular display system and its practical properties. *Appl. Microbiol. Biotechnol.*, **75**(3):549-555. [doi:10.1007/s00253-006-0835-2]
- Klibanov, A.M., 2001. Improving enzymes by using them in organic solvents. *Nature*, **409**(6817):241-246. [doi:10.1038/35051719]
- Kondo, A., Ueda, M., 2004. Yeast cell-surface display—applications of molecular display. *Appl. Microbiol. Biotechnol.*, **64**(1):28-40. [doi:10.1007/s00253-003-1492-3]
- Kristensen, J.B., Xu, X.B., Mu, H.L., 2005. Diacylglycerol synthesis by enzymatic glycerolysis: screening of commercially available lipases. *J. Am. Oil Chem. Soc.*, **82**(5): 329-334. [doi:10.1007/s11746-005-1074-5]
- Kurland, C.G., 1991. Codon bias and gene-expression. *FEBS Lett.*, **285**(2):165-169. [doi:10.1016/0014-5793(91)80797-7]

- Lithwick, G., Margalit, H., 2003. Hierarchy of sequence-dependent features associated with prokaryotic translation. *Genome Res.*, **13**(12):2665-2673. [doi:10.1101/gr.1485203]
- Lo, S.K., Baharin, B.S., Tan, C.P., Lai, O.M., 2004. Lipase-catalysed production and chemical composition of diacylglycerols from soybean oil deodoriser distillate. *Eur. J. Lipid Sci. Technol.*, **106**(4):218-224. [doi:10.1002/ejlt.200300888]
- Matsumoto, T., Takahashi, S., Kaieda, M., Ueda, M., Tanaka, A., Fukuda, H., Kondo, A., 2001. Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel production. *Appl. Microbiol. Biotechnol.*, **57**(4):515-520.
- Murai, T., Ueda, M., Shibasaki, Y., Kamasawa, N., Osumi, M., Imanaka, T., Tanaka, A., 1999. Development of an arming yeast strain for efficient utilization of starch by co-display of sequential amylolytic enzymes on the cell surface. *Appl. Microbiol. Biotechnol.*, **51**(1):65-70. [doi:10.1007/s002530051364]
- Prim, N., Blanco, A., Martinez, J., Pastor, F.I.J., Diaz, P., 2000. *estA*, a gene coding for a cell-bound esterase from *Pae-nibacillus* sp. BP-23, is a new member of the bacterial subclass of type B carboxylesterases. *Res. Microbiol.*, **151**(4):303-312. [doi:10.1016/S0923-2508(00)00150-9]
- Proshkin, S., Rahmouni, A.R., Mironov, A., Nudler, E., 2010. Cooperation between translating ribosomes and RNA polymerase in transcription elongation. *Science*, **328**(5977):504-508. [doi:10.1126/science.1184939]
- Shibasaki, S., Maema, H., Ueda, M., 2009. Molecular display technology using yeast-arming technology. *Anal. Sci.*, **25**(1):41-49.
- Sinclair, G., Choy, F.Y.M., 2002. Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. *Protein Expr. Purif.*, **26**(1):96-105. [doi:10.1016/S1046-5928(02)00526-0]
- Tamalampudi, S., Talukder, M.R., Hama, S., Numata, T., Kondo, A., Fukuda, H., 2008. Enzymatic production of biodiesel from *Jatropha* oil: a comparative study of immobilized-whole cell and commercial lipases as a biocatalyst. *Biochem. Eng. J.*, **39**(1):185-189. [doi:10.1016/j.bej.2007.09.002]
- Thompson, J.R., Register, E., Curotto, J., Kurtz, M., Kelly, R., 1998. An improved protocol for the preparation of yeast cells for transformation by electroporation. *Yeast*, **14**(6):565-571. [doi:10.1002/(SICI)1097-0061(19980430)14:6<565::AID-YEA251>3.0.CO;2-B]
- Washida, M., Takahashi, S., Ueda, M., Tanaka, A., 2001. Spacer-mediated display of active lipase on the yeast cell surface. *Appl. Microbiol. Biotechnol.*, **56**(5-6):681-686. [doi:10.1007/s002530100718]
- Zaks, A., Klivanov, A.M., 1988. Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.*, **263**(7):3194-3201.