Title
EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into Saccharomyces cerevisiae via CRISPR-Cas9.

Permalink
https://escholarship.org/uc/item/1xc9v9rk

Journal
Biotechnology journal, 11(8)

ISSN
1860-6768

Authors
Jessop-Fabre, Mathew M
Jakočiūnas, Tadas
Stovícek, Vratislav
et al.

Publication Date
2016-08-01

DOI
10.1002/biot.201600147

Peer reviewed
Introduction

The yeast *S. cerevisiae* is widely applied in industrial biotechnology for production of fuels, chemicals, and pharmaceutical ingredients and it is also used for fundamental research as a eukaryotic model organism [1–3]. Genetic manipulation of *S. cerevisiae* is greatly facilitated by its efficient inherent homologous recombination (HR) machinery for DNA double-strand break (DSB) repair [4]. As a result, many genome engineering tools have been developed.
opened that take advantage of HR for targeted integration of heterologous DNA into the yeast genome [5, 6]. Chromosomal gene integration has several benefits over plasmid-based approaches, such as increased strain stability, better control of gene expression level and lower population heterogeneity [5]. However, though the HR machinery has a high fidelity, selection is still necessary to identify the colonies that have been successfully engineered. This has the fundamental drawback that selection markers need to be recycled if multiple genome edits are needed. Also, both dominant and auxotrophic markers have been reported to have an effect on cell physiology [7, 8]. Moreover, antibiotic-based selection markers are undesirable in industrial strains due to the risk of spreading drug resistance. A way to improve homology-directed chromosomal integration of heterologous gene fragments is by using DNA endonucleases for targeted DSB as this has been shown to increase recombination events by approximately three orders of magnitude [9–12]. Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Type II system from bacteria has been adopted for genetic engineering of yeast [13]. This system consists of two components: guide RNA (gRNA) and CRISPR-associated endonuclease (Cas9). Upon expression in the cell, the gRNA/Cas9 complex is recruited to DNA by base-pairing of the gRNA recognition sequence (typically 20 bp) and the complementary DNA strand, after which the Cas9 introduces a DSB break. The target DNA sequence must be immediately followed by a protospacer adjacent motif (PAM) (i.e. NGG for Streptococcus pyogenes-derived Cas9); Cas9 makes a blunt cut three nucleotides upstream of the PAM site [14]. There have been many recently published examples of CRISPR-Cas9-based tools for yeast genome engineering [15–21] that are reviewed in Jakociūnas et al. [22]. The work presented here builds on the previously reported method EasyClone for single-step targeted integration of multiple expression cassettes [5, 6]. The EasyClone method allows targeted genomic integration of up to three vectors in a single transformation event using auxotrophic or dominant selection markers. These target sites are located in the intergenic regions and are interspersed by essential genes to ensure that the integrated DNA fragments are not at risk of being removed by HR [23]. Moreover it has been confirmed that integration into these sites ensures a high-level of expression and does not interfere with cellular growth [23]. The selection markers can be removed via Cre-LoxP-mediated recombination, which requires one extra transformation with CreA-vector, cultivation to induce the CreA expression and finally screening of the resulting clones on selection plates to confirm the loss of the marker; the marker removal procedure has a turnaround time of about five days. Once the selection markers are eliminated, the strain is ready for the next integration event or for other genetic modifications. Marker removal, as well as being time consuming, can also cause genome instability [24, 25].

Here we present the EasyClone-MarkerFree method, which takes advantage of CRISPR-Cas9 for introduction of double-strand DNA breaks in the defined integration sites, which causes very efficient integration of expression cassettes and thus bypasses the need for selection. The gRNA helper vectors, expressing gRNA molecules that recruit Cas9 to the particular chromosomal locations, can be easily removed from the strain by growth on non-selective medium, after which the strain is ready for the next modification. This shortens the turnaround time to only one or two days. Additional advantages are: simplified experimental design for cloning, because the choice of selection markers does not need to be taken into consideration; elimination of the effect of the selection markers on strain physiology; and moreover a possibility to iterate gene integrations with other edits guided by CRISPR/Cas9, e.g. gene deletions. The use of predefined and characterized chromosomal target sites dispenses with the need for the experimenter to identify suitable regions in which to integrate their genes of interest. Furthermore, we demonstrate the utility of the method by engineering 3-hydroxypropionic acid producing strains with improved precursor supply strategies.

2 Materials and methods

2.1 Strains

Two different strains of S. cerevisiae were used: the haploid laboratory strain CEN.PK113-7D (MATα URA3 HIS3 LEU2 TRP1 MAL2-8 c SUC2, obtained from Peter Köttler, Johann Wolfgang Goethe University Frankfurt, Germany), and the diploid industrial Ethanol Red strain (MATαα, obtained from Fermentis, A Lesaffre division). E. coli strain DH5α alpha was used to clone, propagate, and store the plasmids.

2.2 Construction of plasmids

The codon-optimized genes, encoding the pyruvate dehydrogenase complex and lipote-protein ligase from Enterococcus faecalis, were ordered from GenScript (sequences can be found in Supporting information, Table S5). The genes encoding ATP-dependent citrate lyase and the mitochondrial citrate transport protein, were amplified from the genomic DNA of Yarrowia lipolytica DSM-8218, obtained from the DSMZ collection (www.dsmz.de). All of the primers, biobricks and plasmids constructed and used in this study can be found in Supporting information, Tables S1–S3.

The EasyClone-MarkerFree vectors were created by amplifying the EasyClone 2.0 vectors [6] with primers that were designed to attach to either side of the selection markers, creating a fragment that no longer contained the marker. These fragments were then ligated to form the
marker-less vectors. Seven of the resulting vectors (named “Intermediate vectors” in Table S3) contained PAM sites in the integration regions, which were removed by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturers’ protocol.

gRNA cassettes targeting particular integration loci (chromosomal coordinates can be found in Supporting information, Table S4) were ordered as double-stranded gene blocks from IDT DNA. These cassettes were amplified using primers 10525(TJOS-62 [P1F]) and 10529(TJOS-65 [P1R]) and USER-cloned into pCIB2926 (pTAJAK-71) [15] to give single gRNA helper vectors. For construction of triple gRNA helper vectors, three gRNA cassettes were amplified using three primer pairs (10526[TJOS-62 [P1F]) and 10530(TJOS-66 [P2F]) for the first, 10526(TJOS-63 [P2F]) and 10531(TJOS-67 [P3R]) for the second, and 10527(TJOS-64 [P3F]) and 10529(TJOS-65 [P1R]) for the third gRNA cassette) and cloned into pCIB2926 (p-TAJAK-71) [15]. Single gRNA helper vectors for Ethanol Red were constructed by PCR amplification of the template plasmid pCIB3041 using primers indicated in Supporting information, Table S1 as described in [17]. All of the cloning steps for creating gRNA helper vectors and EasyClone-MarkerFree vectors were performed in E. coli. Correct cloning was confirmed by Sanger sequencing.

For expression of the Cas9 gene we used an episomal vector pCIB2312 with CEN-ARS replicon and KanMX resistance marker [17].

The EasyClone-MarkerFree vectors for expression of fluorescent protein or 3HP pathway genes were cloned as described in [5, 26]. The vectors were linearized with NotI, the integration fragment (part of the expression vector without E. coli ori and AmpR) was gel-purified and transformed, along with a gRNA helper vector, into yeast carrying the Cas9 plasmid (pCIB2312) via the lithium acetate method [27]. After the heat shock the cells were recovered for two hours in YPD medium and then plated on YPD agar containing 200 mg/L G418 and 100 mg/L nourseothricin. For yeast transformations with a single vector we routinely use 500 ng of the linear integration fragment along with 500 ng of the relevant gRNA helper plasmid. For yeast transformations with three vectors we use 1 µg of linear integration fragments and 1 µg of triple gRNA helper plasmid. Correct integration of the vectors into the genome was verified by colony PCR using primers listed in Supporting information, Table S1.

2.3 Media and growth conditions

The E. coli strains containing the plasmids were grown in Luria–Bertani (LB) media supplemented with 100 µg/mL ampicillin. Yeast strains were grown in liquid YPD media, or on YPD agar plates with 20 g/L glucose (Sigma–Aldrich). When needed the medium was supplemented with antibiotics G418 (200 mg/L) for selection of the Cas9 plasmid, and/or nourseothricin (100 mg/L) for selection of the gRNA plasmid. Synthetic complete medium with 20 g/L glucose was prepared using drop-out powder from Sigma–Aldrich.

The strains were screened for production of 3HP as following. Single colonies were grown in 500 µL synthetic complete media in 96 deep-well plates with air-penetrable lids (EnzyScreen, Germany) at 30°C with 250 rpm shaking overnight. The overnight cultures were then inoculated into 50 mL of either mineral media (MM) with 60 g/L glucose [5], or a simulated fed-batch media (feed-in-time, FIT) in 500 mL-shake flasks with baffles to a starting OD600 of 0.1. The feed-in-time (FIT) medium M-Sc. syn-1000 synthetic FIT was purchased from M2P labs GmbH (Germany). All the media for 3HP cultivations were supplemented with 500 ng/mL of DL-α-lipoic acid (Sigma Aldrich). The strains were cultivated in MM or FIT media at 30°C with 250 rpm for 168 h. Samples were taken every 12 h (every 1–2 h during the exponential phase in MM), with the optical density OD600 being measured on a 20-fold diluted culture broth. Samples were then centrifuged at 3000 xg for 30 min and the supernatant was stored at -20°C until HPLC analysis. Cultivations were carried out in triplicate.

2.4 Analytical procedures

Strains expressing gfp were analyzed using a BioTek Synergy MX microplate reader. Excitation wavelength was set at 485 nm, and emission was read at 530 nm. Optical density was measured at 600 nm. Concentration of 3-hydroxypropionic acid in the broth was measured by HPLC, using a method previously reported in [28].

2.5 Integration Stability

Strains that had been transformed with gfp-containing vectors were grown in 500 µL of YPD in 96 deep-well plates with air-penetrable lids at 30°C with 250 rpm shaking overnight. The following morning, 50 µL of each culture was passed to 500 µL of fresh YPD. This was repeated for a total of five passages. At the end of the passages, the culture was plated onto YPD agar, and the fluorescent colonies were counted.

2.6 Determination of gene copy number by qPCR

Quantitative PCR was performed on the Ethanol Red strain containing the genes from Enterococcus faecalis. Primers were designed using the PrimerQuest® tool (https://eu.idtdna.com/Primerquest/Home/Index). Details of the primers used can be found in Supporting information, Table S1. Genomic DNA was prepared using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research) according to the manufacturer’s protocol. Each reaction contained 10 µL of 2xSYBR Green qPCR MasterMix (Life
order to obtain vector backbones with sticky ends. The 
Bsm by linearization with SI and nicking with Nb.
cloning, the EasyClone-MarkerFree vectors are prepared
The suggested workflow is illustrated on Fig. 1. For USER
to remove the selection markers and, where necessary, to
3.2 Workflow
The suggested workflow is illustrated on Fig. 1. For USER
to remove the selection markers and, where necessary, to
Figure 1. An overview of the EasyClone-MarkerFree method. The BioBricks encoding genes and promoters are generated by PCR-amplification with uracil-containing primers (a). The BioBricks are assembled with the integration vectors via USER cloning (b) and the reaction mixture is transformed into *E. coli* (c). The resulting plasmids are isolated from *E. coli* and confirmed by sequencing, then they are linearized and transformed into Cas9-expressing *S. cerevisiae* along with helper gRNA vector, which causes double-stranded breaks at the designated integration sites (d–e). Yeast cells are selected on plates with G418 and nourseothricin. The linear integration fragments along with gRNA helper vector are transformed into *S. cerevisiae* expressing Cas9. The transformants are selected on plates with G418 and nourseothricin. (f) The gRNA helper vector is removed by growing the cells on non-selective medium. The strain is now ready for a new round of genetic modifications.

**G**, gene; **P**, promoter; **T**, terminator; **H**, homology region. A detailed protocol is provided in Supporting information.
their genomes, while for the EasyClone system with selection markers, the integration cassette would always be integrated in the genome, however sometimes it would not be targeted to the designed integration site. Further, the transformed colonies were each checked for fluorescence, with 99% of the colonies showing fluorescence (84–559 colonies were obtained and tested per transformation). Fluorescence levels showed some smaller variations for different integration sites (Fig. 2) as we also observed previously [6]. Another observation was that among those clones with an integrated fragment, all clones were fluorescent, which differs from the results we obtained with CRISPR-Cas9-mediated insertion of expression fragments with short homology arms (40 bp), where only half of the clones were fluorescent [17]. We suggest that the high fidelity of correct integration is due to the long homology arms of ~0.5 kb present in the EasyClone vectors.

We further tested the stability of the integrations of the gfp gene in each of the sites. After five passages, the population retained 100% of fluorescent colonies, reinforcing the notion that these sites are stable due to their location in-between essential genes.

The simultaneous targeting of three integration sites, assisted with triple gRNA helper vectors, resulted in 60–70% targeting efficiencies, which is also comparable with previously reported efficiencies for CRISPR-Cas9 methods for multi-loci integration [16, 18]. We previously observed for the EasyClone system with selection markers that targeting efficiency into the same chromosome was only 44%, however the efficiency is higher for simultaneous integration into different chromosomes (unpublished results). We believe that simultaneous targeting of three sites that are closely positioned on the same chromosome, may lead to genome instability. Therefore to achieve the best targeting efficiencies, triple gRNA helper vectors were designed to target distinct chromosomes.

### 3.4 Application of EasyClone-MarkerFree vector toolkit for engineering of precursor supply for 3-hydroxypropionic acid production

As a test case, a pathway to produce 3HP was inserted into two different S. cerevisiae strains, CEN.PK113-7D and ethanol Red, and the strains were further engineered for improved acetyl-CoA supply. The basic pathway strains were constructed by inserting a modified acetyl-CoA carboxylase ACC1<sup>966A,S1157A</sup> and a malonyl-CoA reductase MCR from Chloroferoxs auranticus. Three different strategies to boost acetyl-CoA production in the cytoplasm were compared (Fig. 3A). One of the strategies relied on expression of a functional bacterial pyruvate dehydrogenase complex from Enterococcus faecalis in the cytoplasm, which required expression of genes encoding subunits E1α, E1β, E2, and E3 of PDH complex, as well as two genes involved in lipoylation of E2 [30]. Another strategy was based on overexpression of the pyruvate dehydrogenase bypass, namely pyruvate decarboxylase PDC1, aldehyde dehydrogenase ALD6 and acetyl-CoA synthase from Salmonella enterica <i>ACS</i><sub>SE</sub> [31]. The third strategy encompassed overexpression of ATP-dependent citrate lyase, consisting of two subunits ACL1 and ACL2, and a mitochondrial citrate transporter protein from Y. lipolytica. The genes required for different strategies were cloned into three EasyClone-MarkerFree plasmids and transformed into yeast strains expressing basic 3HP pathway along with the triple gRNA helper vector (pCIB3052 for CEN.PK and pCIB4668 for ethanol Red).

Overexpression of the cytoplasmic PDH complex proved to be the most successful strategy in CEN.PK, with an improvement in 3HP final titer of 19% over the basic strain in mineral medium, and 95% in the simulated fed-batch medium (Fig. 3). The PDH bypass showed no improvement. The ACL strategy performed worse than the basic pathway in MM, but interestingly showed an improvement of 60% in FIT (Fig. 3B). Growth profiles can be found in Supporting information, Fig. S1.

In the diploid industrial strain (Ethanol Red), the PDH complex also performed well, with an improvement of 23% over the basic strain (Supporting information, Fig. S2). These results confirm that the bacterial PDH complex from Enterococcus faecalis is a robust and efficient way to improve cytosolic acetyl-CoA levels in yeast. We confirmed that EasyClone-MarkerFree method integrates the expression cassettes on both homologous chromosomes, when applied in diploid strains. We performed qPCR on the diploid industrial strain (Ethanol Red) carrying the genes for the PDH complex. All of the six genes were shown to have a copy number of 2. The PDH bypass seems to have performed poorly, contradicting previous...
works. This may be due to the low activity of the 3HP biosynthetic genes in this work, as we only integrated ACC1 and MCR in single copies. In other studies, where we expressed ACC1 and MCR from either a high-copy episomal vector or integrated them in multiple copies into the genome, the overexpression of PDH bypass did have a positive effect on the 3HP production [32–34].

4 Concluding remarks

The EasyClone-MarkerFree vector toolkit can be used to simultaneously introduce one to three integration cassettes into the genome of S. cerevisiae without the use of selection markers. Using standardized BioBricks, the integration cassettes can be constructed for overexpression of one or two genes per integration site. In this study we successfully integrated up to six genes in a single transformation with 60–70% targeting efficiency. The system is well suited for strain construction via multiple iterative metabolic engineering cycles. We have shown that it performs well in both the haploid laboratory CEN.PK strain, and also in the diploid industrial Ethanol Red strain. The EasyClone-MarkerFree vector toolkit can be obtained from AddGene.

The work was funded by the Novo Nordisk Foundation. VS and IB acknowledge funding for the BioREFINE-2G project by the European Commission in the 7th Framework Programme (Project no. FP7-613771).

The authors declare no financial or commercial conflict of interest.

5 References

[1] Botstein, D., Chervitz, S. A., Cherry, J. M., Yeast as a model organism. Science 1997, 277, 1259–1260.
[2] Borodina, I., Nielsen, J., Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. Biotechnol. J. 2014, 9, 609–620.
[3] Jensen, M. K., Kesseling, J. D., Recent applications of synthetic biology tools for yeast metabolic engineering. FEMS Yeast Res. 2015, 15, DOI: 10.1111/1567-1364.12185.
[4] Schiestl, R. H., Petes, T. D., Integration of DNA fragments by illegitimate recombination in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 1991, 88, 7585–7589.
[5] Jensen, N. B., Strucko, T., Kildegaard, K. R., David, F. et al., EasyClone: Method for iterative chromosomal integration of multiple genes in Saccharomyces cerevisiae. FEMS Yeast Res. 2014, 14, 238–248.
[6] Stovicke, V., Borja, G. M., Forster, J., Borodina, I., EasyClone 2.0: Expanded toolkit of integrative vectors for stable gene expression in industrial Saccharomyces cerevisiae strains. J. Ind. Microbiol. Biotechnol. 2015, 42, 1519–1531.
[7] Pronk, J. T., Auxotrophic yeast strains in fundamental and applied research. Appl. Environ. Microbiol. 2002, 68, 2095–2100.
[8] Alam, M. T., Zeleznikai, A., Mülleder, M., Shilaha, P. et al., The metabolic background is a global player in Saccharomyces gene expression epistasis. Nat. Microbiol. 2016, 1, 1–10.
[9] Storici, F., Durham, C. L., Gordenin, D. A., Rehmick, M. A., Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. Proc. Natl. Acad. Sci. USA 2003, 100, 14994–14999.
[10] Wingler, L. M., Cornish, V. W., Reiterative recombination for the in vivo assembly of libraries of multigene pathways. Proc. Natl. Acad. Sci. USA 2011, 108, 15135–15140.
[11] Kuijpers, N. G., Chirompi, S., Voe, T., Solis-Escalante, D. et al., One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in Saccharomyces cerevisiae. FEMS Yeast Res. 2013, 13, 769–781.
[12] Kuijpers, N. G., Solis-Escalante, D., Bosman, L., van den Broek, M. et al., A versatile, efficient strategy for assembly of multi-fragment
expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination sequences. *Microb. Cell Fact.* 2013, 12, 47.

[13] DiCarlo, J. E., Norville, J. E., Mali, P., Rice, X. et al., Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res.* 2013, 41, 4336–4343.

[14] Jinek, M., Chylinski, K., Fonfara, I., Hauer, M. et al., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012, 337, 816–821.

[15] Jakociūnas, T., Bonde, I., Herrgård, M. J., Harrison S. J. et al., Multi-gene pathway in a versatile yeast expression platform. *Microbial production of indolylglucosinolate through engineering of* 34–59.

[16] Mans, R., van Rossum, H. M., Wijsman, M., Backx, A. et al., CRISPR/Cas9: A molecular Swiss army knife for simultaneous introduction of synergistic alleles and metabolic pathways in yeasts via CRISPR/Cas9. *Metab. Eng.* 2015, 32, 711–720.

[17] Jakociūnas, T., Jensen, M. K., Keasling, J. D., CRISPR/Cas9 advances engineering of microbial cell factories. *Metab. Eng.* 2016, 34, 44–59.

[18] Mikkelsen, M. D., Buron, L. D., Salomonsen, B., Olsen, C. E. et al., Microbial production of indolylglucosinolate through engineering of a multi-gene pathway in a versatile yeast expression platform. *Metab. Eng.* 2012, 14, 104–111.

[19] Delneri, D., Tomlin, G. C., Wilson, J. L., Hutter, A. et al., Exploring redundancy in the yeast genome: An improved strategy for use of the cre-loxP system. *Gene.* 2000, 252, 127–135.

[20] Solis-Escalante, D., Kuijpers, N. G. A., van der Linden, F. H., Pronk, J. T. et al., Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double-strand DNA breaks in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2014, 14, 741–754.

[21] Nour-Eklin, H. H., Geu-Flores, F., Håkkinen, B. A., USER cloning and USER fusion: The ideal cloning techniques for small and big laboratories. *Methods Mol. Biol.* 2010, 643, 185–200.

[22] Gietz, R. D., Schiestl, R. H., High-efficacy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* 2007, 2, 31–34.

[23] Borodina, I., Kildegaard, K. R., Jensen, N. B., Blücher, T. H. et al., Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via beta-alanine. *Metab. Eng.* 2015, 27, 57–64.

[24] Li, M., Borodina, I., Application of synthetic biology for production of chemicals in yeast *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 2014, DOI: 10.1111/1567-1364.12213.

[25] Kozak, B. U., van Rossum, H. M., Luttik, M. A., Akeroyd, M. et al., Engineering acetyl coenzyme A supply: Functional expression of a bacterial pyruvate dehydrogenase complex in the cytosol of *Saccharomyces cerevisiae*. *MBio* 2014, 5, e01696–e01614.

[26] Shiha, Y., Paradise, E. M., Kirby, J., Ro, D. K., Keasling, J. D., Engineering the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab. Eng.* 2007, 9, 160–168.

[27] Chen, Y., Bao, J., I-Kwon, K., Stewees, V., Nielsen, J., Coupled incremental precursor and co-factor supply improves 3-hydroxypropionic acid production in *Saccharomyces cerevisiae*. *Metab. Eng.* 2014, 22, 104–109.

[28] Jensen, N. B., Borodina, I., Chen, Y., Maury, J. et al., Microbial production of 3-hydroxypropionic acid. *WO Patent 198831 A1*, 2014.

[29] Kildegaard, K. R., Wang, Z., Chen, Y., Nielsen, J., Borodina, I., Production of 3-hydroxypropionic acid from glucose and xylose by metabolically engineered *Saccharomyces cerevisiae*. *Metab. Eng. Comm.* 2015, 2, 132–136.
Meeting report
Metabolic Engineering Summit Beijing
Xiangbin Chen
http://dx.doi.org/10.1002/biot.201500664

Commentary
Toward improved host cell protein impurity assessment
Jong Yoon Baik, Kelvin H. Lee
http://dx.doi.org/10.1002/biot.201600223

Review
Recent advances in exploiting ionic liquids for biomolecules: Solubility, stability and applications
Magaret Sivapragasam, Muhammad Moniruzzaman and Masahiro Goto
http://dx.doi.org/10.1002/biot.201500603

Research Article
Quantitative definition and monitoring of the host cell protein proteome using iTRAQ – a study of an industrial mAb producing CHO-S cell line
Lesley M. Chiverton, Caroline Evans, Jagroop Pandhal, Andrew R. Landels, Byron J. Rees, Peter R. Levison, Phillip C. Wright and C. Mark Smales
http://dx.doi.org/10.1002/biot.201500550

Research Article
Generic HPLC platform for automated enzyme reaction monitoring: Advancing the assay toolbox for transaminases and other PLP-dependent enzymes
Tim Börner, Carl Grey and Patrick Adlercreutz
http://dx.doi.org/10.1002/biot.201500587

Research Article
Hyperosmotic stimulus study discloses benefits in ATP supply and reveals miRNA/mRNA targets to improve recombinant protein production of CHO cells
Jennifer Pfizenmaier, Lisa Junghans, Attila Teleki and Ralf Takors
http://dx.doi.org/10.1002/biot.201500606

Research Article
Stirred tank bioreactor culture combined with serum/-xenogeneic-free culture medium enables an efficient expansion of umbilical cord-derived mesenchymal stem/stromal cells
Amanda Mizukami, Ana Fernandes-Platzgummer, Joana G. Carmelo, Kamilla Swiech, Dimas T. Covas, Joaquim M. S. Cabral and Cláudia L. da Silva
http://dx.doi.org/10.1002/biot.201500532

Research Article
Mono- and dichromatic LED illumination leads to enhanced growth and energy conversion for high-efficiency cultivation of microalgae for application in space
Ines Wagner, Christian Steinweg and Clemens Posten
http://dx.doi.org/10.1002/biot.201500357

Research Article
Improving carbohydrate production of Chlorella sorokiniana NIES-2168 through semi-continuous process coupled with mixotrophic cultivation
Yue Wang, Sheng-Yi Chiu, Shih-Hsin Ho, Zhuo Liu, Tomohisa Hasunuma, Ting-Ting Chang, Kuan-Fu Chang, Jo-Shu Chang, Nan-Qi Ren and Akihiko Kondo
http://dx.doi.org/10.1002/biot.201500270
A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic degradation of polyethylene terephthalate films
Markus Barth, Annett Honak, Thorsten Oeser, Ren Wei, Matheus R. Belisário-Ferrari, Johannes Then, Juliane Schmidt and Wolfgang Zimmermann
http://dx.doi.org/10.1002/biot.201600008

Disulfide-bridging PEGylation during refolding for the more efficient production of modified proteins
Claire Ginn, Ji-won Choi and Steve Brocchini
http://dx.doi.org/10.1002/biot.201600035

Identifying and retargeting transcriptional hot spots in the human genome
Joseph K. Cheng, Amanda M. Lewis, Do Soon Kim, Timothy Dyess and Hal S. Alper
http://dx.doi.org/10.1002/biot.201600015

EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into Saccharomyces cerevisiae via CRISPR-Cas9
Mathew M. Jessop-Fabre, Tadas Jakočiūnas, Vratislav Stovicek, Zongjie Dai, Michael K. Jensen, Jay D. Keasling and Irina Borodina
http://dx.doi.org/10.1002/biot.201600147